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TOPIC HIGHLIGHT

- 1 Clinical efficacy and drug resistance of anti-epidermal growth factor receptor therapy in colorectal cancer
Kocoglu H, Velibeyoglu FM, Karaca M, Tural D
- 8 Robot-assisted surgery for gastric cancer
Procopiuc L, Tudor Ș, Mănuc M, Diculescu M, Vasilescu C
- 18 MicroRNA in pancreatic ductal adenocarcinoma and its precursor lesions
Hernandez YG, Lucas AL

REVIEW

- 30 Antitumor effects of the benzophenanthridine alkaloid sanguinarine: Evidence and perspectives
Gaziano R, Moroni G, Buè C, Miele MT, Sinibaldi-Vallebona P, Pica F
- 40 *Helicobacter pylori* infection and gastric carcinoma: Not all the strains and patients are alike
Figura N, Marano L, Moretti E, Ponzetto A
- 55 State of the art biological therapies in pancreatic cancer
Di Marco M, Grassi E, Durante S, Vecchiarelli S, Palloni A, Macchini M, Casadei R, Ricci C, Panzacchi R, Santini D, Biasco G
- 67 Multimodality treatment strategies have changed prognosis of peritoneal metastases
Lungoci C, Mironiuc AI, Muntean V, Oniu T, Leebmann H, Mayr M, Piso P
- 83 Molecular approach to genetic and epigenetic pathogenesis of early-onset colorectal cancer
Tezcan G, Tunca B, Ak S, Cecener G, Egeli U

MINIREVIEWS

- 99 Novel therapeutic agents in the treatment of metastatic colorectal cancer
Pai SG, Fuloria J
- 105 Long-term outcomes after stenting as a "bridge to surgery" for the management of acute obstruction secondary to colorectal cancer
Suárez J, Jimenez-Pérez J
- 113 Role of self expandable stents in management of colorectal cancers
Cetinkaya E, Dogrul AB, Tirnaksiz MB

- 121 Role of microRNA-7 in digestive system malignancy

Chen WQ, Hu L, Chen GX, Deng HX

ORIGINAL ARTICLE

Retrospective Study

- 128 Impact of *RAS* and *BRAF* mutations on carcinoembryonic antigen production and pattern of colorectal metastases

Cho M, Akiba C, Lau C, Smith D, Telatar M, Afkhami M, Sentovich S, Melstrom K, Fakih M

Contents

World Journal of Gastrointestinal Oncology
Volume 8 Number 1 January 15, 2016

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AIM AND SCOPE

World Journal of Gastrointestinal Oncology (*World J Gastrointest Oncol*, *WJGO*, online ISSN 1948-5204, DOI: 10.4251) is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

WJGO covers topics concerning carcinogenesis, tumorigenesis, metastasis, diagnosis, prevention, prognosis, clinical manifestations, nutritional support, molecular mechanisms, and therapy of benign and malignant tumors of the digestive tract. The current columns of *WJGO* include editorial, frontier, diagnostic advances, therapeutics advances, field of vision, mini-reviews, review, topic highlight, medical ethics, original articles, case report, clinical case conference (Clinicopathological conference), and autobiography. Priority publication will be given to articles concerning diagnosis and treatment of gastrointestinal oncology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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2016 Colorectal Cancer: Global view

Clinical efficacy and drug resistance of anti-epidermal growth factor receptor therapy in colorectal cancer

Hakan Kocoglu, Fatih Mehmet Velibeyoglu, Mustafa Karaca, Deniz Tural

Hakan Kocoglu, Fatih Mehmet Velibeyoglu, Deniz Tural, Department of Medical Oncology, Bakirkoy Education and Research Hospital, 34900 Istanbul, Turkey

Mustafa Karaca, Department of Medical Oncology, Gazi University Faculty of Medicine, 06500 Ankara, Turkey

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Telephone: +90-212-4147171
Fax: +90-212-4147172

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Abstract

Colorectal cancer (CRC) ranked third in cancer related death and its incidence has been increasing worldwide. In recent decades important therapeutic advances have

been developed in treatment of metastatic CRC (mCRC), such as monoclonal antibodies against epidermal growth factor receptor (anti-EGFR), which provided additional clinical benefits in mCRC. However, anti-EGFR therapies have limited usage due to approximately 95% of patients with *KRAS* mutated mCRC do not response to anti-EGFR treatment. Thus, *KRAS* mutation is predictive of nonresponse to anti-EGFR therapies but it alone is not a sufficient basis to decide who should not be received such therapies because; approximately fifty percent (40%-60%) of CRC patients with wild-type *KRAS* mutation also have poor response to anti-EGFR based treatment. This fact leads us to suspect that there must be other molecular determinants of response to anti-EGFR therapies which have not been identified yet. Current article summarizes the clinical efficacy of anti-EGFR therapies and also evaluates its resistance mechanisms.

Key words: Colorectal cancer; Epidermal growth factor receptor; *KRAS* mutation; Anti-epidermal growth factor receptor antibody; Drug resistance

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Core tip: Molecular targeting agents, such as monoclonal antibodies against epidermal growth factor receptor (anti-EGFR), provide additional clinical benefits in metastatic colorectal cancer (CRC). However, anti-EGFR therapies have limited usage due to approximately 95% of patients with *KRAS* mutated metastatic CRC do not response to anti-EGFR treatment. Thus, *KRAS* mutation is predictive of nonresponse to anti-EGFR therapies but it alone is not a sufficient basis to decide who should not be received such therapies because approximately fifty percent (40%-60%) of CRC patients with wild-type *KRAS* mutation also have poor response to anti-EGFR based treatment. This fact leads us to suspect that there must be other molecular determinants of response to anti-EGFR therapies which have not been identified yet. Current article summarizes the clinical efficacy of

anti-EGFR therapies and also evaluates its resistance mechanisms.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers in both genders (second in females and third in males)^[1]; and it is also ranked third in cancer related death in both genders with approximately 15.1 deaths per 100000^[2,3]. While the mortality rate of CRC has been decreasing in Western countries, its incidence has been increasing worldwide, except United States^[4]. Despite of decreasing death rates, approximately fifty percent of patients with CRC are diagnosed with metastatic disease in their initial assessments^[5].

Several chemotherapeutic agents [*e.g.*, pyrimidine analogs (*e.g.*, 5-fluorouracil), platinum-based antineoplastic agents, and topoisomerase inhibitors] have become available in the past and thus survival rate of CRC patients significantly increased. Also, recently developed molecular targeting agents, such as monoclonal antibodies against epidermal growth factor receptor (EGFR) (*e.g.*, cetuximab and panitumumab)^[6,7], provided additional clinical benefits in metastatic CRC (mCRC)^[8-10].

In several types of cancer, including CRC, EGFR is overexpressed or amplified. Monoclonal antibodies keep EGFR in an inactive state by binding to and occluding the ligand-binding site of EGFR when the ligand is unbound (acting as competitive antagonists). This leads an inhibition of intracellular signaling pathways of EGFR (RAS/RAF/MAPK and PI3K/PTEN/AKT) that involved in several cellular activities including cell proliferation, motility, invasion, and survival^[11].

KRAS, a signal transduction molecule, transduces the signal from ligand-bound EGFR to the nucleus. Prospective randomized trials elucidated that presence of mutation in *KRAS* gene leads to non-response to anti-EGFR based treatment^[6-10,12-14]. Therefore, it is highly recommended that *KRAS* mutation status should be known before initiating anti-EGFR based treatment in mCRC patients. Thus, *KRAS* mutation is predictive of nonresponse to anti-EGFR therapies but it alone is not a sufficient basis to decide who should not be received such therapies because almost 60% of CRC patients with wild-type (WT) *KRAS* mutation also have poor response to anti-EGFR based treatment^[15]. This fact leads us to suspect that there must be other molecular determinants of response to anti-EGFR therapies which have not been identified yet. Current article summarizes

the clinical efficacy of anti-EGFR therapies and also evaluates its resistance mechanisms.

CLINICAL EFFICACY OF ANTI-EGFR ANTIBODY IN MCRC

Both Cetuximab, an IgG1 type chimeric monoclonal antibody, and panitumumab, an IgG2 type fully human monoclonal antibody, induce apoptosis by inhibiting downstream signaling pathways of EGFR (RAS/RAF/MAPK and PI3K/PTEN/AKT). Also, these molecules, especially cetuximab, activate antibody-dependent cellular cytotoxicity which consequently improves their cytotoxic actions and therapeutic effectiveness^[16].

The recent published randomized non-inferiority phase III study showed median overall survival (OS) was similar in patients with mCRC who treated with panitumumab alone and with cetuximab alone^[17]. The incidences of any grade and grade 3-4 adverse events were similar in both treatment groups, however, the incidence of grade 3-4 infusion reaction was lower and grade 3-4 hypomagnesaemia was higher in panitumumab group than in cetuximab group^[18]. In some studies, cetuximab and panitumumab have been investigated in combination with FOLFIRI (folinic acid, fluorouracil, and irinotecan) and FOLFOX (folinic acid, fluorouracil, and oxaliplatin) as initial therapy option for treatment of mCRC. And a meta-analysis of these 14 randomized studies concluded that there is a clear benefit to the use EGFR inhibitors in patients with WT *KRAS* mCRC^[18]. An updated analysis (CRYSTAL trial) demonstrated that adding cetuximab to FOLFIRI as first-line therapy improves survival in patients with WT *KRAS* mCRC^[19]. Also another randomized phase III study showed that the combination of panitumumab and FOLFIRI significantly improves progression-free survival (PFS), but not OS, in mCRC patients with WT *KRAS*^[9]. Three other trials have evaluated the addition of cetuximab to FOLFOX in first line treatment of patients WT *KRAS* mCRC. In randomized phase II OPUS study, combination of FOLFOX and cetuximab was associated with increased response rate and PFS. However, this treatment had no benefit in median OS^[12]. In the Medical Research Council (MRC) COIN study, adding cetuximab to oxaliplatin-based chemotherapy in patients with WT *KRAS* mCRC increased response rate with no benefit in PFS or OS^[20]. Similarly, another phase III study (NORDIC-VII) showed that cetuximab did not add significant benefit when combined with FOLFOX in treatment of patients with WT *KRAS* mCRC^[21]. In contrast to earlier studies, the recent published randomized phase III CALGB/SWOG 80405 trial demonstrated that addition of cetuximab to FOLFOX or FOLFIRI chemotherapy was significantly increased PFS and OS in treatment of patients with all RAS-WT mCRC^[22]. In the study by Douillard *et al.*^[23] (the PRIME study), which compared panitumumab plus FOLFOX and FOLFOX alone in mCRC patients with WT *KRAS*/NRAS,

panitumumab plus FOLFOX group showed a statistically significant improvement in PFS and OS.

Based on this knowledge, all patients with newly diagnosed mCRC should be tested for *KRAS* mutation. Also screening of *KRAS* mutations seems essential in mCRC patients to initiate anti-EGFR based treatment. But *KRAS* mutation alone is not a sufficient basis to decide who should not be received such therapies because almost 60% of CRC patients with WT *KRAS* mutation also have poor response to anti-EGFR based treatment^[15]. Also 5%-9% of CRC patients have a specific mutation in *BRAF* gene (V600E)^[24,25]. But the use of *BRAF* as a predictive marker for response to anti-EGFR based treatment is unclear. This fact leads us to suspect that there must be other molecular determinants of response to anti-EGFR therapies which have not been identified yet.

MECHANISMS OF RESISTANCE TO ANTI-EGFR TREATMENT

KRAS/NRAS/BRAF mutations

Approximately 40% of CRC patients have mutation in exon 2 of the coding of the *KRAS* gene^[26,27]. Prospective randomized studies showed that *KRAS* mutations are predictive of non-response to anti-EGFR based treatment^[6-10,12-14]. These studies showed that tumors with a mutation in codon 12 or 13 of exon 2 of the *KRAS* gene are essentially unresponsive to anti-EGFR based treatment. Recent studies demonstrated that mutation in *KRAS* outside of exon 2 and mutation in *NRAS* are also predictive for unresponsiveness to anti-EGFR treatment^[23,28]. Recently, a study assessed the superiority of FOLFOX plus panitumumab to FOLFOX alone according to *RAS* (*KRAS* or *NRAS*) or *BRAF* (B-type Raf kinase) mutation status. In that study, 17% of patients with non-mutated *KRAS* exon 2 had other *RAS* mutation which has been shown to be associated with inferior survival with panitumumab plus FOLFOX treatment^[23]. Cetuximab or panitumumab treatments seem to be eligible for selected patients with WT *KRAS* tumors who also have *BRAF*-WT mutations^[29].

BRAF oncogene encodes *BRAF* protein which is a member of *RAS/RAF/MAPK* (mitogen-activated protein kinase) pathway^[27]. Mutations in *BRAF* and *KRAS* genes are mutually exclusive^[30]. Approximately 9% (5%-9%) of patients with CRC have a mutation in *BRAF* gene (V600E)^[24,25]. CRYSTAL and PETACC-3 studies demonstrated that patients with *BRAF* mutation have a worse prognosis than those with the WT tumors^[19,31]. However, the use of *BRAF* as a predictive marker is unclear. CRYSTAL study elucidated that *BRAF* mutation does not seem to be strong predictive biomarker for the addition cetuximab to FOLFIRI in the first line treatment of WT mCRC^[19]. Also, subset analysis of the PRIME study found that *BRAF* mutation indicates poor prognosis but it may not be predictive of the benefit of adding panitumumab to FOLFOX in the first line treatment of

mCRC^[8]. Tol *et al*^[25] demonstrated that *BRAF* mutation is a negative indicator for prognosis in mCRC patients and in contrast to *KRAS* mutation, this feature is not restricted to the outcome of the cetuximab. In subsequent lines of therapy elucidated that *BRAF* mutation is a marker of resistance to anti-EGFR treatment in the non-first line setting of mCRC^[29,32,33].

Vemurafenib is orally administered selective inhibitor of *BRAF* V600 kinase but using it alone in *BRAF*-mutated CRC patients results insufficient activity^[34]. Studies suggested that feedback activation of EGFR signaling might be responsible of the resistance to Vemurafenib in CRC^[35,36]. In a cohort study by Hyman *et al*^[37], median PFS and OS did not change with vemurafenib monotherapy or vemurafenib and cetuximab combination therapy in patients with CRC (Table 1).

HYPERACTIVATION OF PI3K-PTEN AXIS

Interestingly, 41% of patients do not have *KRAS* or *BRAF* mutation, and they do not respond to anti-EGFR treatment^[29]. Some studies suggested that anti-EGFR downstream pathways other than *RAS/RAF/MAPK* [e.g., phosphoinositide 3-kinase/phosphatase and tensin homolog pathway (PI3K/PTEN)], might be responsible for the resistance to anti-EGFR based therapy. It was shown that mutation in *PI3KCA* (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha) or loss of *PTEN* is associated with resistance to anti-EGFR based treatment^[38-40]. Tural *et al*^[41] investigated the effect of oncogenic activation of the members of EGFR downstream pathways (e.g., *PI3K*, *PTEN* and *BRAF*) on response to anti-EGFR therapy. They have showed that *PI3K* expression and *PTEN* loss might be used as predictive to the response to anti-EGFR treatment in mCRC patients with WT *KRAS*. According to this study, *BRAF* negative, *PTEN* expressing and *PI3K* non-expressing CRCs have higher response rate and longer PFS and OS than all others. Most studies evaluated *PI3K* mutation in response to cetuximab based treatments in CRC patients^[38,42-45]. In these studies, *PI3K* mutation has been suggested as predictive of resistance to anti-EGFR-based therapies. On the other hand, the role of *PI3K* mutation in response is conflict. Perrone *et al*^[38] has investigated *PI3KCA* gene mutations in CRC patients and they suggested that mutation in *PI3KCA* causes resistance to anti-EGFR therapies. Also Prenen *et al*^[45] analyzed *PI3KCA* and *KRAS* mutations status in chemo-refractory mCRC patients who treated with anti-EGFR based treatment and they did not determine any correlation between *PI3KCA* mutation and response to anti-EGFR treatment. Nevertheless, most of studies have suggested that *PTEN* inactivation is a negative predictor of response to anti-EGFR therapy^[38-40]. Bardellie *et al*^[46] stated that *PI3K* expression and *PTEN* loss are correlated with decreased survival and are predictors of poor response to anti-EGFR therapy. Based on these studies, it is well known that activating mutation in *PI3KCA* or inactivation of *PTEN* phosphates

Table 1 Clinical trials of targeted agents in combination with chemotherapy as first-line treatments for metastatic colorectal cancer

Ref.	Year	Population	Patient number	Regimen	Median PFS (mo)	P ¹	Median OS (mo)	P ¹	Response rate (%)	P ¹
CRYSTAL ^[19]	2009	All	599	FOLFIRI	8.0	0.048	18.6	0.31	38.7	0.0038
			599	FOLFIRI + Cetuximab	8.9		19.9		46.9	
		KRAS WT	350	FOLFIRI	8.4	0.0012	20	0.0093	39.7	< 0.001
		subgroup	316	FOLFIRI + Cetuximab	9.9		23.5		57.3	
		KRAS MT	183	FOLFIRI	7.7	0.26	16.7	0.75	36.1	0.35
OPUS ^[12]	2009	subgroup	214	FOLFIRI + Cetuximab	7.4		16.2		31.3	
		All	168	FOLFOX4	7.2	0.62	18	0.91	36	0.064
			169	FOLFOX4 + Cetuximab	7.2		18.3		46	
		KRAS WT	97	FOLFOX4	7.2	0.0064	18.5	0.39	34	0.0027
		subgroup	82	FOLFOX4 + Cetuximab	8.3		22.8		57	
COIN ^[20]	2011	KRAS MT	59	FOLFOX4	8.6	0.0153	17.5	0.2	53	0.029
		subgroup	77	FOLFOX4 + Cetuximab	5.5		13.4		34	
		KRAS WT	367	FOLFOX/XELOX	8.6	0.60	17.9	0.68	57	0.049
		group	362	FOLFOX/XELOX + Cetuximab	8.6		17		64	
		KRAS WT	127	FOLFOX	9.2	0.056	-	-	-	-
NORDIC-VII ^[21]	2012	group	117	FOLFOX + Cetuximab	9.0		-		-	
		KRAS WT	240	XELOX	8.0	0.56	-	-	-	-
		group	245	XELOX + Cetuximab	8.4		-		-	
		KRAS MT	268	FOLFOX/XELOX	-	-	14.8	0.8	-	-
		group	297	FOLFOX/XELOX + Cetuximab	-		13.6		-	
CALGB/SWOG ^[22]	2014	All	185	Nordic FLOX (control group)	7.9	-	20.4	-	41	-
			194	FLOX + Cetuximab	8.3	0.31	19.7	0.67	49	0.15
			187	intermittent FLOX + Cetuximab	7.3	NA	20.3	0.79	47	NA
		KRAS WT	97	Nordic FLOX (control group)	8.7	-	22	-	47	-
		subgroup	97	FLOX + Cetuximab	7.9	0.66	20.1	0.48	46	0.89
PRIME ^[8]	2010		109	intermittent FLOX + Cetuximab	7.5	NA	21.4	0.66	51	NA
		KRAS MT	58	Nordic FLOX (control group)	7.8	-	20.4	-	40	-
		subgroup	72	FLOX + Cetuximab	9.2	0.07	21.1	0.89	49	0.31
			65	intermittent FLOX + Cetuximab	7.2	NA	20.5	0.84	42	NA
		KRAS WT	578	FOLFIRI or mFOLFOX6 + Cetuximab	10.45	NA	29.93	0.34	-	-
Hyman <i>et al</i> ^[37]	2015	group	559	FOLFIRI or mFOLFOX6 + Bevacizumab	10.84		29.04		-	
		KRAS WT	331	FOLFOX4	8.0	0.02	19.7	0.072	48	0.068
		group	325	FOLFOX4 + Panitumumab	9.6		23.9		55	
		KRAS MT	219	FOLFOX4	8.8	0.02	19.3	0.068	40	-
		group	221	FOLFOX4 + Panitumumab	7.3		15.5		40	
Reidy <i>et al</i> ^[51]	2010	BRAF V600	10	Vemurafenib	4.5	-	9.3	-	0	-
		group	27	Vemurafenib + Cetuximab	3.7		7.1		4	
		All	23	IMC-A12 (anti-IGF-1R antibody)	5.9	-	5.2	-	0	-
			21	IMC-A12 (anti-IGF-1R antibody) + Cetuximab	6.1		4.5		5	
		KRAS WT	20	IMC-A12 (anti-IGF-1R antibody) + Cetuximab	9.4		10.9		0	

¹95% CI. PFS: Progression-free survival; OS: Overall survival; All: All patients group; WT: Wild type; MT: Mutant type; NA: Not available; KRAS: KRAS exon 2, codons 12 and 13; FOLFIRI: Irinotecan, fluorouracil, and leucovorin; FOLFOX: Fluorouracil, leucovorin, and oxaliplatin; XELOX: Capecitabine and oxaliplatin; FLOX: Fluorouracil, leucovorin, and oxaliplatin.

can deregulate PI3K signaling pathway^[46]. Two studies demonstrated that PI3KCA mutation and PTEN loss which cause PI3K pathway activation are significant predictors of response to anti-EGFR treatment^[38,42]. Also, Tural *et al*^[41] indicated that PI3K expression and PTEN loss together are correlated with significantly worse outcome.

HYPEREXPRESSION OR HYPERACTIVATION OF TYPE 1 INSULIN LIKE GROWTH FACTOR RECEPTOR

The type 1 insulin like growth factor receptor (IGF-

1R) belongs to the class of tyrosine kinase receptors. IGF-1R functions by activating downstream signaling pathways which include MAPK and PI3K/AKT. Previous studies showed that IGF-1R overexpression results neoplastic transformation of cultured cells^[47]. Also IGF-1R overexpression was seen in several types of human tumors^[48] and its downregulation has been shown to be able to inhibit the growth of these cells^[49]. These findings make IGF-1R an attractive candidate as therapeutic target in anti-tumor therapies. A previous study showed that combination therapy of antibodies against to IGF-1R and anti-EGFR results in further inhibition of CRC cell line growth^[50]. A phase II study evaluated the safety and the efficacy of human anti-IGF-1R monoclonal antibody

(either alone or in combination with cetuximab) in mCRC patients, and both treatment modalities was reported as insufficient in chemorefractory mCRC patients^[51] (Table 1).

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2016 Gastric Cancer: Global view

Robot-assisted surgery for gastric cancer

Livia Procopiuc, Ștefan Tudor, Mircea Mănuș, Mircea Dicușcu, Cătălin Vasileșcu

Livia Procopiuc, Mircea Mănuș, Mircea Dicușcu, Cătălin Vasileșcu, "Carol Davila" University of Medicine and Pharmacy, 020022 Bucharest, Romania

Ștefan Tudor, Cătălin Vasileșcu, Department of General Surgery and Liver Transplantation, Fundeni Clinical Institute, 022238 Bucharest, Romania

Mircea Mănuș, Mircea Dicușcu, Department of Gastroenterology and Hepatology, Fundeni Clinical Institute, 022238 Bucharest, Romania

Author contributions: Procopiuc L, Tudor Ș, Mănuș M, Dicușcu M and Vasileșcu C wrote the manuscript; Vasileșcu C critically revised and approved the final version of the manuscript.

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Correspondence to: Cătălin Vasileșcu, Associate Professor, Department of General Surgery and Liver Transplantation, Fundeni Clinical Institute, 258 Fundeni Street, 022238 Bucharest, Romania. catvasilescu@gmail.com
Telephone: +40-722-207260
Fax: +40-213-188811

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Abstract

Minimally invasive surgery for gastric cancer is a relatively new research field, with convincing results mostly stemming from Asian countries. The use of the robotic surgery platform, thus far assessed as a safe procedure, which is also easier to learn, sets the background for a wider spread of minimally invasive technique in the treatment of gastric cancer. This review will cover the literature published so far, analyzing the pros and cons of robotic surgery and highlighting the remaining study questions.

Key words: Gastric cancer survival; Robotic surgery; Gastric cancer surgery; Lymphadenectomy; Minimally invasive surgery

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Core tip: An important problem remains regarding the selection of the appropriate technique for a given gastric cancer case. Encouraging results are being published using the robotic technique, but the lack of homogenous study groups in terms of staging, comorbidities and adjuvant and neoadjuvant therapies makes it hard to establish a clear indication for the robotic gastrectomy in gastric cancer. Carefully weighing the treatment options is especially important since there are more and more groups publishing acceptable results with the robotic technique.

Procopiuc L, Tudor Ș, Mănuș M, Dicușcu M, Vasileșcu C.

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INTRODUCTION

Surgery is unanimously considered the mainstay curative treatment in gastric cancer. Technically, the possibilities range from open surgery to minimally invasive methods like laparoscopy or robotic surgery. However, the newer laparoscopic techniques have only proven their effectiveness in early gastric cancer^[1]. The current challenge for robotic surgery in gastric cancer is to prove its benefit as a treatment option, ideally in the form of a survival advantage. Up until now studies only proved its non-inferiority compared with existent techniques.

Technologic progress has clearly had an impact in medicine and surgery, in particular. However the newest developments in the field of technology are not always the best ones and examples can easily be found in the last decades. Rejecting a new technique altogether is, however, not an option in the field of surgery. It would possibly mean closing the roads to a new development that could allow for patients to benefit from procedures which are not easily or not at all undertaken at the moment.

MINIMALLY INVASIVE SURGERY FOR GASTRIC CANCER

The existence of so many treatment options for gastric cancer suggests that currently there is no consensus regarding the adequate therapeutic conduct. Thus far, the following objectives for gastric cancer surgery have been made clear and should be pursued in any case: (1) If surgery can be performed, it must proceed, usually as a part of multimodal cancer treatment^[1]. The surgical approach is based on the Virchow-Halsted theory of centrifugal dissemination of carcinomas. This mechanistic theory dating from the end of the 19th century is based on the fact that cancer was believed to begin in the target organ and then spread in an orderly fashion through lymphatic drainage routes invading lymph nodes along the way^[2,3]; (2) The tumor must be resected according to oncological safety limits^[1]; and (3) An adequate lymphadenectomy must be performed. Its extent varies depending on the location and stage of the tumor^[1]. Reaching these objectives correlates with a higher survival rate and a lower rate of recurrence^[4,5].

Modern day gastric cancer treatment was definitely impacted by technological progress. The laparoscopy revolution was quickly introduced in this field, with the first laparoscopic gastrectomy performed by Kitano *et al.*^[6]. Experience accumulated with bariatric surgery must not be neglected either, as it led to an improvement

in the technique required to perform intracorporeal anastomoses. The consequence was a rapid development of laparoscopic surgery for gastric cancer beginning, of course, with the early stages. On the other hand, the treatment options for gastric cancer were also enriched by the development of endoscopy, which limited the indications for video-assisted surgery.

Nonetheless, minimally invasive surgery failed to disseminate with great speed worldwide owing mostly to the fact that it is a technically demanding procedure. It is currently particularly favored in Asian countries^[7,8] where it is gaining terrain as a treatment for early gastric cancer, but it is interesting to note that laparoscopic surgery for gastric cancer is still an investigational procedure even in countries like Japan^[1]. To advance this type of surgery into the category of standard procedures, results of large randomized controlled studies like KLASS-01^[9], KLASS-02^[10] and JCOG 0912^[11] comparing the results of open and laparoscopic surgery are still awaited.

At the moment, the benefits of laparoscopy are still being debated, despite all the published studies which seemingly accrue "pro" arguments at a constant rate. In our opinion, the main objection is that these studies presenting good postoperative as well as oncological outcomes, mostly come from highly experienced large-volume surgical centers, which offer a standard of care that is not easily reproducible everywhere in the world.

ROBOTIC SURGERY

The robotic technologies were brought about to circumvent some of the difficulties of laparoscopic surgery. The laparoscopic procedures for gastric cancer have indeed been associated with improved postoperative outcomes and oncological results^[12-15], but the platform itself imposes a series of technical shortcomings. The two-dimensional views coupled with the fulcrum effect and the inherent tremor reduce the surgical range of motion and prolong the learning curve especially for large scale procedures such as gastrectomy. The robotic system comes with a three-dimensional view enabling depth perception, the EndoWrist[®] technology which allows for seven degrees of freedom and tremor filtration. Additionally, images can be enlarged enabling the performance of delicate steps such as lymph node dissection along great vessels which are essential in achieving a D2 dissection, suturing or knotting. These features could enable the performance of relatively complicated procedures such as function-preserving gastrectomy or extended resections for advanced gastric cancer using a minimally invasive method. Nonetheless this technique also has its disadvantages: Costs, duration of the procedures, the necessary trainings.

The use of the robotic platforms in general surgery did not enjoy the same success as it did in urologic surgery, and the field of gastric cancer is no exception. There are a series of shortcomings of the robotic platform explaining this situation. First of all the lack of robotic staplers and robotic seal and cut devices like

LigaSure™ is a considerable inconvenience. Second, due to the costs, the robotic platform cannot be used to cover the whole spectrum of procedures normally performed by a general surgeon^[12].

Current status of robotic surgery in early gastric cancer

Studies evaluating robotic surgery for early gastric cancer alone are scarce and stem mostly from Asian countries. The higher incidence of gastric cancer in these countries, together with the wide extent and increased efficacy of the national gastric cancer screening programs fueled the search for minimally invasive treatment modalities for the early stages of the disease. This led not only to the development of endoscopic resection, but also to a large pool of surgeons well versed in minimally invasive gastrectomies. The encouraging results published in small non-randomized comparative studies of laparoscopic vs open surgery for early gastric cancer^[13-15] were followed by the increased use of laparoscopy in clinical practice. Japan reports that at least 20% of the gastrectomies for early gastric cancer in its hospitals are now being performed laparoscopically^[1]. The need for better statistical evidence supporting the minimally invasive treatment of early gastric cancer was answered by starting two major randomized controlled trials which are now underway in Japan and South Korea comparing laparoscopy and open surgery^[9,11].

Following the foot-steps of laparoscopic surgery, robotics was first introduced in the treatment of early stage patients by the same surgeons who had acquired experience in the field of laparoscopic gastrectomies. After the first robotic gastrectomy reported in 2003 by Hashizume *et al*^[16], a series of encouraging reports on robotic surgery for gastric carcinomas began to appear in literature (Table 1).

In keeping with the trends of gastric cancer incidence in the eastern and western continents, Asian studies focus on mixed cohorts of gastric cancer patients with a high prevalence of the early stages or on early gastric cancer patients alone (Table 1). The largest cohort of early-stage gastric cancer to date was published by Woo *et al*^[17]. A total of 827 patients were included in this nonrandomized comparative study of robotic (236 patients) and laparoscopic surgery (591 patients) for stage Ia and Ib gastric carcinomas. The total operative time was significantly increased for the robotic procedures compared with laparoscopy (219.5 min vs 170.7 min, $P < 0.001$), but the robotic group also showed a lower estimated blood loss (91.6 mL vs 147.8 mL, $P = 0.02$). The length of hospital stay was slightly in favor of the laparoscopic group (7 d vs 7.7 d, $P = 0.004$) and there were no differences regarding morbidity and mortality. In terms of oncological principles, the number of retrieved lymph nodes was not different and all the patients in the robotic group had negative resection margins^[17].

Other studies comparing robotic surgery to laparoscopy in the treatment of gastric cancer show the same operative outcomes. The operative times are always

significantly longer for the robotic group (Table 2). This has been attributed to longer docking times necessary for the robot. However, a learning curve effect can be derived from the two studies separating the laparoscopic surgery group into an initial and a recent subgroup^[18,19]. The operating times reported for the initial laparoscopic technique subgroup are even longer than those of the robotic subgroup. That is no longer the case for the recent laparoscopy subgroup which yields the shortest operating time between the three subgroups (Table 2). In the study of Song *et al*^[19] the difference between these mean operative times were 289.5, 230 and 134 min, respectively with a statistically significant difference. The decrease of the mean operative times between the initial and the latter robotic cases (231 min vs 208 min) in the large cohort published by Woo *et al*^[17] indicates that shortening the operating times is also a matter of exercise, as was the case when the laparoscopic gastrectomies were introduced.

Regarding the estimated blood loss and the number of retrieved lymph nodes, there are conflicting results stemming from most of the cohorts comparing laparoscopy to robotic surgery (Table 2). A meta-analysis performed by Shen *et al*^[20] including the studies which also appear in our retrospective tables (Tables 1-3) comparing robotics and laparoscopy also found no statistically significant difference on the number of retrieved lymph nodes. However, a significantly lower blood loss was found in favor of the robotic group.

Current status of robotic surgery in advanced gastric cancer

Papers stemming from Europe, on the other hand, have a large prevalence of advanced gastric cancer cases in their study groups. In the largest study up to date (5839 patients) comparing robotic (436 patients), laparoscopic (861 patients) and open surgery (4542 patients) performed for stage I, II and III gastric cancer by Kim KM *et al*^[47], overall safety of these three types of surgery was the main focus. The overall complication rate was the same between the three groups (OG 10.7% LG 9.4% and RG 10.1%, $P = 0.494$) and so was their severity ($P = 0.424$). However, robotic surgery was prone to complications related to leaks ($P = 0.017$), whereas ileus and abscesses were more prevalent in open surgery ($P = 0.001$, $P = 0.013$ respectively). The authors explain that stapling lines were not reinforced with sutures in minimally invasive surgery, as opposed to open surgery and that the patients included in the open surgery group were mainly patients with more advanced disease for whom the complexity of the resections was higher. The robotic group showed a faster recovery with a shorter time to starting the soft diet and a shorter postoperative stay ($P < 0.001$ for both parameters) (Table 3). This study also showed an increased duration of the procedure compared to laparoscopic and open surgery (224 min vs 176 min vs 158 min, $P < 0.001$) combined with a lower estimated blood loss for the robotic group ($P < 0.001$). The

Table 1 Summary of studies reporting use of robotic surgery for gastric cancer

Ref.	Year	Type of study	Type of surgery	Stage ¹	Type of resection	No. of patients			
						Total	R	L	O
Patriti <i>et al</i> ^[21]	2008	CS	R	6 patients I , 6 patients II , 1 patient III	8 DG, 4 TG, 1 PG	13	13		
Lee <i>et al</i> ^[22]	2011	CS	R	I	DG	12	12		
D'Annibale <i>et al</i> ^[23]	2011	CS	R	17 patients I , 6 patients II , 1 patient III	11 TG, 13 DG	24	24		
Isogaki <i>et al</i> ^[24]	2011	CS	R	N/A	46 DG, 14 TG, 1 PG	61	61		
Kim <i>et al</i> ^[25]	2013	CS	R	11 patients I , 1 patient III	N/A	12	12		
Liu <i>et al</i> ^[26]	2013	CS	R	26 patients I , 32 patients II , 46 patients III	38 DG, 54 TG, 12 PG	104	104		
Park <i>et al</i> ^[27]	2013	CS	R	178 patients I , 22 patients II or more advanced	154 STG, 46 TG	200	200		
Tokunaga <i>et al</i> ^[28]	2014	CS	R	I A	18 DG	18	18		
Anderson <i>et al</i> ^[29]	2007	CS	R	Early GC	7 STG	7	7		
Song <i>et al</i> ^[30]	2009	CS	R	Early GC	67 STG, 33 TG	100	100		
Hur <i>et al</i> ^[31]	2010	CS	R	N/A	5 STG, 2 TG	7	7		
Uyama <i>et al</i> ^[32]	2012	CS	R	18 patients I A, 7 patients II A to III C	25 DG	25	25		
Yu <i>et al</i> ^[33]	2012	CS	R	N/A	29 DG, 12 TG	41	41		
Jiang <i>et al</i> ^[34]	2012	CS	R	24 patients I , 28 patients II , 68 patients III ²	62 DG, 35 TG, 23 PG	120	120		
Hyung <i>et al</i> ^[18]	2007	NC	R vs L	N/A	N/A	30	10	20	
Song <i>et al</i> ^[19]	2009	NC	R vs L	R: 20 patients I , L: 37 patients I , 3 patients II	R: 20 DG, L: 40 DG	60	20	40	
Pugliese <i>et al</i> ^[35]	2010	NC	R vs L	37 patients early GC, 33 patients advanced GC	64 STG	64	16	48	
Woo <i>et al</i> ^[17]	2011	NC	R vs L	827 patients I a or I b	R: 172 DG, 62, 2 CT; L: 481 DG, 108 TG, 2 CT	827	236	591	
Eom <i>et al</i> ^[36]	2012	NC	R vs L	R: 25 patients I , 3 patients II , 2 patients III, L: 56 patients I , 6 patients II	DG both groups	92	30	62	
Park <i>et al</i> ^[37]	2012	NC	R vs L	R: 27 patients I , 3 patients II ; L: 108 patients I , 11 patients II , 1 patient III	DG both groups	150	30	120	
Yoon <i>et al</i> ^[38]	2012	NC	R vs L	R: 29 patients I , 7 patients II , L: 55 patients I , 7 patients II , 3 patients III	TG both groups	101	36	65	
Kang <i>et al</i> ^[39]	2012	NC	R vs L	R: 82 patients I , 11 patients II , 7 patients III	R: 84 STG, 16 TG	382	100	282	
Hyun <i>et al</i> ^[40]	2013	NC	R vs L	R: 30 patients I , 5 patients II , 3 patients III; L: 67 patients I , 9 patients II , 7 patients III	R: 29 DG, 9 TG; L: 65 DG, 18 TG	121	38	83	
Noshiro <i>et al</i> ^[41]	2014	NC	R vs L	R: 18 patients I , 3 patients II -IV, L: 113 patients I , 47 patients II -IV	DG both groups	181	21	160	
Han <i>et al</i> ^[42]	2014	NC	R vs L	R: 59 patients I , 8 patients II , 1 patient III, L: 66 patients I , 2 patients II	PPG both groups	136	68	68	
Junfeng <i>et al</i> ^[43]	2014	NC	R vs L	R: 29 patients I , 36 patients II , 55 patients III, L: 115 patients I , 98 patients II , 181 patients III	R: 92 DG, 26 TG, 2 PG; L: 261 DG, 118 TG, 15 PG	510	120	394	
Kim <i>et al</i> ^[44]	2014	NC	R vs L	R: 145 patients I , 27 patients II and III; L: 422 patients I , 59 patients II and III	N/A	653	172	481	
Kim <i>et al</i> ^[45]	2010	NC	R vs L vs O	Lower than cT2N1M0	STG all groups	39	16	11	12
Huang <i>et al</i> ^[46]	2012	NC	R vs L vs O	R: 29 patients I , 7 patients II , 3 patients III; L: 55 patients I , 9 patients II , O: 198 patients I , 106 patients II , 282 patients III	R: 32 STG, 7 TG; L: 57 STG, 7 TG; O: 407 STG, 179 TG	689	39	64	586
Kim <i>et al</i> ^[47]	2012	NC	R vs L vs O	R: 3 patients O, 350 patients I , 51 patients II , 32 patients III; L: 8 patients O, 714 patients I , 96 patients II , 43 patients III, O: 28 patients O, 2376 patients I , 823 patients II , 1313 patients III	R: 327 DG, 109 TG, L: 703 DG, 158 TG; O: 3309 DG, 1232 TG	5839	436	861	4542
Pernazza <i>et al</i> ^[48]	2006	NC	R vs O	R: 2 patients O, 20 patients I , 12 patients II , 5 patients III, 6 patients IV	R: 21 DG, 24 TG	90	45	0	45
Caruso <i>et al</i> ^[49]	2011	NC	R vs O	R: 13 patients I , 9 patients II , 4 patients III, 3 patients IV, O: 57 patients I , 18 patients II , 33 patients III, 12 patients IV	R: 16 DG, 12 TG, 1 PG; O: 83 DG, 37 TG	149	29	0	120
Procopiuc <i>et al</i> ^[50]	2015	NC	R vs O	R: 9 patients II , 9 patients III, O: 15 patients II , 14 patients III	R: 7 DG, 10 TG, 1 PG; O: 6 DG, 23 TG	47	18		29

¹Data as reported by the authors from preoperative evaluation; ²Postoperatively obtained staging. CS: Clinical series; NC: Nonrandomized comparative study; R: Robotic surgery; L: Laparoscopic surgery; O: Open surgery; TG: Total gastrectomy; STG: Subtotal gastrectomy; DG: Distal gastrectomy; PG: Proximal gastrectomy; CT: Completion total gastrectomy; PPG: Pylorus-preserving gastrectomy; GC: Gastric cancer.

Table 2 Main operative outcomes in studies reporting use of robotic surgery for gastric cancer

Ref.	OP time (min)	Estimated blood loss (mL)	No. of harvested lymph nodes	Conversions
Patriti <i>et al</i> ^[21]	286	103	28.1	0
Lee <i>et al</i> ^[14]	253	135	46	0
D'Annibale <i>et al</i> ^[23]	267.5	30	28	0
Isogaki <i>et al</i> ^[24]	TG > DG 520 > 388	TG > DG 150 > 61.8	TG approximately equal DG 43 approximately equal 42	0
Kim <i>et al</i> ^[25]	234.7	46.4	42.4	
Liu <i>et al</i> ^[26]	272.52	80.78	23.1	1.8
Park <i>et al</i> ^[27]	248.8	146.1	37.9	3.5
Tokunaga <i>et al</i> ^[28]	331.5	32.5	40	0
Anderson <i>et al</i> ^[29]	420	300	24	0
Song <i>et al</i> ^[30]	231.3	128.2	36.7	0
Hur <i>et al</i> ^[31]	205			
Uyama <i>et al</i> ^[32]	361	51.8	44.3	0
Yu <i>et al</i> ^[33]	TG > DG 285 > 225	TG > DG 180 > 150	34.2	4.8
Jiang <i>et al</i> ^[34]	245	70	22.5	
Hyung <i>et al</i> ^[18]	Initial L > R > Recent L 337 > 253 > 164	-	Recent L > R > Initial L 37.8 > 34 > 29.2	0
Song <i>et al</i> ^[19]	Initial L > R > Recent L 289.5 > 230 > 134 ss	R > Recent L 94.8 > 39.5	Recent L > R > Initial L 42.7 > 35.3 > 31.5	0
Pugliese <i>et al</i> ^[35]	R > L 344 > 235 ss	L > R 148 > 90 ss	L > R 31 > 25	L > R 3 > 2
Woo <i>et al</i> ^[17]	R > L 219.5 > 170.7 ss	L > R 147.9 > 91.6 ss	R > L 39 > 37.4	0 = 0
Eom <i>et al</i> ^[36]	R > L 229.1 > 189.4 ss	R > L 152.8 > 88.3	L > R 33.4 > 30.2	
Park <i>et al</i> ^[37]	R > L 218 > 140 ss	R > L 75 > 60	R approximately equal L 34 approximately equal 35	0
Yoon <i>et al</i> ^[38]	R > L 305.8 > 210.2 ss		R > L 42.8 > 39.4	
Kang <i>et al</i> ^[39]	R > L 202 > 173 ss	L > R 173.4 > 93.2 ss		
Hyun <i>et al</i> ^[40]	R > L 234.4 > 220	R approximately equal L 131.3 approximately equal 130.4	R approximately equal L 32.8 approximately equal 32.6	0 = 0
Noshiro <i>et al</i> ^[41]	R > L 439 > 315 ss	L > R 115 > 96	R > L 44 > 40	R = L 0 = 0
Han <i>et al</i> ^[42]	R > L 258 > 193 ss		L > R 36.5 > 33.4	0
Junfeng <i>et al</i> ^[43]	R > L 234.8 > 221.3 ss	L > R 137.6 > 118.3 ss	R > L 34.6 > 32.7 ss	
Kim <i>et al</i> ^[44]	R > L 206.4 > 167.1 ss	L > R 134.9 > 59.8 ss	R approximately equal L 37.3 approximately equal 36.8	R = L 0 = 0
Kim <i>et al</i> ^[45]	R > L > O 259.2 > 203.9 > 126.7 ss	O > L > R 78.8 > 44.7 > 30.3 ss	O > R > L 43.3 > 41.1 > 37.4	0 = 0
Huang <i>et al</i> ^[46]	R > L > O 430 > 350 > 320	O > L > R 400 > 100 > 50 ss	O > R > L 34 > 32 > 26	
Kim <i>et al</i> ^[47]	R > L > O 226 > 176 > 158 ss	O > L > R 182 > 112 > 85	O > R > L 40.5 > 40.2 > 37.6 ss	
Pernazza <i>et al</i> ^[48]	R > O 293.8 > 224.6		R 34.2	
Caruso <i>et al</i> ^[49]	R > O 290 > 222	O > R 386.1 > 197.6	O > R 31.7 > 28	
Procopiuc <i>et al</i> ^[50]	R > O 320.83 > 243.36 ss	O > R 564.62 > 208.26 ss	O > R 25 > 22	0

R: Robotic surgery; L: Laparoscopic surgery; O: Open surgery; TG: Total gastrectomy; DG: Distal gastrectomy; ss: Statistically significant.

number of harvested lymph nodes was no different between open and robotic surgery.

In the experience of our group, the robotic platform is a versatile tool in the surgical approach of advanced gastric cancer. Our study^[50] enrolled 47 patients who were exclusively advanced gastric cancer patients and went on to receive either open ($n = 29$) or robotic ($n = 18$) surgery. Significantly longer mean operating times (320.83 min vs 243.36 min), but significantly lower blood loss (208.26 mL vs 546.62 mL) and shorter hospital stay (11.04 d vs 8.1 d) were obtained for the robotic group (Table 3). We found no difference in the number of retrieved lymph nodes or the rate of complications. After a mean follow up time of 31.66 mo for the open surgery group and a 24.72 for the robotic surgery group, the Kaplan-Meier analysis of the survival data revealed no statistically significant difference between the two cohorts ($P = 0.177$).

The authors consider that special emphasis needs to be placed on the long-term results of robotic surgery in advanced gastric cancer. The MAGIC trial^[51] published

in 2006 showed a survival benefit for gastric cancer patients receiving epirubicin, cisplatin and fluorouracil perioperatively when compared with patients treated with surgery alone. But the study also reported that 34% of the patients enrolled in the perioperative chemotherapy group, were unable to receive the regimen after surgery owing, among others, to postoperative complications. This creates a need for less invasive surgery like robotic surgery even in the treatment of the advanced gastric cancer patients. Patients would be thus enabled to receive the complete chemotherapy regimen, which would positively impact their survival prognosis^[51].

Another reason to investigate robotic surgery in the treatment of advanced gastric cancer would be the imperfect staging systems currently available. Studies report a considerable amount of patients staged as EGC perioperatively who turn out intraoperatively to suffer from advanced gastric cancer^[52,53]. Given these numbers Pugliese *et al*^[35] even proposed that all gastrectomies be performed including a D2 lymphadenectomy regardless

Table 3 Main postoperative outcomes in studies reporting use of robotic surgery for gastric cancer

Ref.	Time to first flatus (d)	Time to oral feeding (d)	Postoperative hospital stay (d)	Morbidity (%)	Mortality (%)	Follow up time (mo)
Patriti <i>et al</i> ^[21]			11.2	41.4	0	12.2
Lee <i>et al</i> ^[22]	2.4	4.6	6.6	8.3	0	
D'Annibale <i>et al</i> ^[23]		5	6	2	0	48
Isogaki <i>et al</i> ^[24]				4	1	
Kim <i>et al</i> ^[25]			6	0	0	
Liu <i>et al</i> ^[26]	2.5	4.1	6.2	11.8	0	
Park <i>et al</i> ^[27]			8	19	1	
Tokunaga <i>et al</i> ^[28]			8	22.22		
Anderson <i>et al</i> ^[29]		4 (2-8)	4 (3-9)	14.3		
Song <i>et al</i> ^[30]	2.9 ± 0.5	4.2	7.8	13	0	
Hur <i>et al</i> ^[31]						
Uyama <i>et al</i> ^[32]		3.56	12.1	8	0	11
Yu <i>et al</i> ^[33]	3.1	3.7		4.8	0	11
Jiang <i>et al</i> ^[34]			6.3	5	0	
Hyung <i>et al</i> ^[18]	Recent L > Initial L > R 3.3 > 3.1 > 2.9	Initial L > Recent L > R 4.8 > 4.3 > 4	Initial L > R = Recent L 6.9 > 6 = 6			
Song <i>et al</i> ^[19]	Recent L > Initial L = R 3.25 > 3 = 3	Initial L > Recent L > R 4.95 > 4.1 > 4	Initial L > Recent L > R 7.7 > 6.2 > 5.7	Recent L > Initial L = R 10 > 5 = 5		
Pugliese <i>et al</i> ^[35]			R = L 10 = 10 R > L 7.7 > 7 ss	L > R 12.5 > 6.2 L > R 13.7 > 11	R > L 6.2 > 2 R approximately equal L 0.3 approximately equal 0.4	53
Woo <i>et al</i> ^[17]						
Eom <i>et al</i> ^[36]	R = L 3.4 = 3.4		R approximately equal L 7.9 approximately equal 7.8	R > L 13 > 6		
Park <i>et al</i> ^[37]				R > L 17 > 7.5	R = L 0 = 0	
Yoon <i>et al</i> ^[38]	L > R 4.9 > 4.2		L > R 10.3 > 8.8	R > L 16.7 > 15.4		
Kang <i>et al</i> ^[39]			R > L 9.8 > 8.1 ss	R > L 14 > 10.3	R = L 0 = 0	
Hyun <i>et al</i> ^[40]			L > R 11.9 > 10.5	R > L 47.3 > 38.5	R = L 0 = 0	
Noshiro <i>et al</i> ^[41]			L > R 13 > 8 ss	L > R 10 > 9.5	0	
Han <i>et al</i> ^[42]		L > R 5 > 4.4	L > R 9.1 > 8.6	L > R 22.1 > 19.1	R = L 0 = 0	R > L 22.7 > 19.3
Junfeng <i>et al</i> ^[43]	L > R 3.3 > 3.1	L > R 4.1 > 3.9	L approximately equal R 7.9 approximately equal 7.8	R > L 5.8 > 4.3	R > L 32.2 > 30.1	L > R 19 > 15
Kim <i>et al</i> ^[44]			R > L 7.1 > 6.7	R > L 5.2 > 4.2	L > R 0.6 > 0	
Kim <i>et al</i> ^[45]	L > O > R 3.6 > 3.4 > 3.2		O > L > R 6.7 > 6.5 > 5.1 ss		R = L 0 = 0	
Huang <i>et al</i> ^[46]				L > R > O 15.6 > 15.4 > 14.7		
Kim <i>et al</i> ^[47]		O > L > R 5.7 > 4.7 > 4.4 ss	O > L > R 10.2 > 7.8 > 7.5 ss	O > R > L 10.7 > 10.1 > 9.34	0.4 ND	
Pernazza <i>et al</i> ^[48]				R > O 24.5 > 13.3	O > R 8.9 > 4.4	R = O 26 = 26
Caruso <i>et al</i> ^[49]			O > R 13.4 > 9.6	O > R 42.5 > 41.4	O > R 3.3 > 0	O > R 44 > 25
Procopiuc <i>et al</i> ^[50]			O > R 11.04 > 8.1 ss	O > R 27.58 > 22.22	O = R 0 = 0	O > R 31.6 > 24.7

R: Robotic surgery; L: Laparoscopic surgery; O: Open surgery; TG: Total gastrectomy; DG: Distal gastrectomy; ss: Statistically significant; ND: No statistical difference.

of the initial tumoral staging.

TECHNICAL ASPECTS

Combined resections

There has been a lack of studies specifically focused on the possible benefits of robotic multivisceral resections for advanced gastric cancer. Previous research by surgeons experienced in minimally invasive surgery suggests that the precision offered by the robotic platform might be of more use in large, technically-challenging procedures like multivisceral resections, rather than in cases requiring less complex surgery^[54,55].

Lymphadenectomy

To put forth robotic surgery as a viable surgical tech-

nique in gastric cancer treatment, its contribution to performing an extended lymphadenectomy needs to be made clear.

In laparoscopy, one of the major sources of intraoperative bleeding was shown to be lymph node dissection, especially when occurring around the large vessels^[56,57]. In our experience with the robotic platform owing to the elimination of physiologic tremor, the 3D steady view, and the 7 degrees of freedom of the EndoWrist® instruments lymph node dissection along the celiac trunk, the left gastric artery and the hepatic pedicle which are usually associated with increased bleeding, are now performed in a more precise and safe environment^[50].

The cohorts of Hyung^[18] and Song *et al*^[30] both included an initial and a recent laparoscopy group thus

allowing the assessment of the evolution of surgery parameters along the learning curve for this type of surgery and their comparison to the initial experience in robotic surgery. Although not statistically significant, recent laparoscopy showed the highest number of retrieved lymph nodes, with initial robotic cases coming second, in front of the initial laparoscopic cases. This comes to support the view that laparoscopy has a steeper learning curve than robotic surgery and that even inexperienced surgeons may obtain easily reproducible, high quality results faster with the robotic platform. This difference between the two techniques may not be important in the east, where experienced laparoscopic surgeons show no difficulties in quickly adjusting to the robotic platform, but it could bring a significant advantage to the western surgeons who simply cannot benefit from the same training in laparoscopy for gastric cancer due to the particular epidemiology of this disease.

The majority of the studies listed in Tables 1-3 show a higher number of retrieved lymph nodes for robotic procedures, which is an encouraging result given the extent of the preoperative under staging reported until now and the probable need to perform D2 lymphadenectomies for all patients until a reliable method for precise preoperative staging is introduced.

Digestive tract reconstruction

Key moments for the anastomosis are as follows: (1) closure of the duodenal stump; (2) closure of the stomach stump in subtotal gastrectomy or that of the esophageal stump in total gastrectomy; and (3) preparing the jejunum for the gastro-jejuno anastomosis or the eso-jejunoanastomosis. We generally opt for a Roux-en-Y anastomosis^[58].

The reconstruction solutions after total or subtotal gastrectomy can be grouped into two large categories. First, the extracorporeal anastomoses by the robot-assisted surgery require the performance of a minilaparotomy (smaller than 6 cm) through which the ends that need to be anastomosed are brought out and continuity of the digestive tract is reestablished, usually using circular stapler. This technique is not suitable for obese patients for whom the incision may need to be larger than 6 cm to perform the proximal resection and the purse-string suture on the esophageal stump.

To fully take advantage of the minimal invasiveness provided by the robotic platform, several techniques for intracorporeal anastomoses have been developed. They avoid the laparotomy and imply sectioning the esophagus under video control and then performing the anastomosis with a specific technique not requiring an abdominal incision. One option is using the OrViI™ device (Covidien, Mansfield, MA, United States). This consists of a foldable stapler anvil forming a 170° angle with the adjoining PVC tube. The OrViI™ device is introduced through the mouth and into the esophageal stump at which point the anvil is unfolded and connected to the circular stapler introduced abdominally. For this technique our team uses a 21 mm anvil followed by

a Roux-en-Y reconstruction with good postoperative results^[58]. Similar to this is the technique described by Hiki *et al.*^[59] in which the anvil of a circular stapler is attached to a nasogastric tube using sutures and then introduced trans-orally. Another technique was described by Inaba *et al.*^[60] and involves the creation of a side-to-side anastomosis using a linear stapler. Yet another option would be the manual sewing of the anastomosis, which we do not recommend, since it would prolong operating times unnecessarily, given the fact that the available mechanical devices are reliable alternatives.

The role of the assistant surgeon

In a study published by our team^[61], we assessed the role of the patient-side surgeon in robotic surgery. We found obvious benefits for the team when highly-trained assistants were involved in the procedure. Remarkable improvements were seen in handling the robot (docking and undocking times), the speed and precision in manipulating laparoscopic devices like the LigaSure or clip applier devices. Our data show that maintaining the same members of the team throughout more procedures and including assistants who undertook a structured, formal training program are more likely to warrant for fast and safe interventions.

OPEN QUESTIONS OF RESEARCH

An important problem remains regarding the selection of the appropriate technique for a given gastric cancer case. Thus far indications for robotic gastrectomy were: (1) a diagnosis of early gastric cancer without evidence of lymph node involvement; (2) T1 cancer with perigastric lymph node involvement; and (3) serosa-negative gastric cancer without lymph node metastasis. However, many of the patients were understaged preoperatively. This raises the need to study the outcomes of robotic surgery on large patient cohorts in randomized prospective studies not only for early gastric cancer, but also for tumors possibly requiring the D2 lymphadenectomy.

A recently published study surveying gastric cancer surgery techniques in United States academic medical centers^[62] shows that the number of robotic gastrectomies for gastric cancer has remained constant in 2011, 2012 and 2013. The study also mentioned that the robotic technique was utilized in the patients with the highest risk of mortality and severity of illness, in keeping with the fact that minimally invasive surgery has a lower impact on patient performance status and immune response mechanisms postoperatively^[62-64]. Therefore, extending the indications of robotic surgery to advanced gastric cancer is also a valid study point, especially in the West.

The option between endoscopic, laparoscopic, robotic or open surgery must be made based on well-established diagnostic criteria. This is not easy and one must take into account the caveats of evidence based

medicine and randomized controlled trials. The case of the results published by Bonenkamp *et al.*^[65] and Cuschieri *et al.*^[66,67] regarding the survival benefit of the D2 lymphadenectomy and the controversies thereafter have marked a decade of debate regarding the strategies for gastric cancer treatment. Carefully weighing the treatment options is especially important since there are more and more groups publishing acceptable results with the robotic technique.

CONCLUSION

Encouraging results are being published using the robotic technique, but the lack of homogenous study groups in terms of staging, comorbidities and adjuvant and neo-adjuvant therapies makes it hard to establish a clear indication for the robotic gastrectomy in gastric cancer.

Robotic surgery has proven to be safe and feasible thus far, but more convincing large volume prospective studies are needed to put it on the treatment list of early and advanced gastric cancer.

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2016 Pancreatic Cancer: Global view

MicroRNA in pancreatic ductal adenocarcinoma and its precursor lesions

Yasmin G Hernandez, Aimee L Lucas

Yasmin G Hernandez, Department of Internal Medicine, Icahn School of Medicine at Mount Sinai, New York, NY 10029, United States

Aimee L Lucas, Henry Janowitz Division of Gastroenterology, Icahn School of Medicine at Mount Sinai, New York, NY 10029, United States

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Correspondence to: Aimee L Lucas, MD, MS, Assistant Professor of Medicine, Henry Janowitz Division of Gastroenterology, Icahn School of Medicine at Mount Sinai, One Gustave Levy Place, Box #1069, New York, NY 10029, United States. aimee.lucas@mssm.edu
Telephone: +1-212-2410101
Fax: +1-646-5379616

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is the 4th

deadliest cancer in the United States, due to its aggressive nature, late detection, and resistance to chemotherapy. The majority of PDAC develops from 3 precursor lesions, pancreatic intraepithelial lesions (PanIN), intraductal papillary mucinous neoplasm (IPMN), and mucinous cystic neoplasm. Early detection and surgical resection can increase PDAC 5-year survival rate from 6% for Stage IV to 50% for Stage I. To date, there are no reliable biomarkers that can detect PDAC. MicroRNAs (miRNA) are small noncoding RNAs (18-25 nucleotides) that regulate gene expression by affecting translation of messenger RNA (mRNA). A large body of evidence suggests that miRNAs are dysregulated in various types of cancers. MiRNA has been profiled as a potential biomarker in pancreatic tumor tissue, blood, cyst fluid, stool, and saliva. Four miRNA biomarkers (miR-21, miR-155, miR-196, and miR-210) have been consistently dysregulated in PDAC. MiR-21, miR-155, and miR-196 have also been dysregulated in IPMN and PanIN lesions suggesting their use as early biomarkers of this disease. In this review, we explore current knowledge of miRNA sampling, miRNA dysregulation in PDAC and its precursor lesions, and advances that have been made in using miRNA as a biomarker for PDAC and its precursor lesions.

Key words: Pancreatic cancer; MicroRNA; Biomarkers; Pancreatic intraepithelial lesions; Intraductal papillary mucinous neoplasm

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Core tip: Reliable biomarkers are needed to detect pancreatic ductal adenocarcinoma (PDAC) early in order to decrease mortality. In this review, we discuss what the current knowledge is on microRNA (miRNA) in PDAC and its precursor lesions. MiR-21, miR-155, miR-196, miR-210 are dysregulated in tissue, serum, cyst fluid, and stool of PDAC patients. MiR-21, miR-155, and miR-196 are dysregulated in intraductal papillary

mucinous neoplasm and pancreatic intraepithelial lesions demonstrating that these miRNAs may serve as potential biomarkers for early stage lesions and cancer.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the 4th deadliest cancer in the United States, due to its aggressive nature, late detection, and resistance to chemotherapy^[1,2]. The majority of PDAC develops from 3 precursor lesions, pancreatic intraepithelial lesions (PanIN), intraductal papillary mucinous neoplasm (IPMN), and mucinous cystic neoplasm (MCN)^[3]. The cystic precursor lesions of the pancreas are detectable by certain imaging modalities such as Endoscopic ultrasound (EUS)^[4-6], Magnetic Resonance Imaging of the Abdomen with Cholangiopancreatography^[7], and computerized tomography scan^[8,9]. To date, there is no modality that clearly detects PanIN lesions, although studies have suggested a correlation between multifocal PanIN and lobular atrophy of the pancreas on EUS^[10].

Early detection and surgical resection can increase PDAC 5-year survival rate from 6% for Stage IV to 50% for Stage I^[11,12]. Detection and surgical removal of precursor lesions has the potential to be curative. Because of this, there has been much research focused on identification of individuals at high-risk of PDAC, detection of early stage lesions, and on the discovery of reliable biomarkers of this deadly disease. Carbohydrate antigen (CA) 19-9 is a poor biomarker of PDAC, as it is elevated in 30%-40% of benign diseases of the pancreas^[13,14] with a sensitivity of 79% (70%-90%) and specificity of 82% (68%-91%)^[15] for PDAC.

MicroRNA (miRNA) expression has been studied in tumor detection, cancer development and progression, and prognosis^[16]. MiRNAs are small noncoding RNAs (18-25 nucleotides) that regulate gene expression by affecting translation of messenger RNA (mRNA)^[16-18]. MiRNA function to stabilize mRNA transcripts *via* post-transcriptional gene silencing *via* inhibition of the translation process or cleavage of their target mRNAs^[16,19]. Over the last decade, the role of miRNA in cancer development and detection has evolved. MiRNA is very stable in tissue, plasma, stool, and other fluids and can be quantified in very small sample sizes, making it an excellent potential biomarker for the detection of PDAC. Current priorities include: (1) identification of miRNAs that are reliably dysregulated in PDAC; (2) determining which sample source(s) are easily accessible and have the highest yield for detecting these biomarkers; and (3) development of novel ways in which to use this

information to detect early onset PDAC and precursor lesions. Array-based analysis is used to evaluate the expression levels of thousands of miRNAs in various tissue types. Subsequent trials have validated these findings by performing quantitative real time PCR (QRT-PCR) and performed receiver operating characteristics (ROC) analysis to determine the sensitivity and specificity of these miRNAs as potential biomarkers for early or advanced disease. Some miRNAs, such as miR-21, miR-155, miR-196a, and miR-210 have stood out as potential biomarkers of this highly fatal disease^[20-24]. Our review is aimed at exploring current knowledge of miRNA sampling, miRNA dysregulation in PDAC and its precursor lesions, and advances that have been made in using miRNA as a biomarker for PDAC and its precursor lesions.

ROLE OF MIRNA IN PDAC DETECTION: SAMPLES FROM TISSUE, SERUM, PANCREATIC JUICE, STOOL AND SALIVA

Attention has been paid to circulating serological signatures, autoantibodies, epigenetic markers, circulating tumor cells (TCs), and miRNAs in order to detect PDAC at an earlier stage of disease^[25,26]. The use of miRNA for diagnosis and screening is still an evolving field; in the right patient population, an ideal miRNA test would be highly sensitive and specific, minimally-invasive and cost-effective. MiRNA expression in PDAC was first examined in PDAC tissue cells^[27]. Now miRNA has been found in serum, blood, whole plasma, stool, saliva, and cyst fluid (Table 1). Current knowledge is described below.

MiRNA in whole pancreas tissue or PDAC biopsies

Szafranska *et al.*^[27] performed the first analysis comparing miRNA expression in normal pancreas tissue, chronic pancreatitis (CP) tissue and PDAC tissue. On imaging, it can be challenging to distinguish CP from PDAC given the thick stroma and inflammation that may be found in both of these conditions. Furthermore, it is unclear if the aberrant expression of particular miRNAs is secondary to the desmoplastic reaction in CP and PDAC, and not related to tumorigenesis itself. They and others have found that miRNA-216 and miRNA-217 are significantly down-regulated in PDAC and miRNA-143, miR-145, miR-146a, miR-148a, miR-150, miR-155, miR-196a, miR-196b, miR-210, miR-222, miR-223, miR-31 are up-regulated in PDAC^[24,27-29]. However, this study also demonstrated that dysregulation of miRNA-196a, miR-196b, miR-203, miR-210, miR-222, miR-217, and miR-375 were found only in PDAC, whereas miRNA-29c, miR-96, miR-143, miR-145, miR-148b, and miR-150 were abnormally expressed in both CP and PDAC. This may suggest that the latter are responsible for causing the desmoplastic reaction as opposed to tumorigenesis.

Table 1 MiRNA in pancreatic ductal adenocarcinoma

MiRNA	Whole pancreas	Serum and plasma	Saliva	Stool	Pancreatic juice
miR-10b		↑[54]			
miR-16		↑[52]			
miR-18a		↑[29,56,57]			
miR-20a		↑[55,134]			↓[62]
miR-21	↑[22,24,27,32,34,38,55,71]	↑[55,71]		↑[63]↔[71]	↑[61,62,71]
miR-24		↑[55]			
miR-27a-3p		↑[134]			↑[62]
miR-29c	↓[27]				
miR-30a-3p	↓[27]				
miR-30c		↓[37]			
miR-31	↑[27]				
miR-34a		↑[37]			
miR-96	↓[27,135]				↓[62]
miR-99a		↑[52]			
miR-101	↑[32]				
miR-106b		↑[54]			
miR-130b	↓[27]				
miR-135b	↑[31]				
miR-139-3p		↓[37,58]			
miR-141	↓[27]				
miR-143	↑[27,71]			↓[71]	
miR-145	↑[27]				
miR-146a	↑[27]				↑[62]
miR-148a	↓[27,29]				
miR-148b	↓[27-29]				
miR-150	↑[27]				
miR-155	↑[22-24,27,32,66,71]	↑[22,54]		↑[63]↓[71]	↑[61,71]
miR-181a,b,d	↑[24]			↑[72]	
miR-185		↑[52,55,134]			
miR-191		↑[55]			
miR-192		↑[37,58]			
miR-194		↑[37]			
miR-196a	↑[22,23,27,49,52,71]	↑[21,22,52,71]		↑[72]↓[71]	↑[62,71]
miR-196b	↑[27]				↑[60]
miR-200a		↑[136]			↑[62]
miR-200b		↑[136]			
miR-205					
miR-210	↑[27,71,137,138]	↑[137]		↑[72], ↔ [71]	↑[60,71]
miR-212	↑[38]	↑[37]			
miR-216	↓[27,38]			↓[63]↓[71]	
miR-217	↓[27]				↓[62]
miR-222	↑[24,27,38]				
miR-223	↑[27]				
miR-375	↓[27,71]			↔[71]	
miR-492		↓[59]			↑[60]
miR-494	↓[27,74]				
miR-508-5p		↓[37]			
miR-513a-5p		↓[37]			
miR-602		↑[37]			
miR-630		↓[37]			
miR-663a		↓[59]			
miR-801		↑[37]			
miR-887		↓[37]			
miR-923		↓[37]			
miR-940			↑[74]	↑[74]	
miR-1290	↑[30]				
miR-1427					↑[60]
miR-3679-5p			↓[74]	↓[72,74]	

miR: MicroRNA; ↑: Up-regulated; ↓: Down-regulated; ↔: Unchanged.

MiR-1290 is elevated in early stage PDAC compared to normal controls^[30]. Additionally, miR-135b has been shown to be an effective biomarker for distinguishing PDAC from CP with high sensitivity and specificity^[31].

MiR-21, MiR-155, and miR-196 have been demonstrated by multiple groups to differentiate PDAC from non-cancerous lesions of the pancreas^[20-24,27,32]. Special attention has been placed on the role of miR-21 in

PDAC, as it has been implicated in tumorigenesis, TC invasion, the desmoplastic reaction, and metastasis of TC^[33-36]. Further studies did not demonstrate that miR-21 expression in stromal cells correlated with tumor stage.

MiR-192 has also been found to be present in pancreatic TC, but is seldom seen in stromal cells and not found in adjacent normal pancreas tissue^[37]. In this same study, miR-194 expression was detected in PDAC tissue, but not found in the surrounding normal pancreatic tissue. Unfortunately, despite these findings, no significant difference was found between the serum levels of miR-194 in patients with PDAC and healthy controls.

One proposed mechanism for PDAC development includes signaling between the molecular markers of the desmoplastic reaction and TCs^[38-41]. Liffers *et al.*^[29] demonstrated that miR-148a is down-regulated in microdissected PDAC tissue and when over-expressed prevents tumor growth. This suggests that miR-148a may have a crucial role in the molecular signaling by which tumorigenesis occurs. While it is important to find biomarkers that are deregulated in PDAC, it is also important to understand which miRNAs are involved in these aberrant signaling pathways.

MiRNA in serum and plasma of PDAC patients

MiRNAs are known to have organ-specific expression in many human cancers^[42,43]. Less than a decade ago, studies found that miRNA could reliably be detected in the serum in both animal models and humans^[44,45], and since that time, there has been much research dedicated to identifying which miRNAs have differential expression and the implications of these findings in the detection, staging, treatment, and prognosis of cancers^[46-50].

Attempts to use miRNA biomarkers in conjunction with CA19-9 have yielded mixed results. A study examining 847 different miRNAs in patients with PDAC found increased expression of miR-375 in PDAC as opposed to controls. MiR-375 did not improve detection nor predict prognosis in patients with PDAC when compared to CA19-9 alone^[51]. Liu *et al.*^[52] found that using serum miR-16 and miR-196a in combination with CA19-9 increased detection of PDAC and Stage I lesions when compared to either modality alone, which suggests that miR-16 and miR-196a may be deregulated early in PDAC. These biomarkers were also up-regulated in studies performed on pancreas tissue, demonstrating that miR-16 and miR-196a can be used as peripheral biomarkers of PDAC. Gao, *et al.*^[53] also demonstrated that miR-16, when combined with CA19-9, served as a potential biomarker for detection of PDAC when compared to patients with CP.

One limitation of CA19-9 as a biomarker is that it is elevated in a large portion of patients with benign pancreatic diseases. Because of this limitation, studies have evaluated the miRNA expression of patients with PDAC compared to those with benign diseases such as CP or choledocolithiasis. They found that miR-10b,

miR-155, and miR-106b were consistently elevated in the serum of patients with PDAC but not in those with benign pancreatic disease^[54]. Liu *et al.*^[55] have demonstrated that up-regulation of miR-20a, miR-21, miR-24, miR-25, miR-99a, miR-185, and miR-191 can be used to distinguish PDAC from healthy controls and CP. Additionally, miR-135b has been shown to be an effective biomarker for distinguishing PDAC from CP with high sensitivity and specificity^[31]. MiR-18a levels have also been shown to have increased expression in patients with PDAC and interestingly decrease after surgical resection suggesting that miR-18a levels may be a good marker to not only detect disease but also to monitor disease recurrence^[29,56,57]. Zhang *et al.*^[37] also demonstrated that miR-194, miR-192, miR-602, miR-801, miR-212, miR-34a are up-regulated in PDAC, while miR-923, miR-139-3p, miR-513a-5p, miR-630, miR-30c-1, miR-887, miR-508-5p, and miR-139a-5p were down-regulated in PDAC specimens^[37,58]. From these data, they demonstrated that miR-192 is neither present in the stromal cells of the pancreas nor the serum, but it is up-regulated in PDAC TCs and is involved in cell proliferation of PANC-1 TC lines *in vitro*^[58]. Lin *et al.*^[59] performed microarray on 1711 serum miRNAs and found that 23 were down-regulated and 22 were up-regulated in the serum of PDAC patients when compared to normal controls. Of these, miR-492 and miR-663a were found to have decreased expression that was statistically significant in PDAC; however, only miR-663a was found to have a positive correlation with stage of disease^[59]. Further studies are needed to determine which miRNAs will be clinically relevant.

Pancreatic juice miRNA

Pancreatic juice sampling requires an invasive endoscopic procedure, but studying the miRNA concentrations of patients with PDAC, benign pancreatic lesions, and healthy controls can shed light on potential biomarkers for detecting disease as they are found in high concentration in cyst fluid. As EUS and endoscopic retrograde cholangiopancreatogram (ERCP) are two methods by which pancreatic masses are frequently detected and sampled, these specimens could be sent for miRNA analysis in order to determine the malignant potential of these lesions. Wang *et al.*^[60] performed microarray of 49 miRNAs on secretin-stimulated pancreatic juice of a group of patients with PDAC, CP, and normal controls. They demonstrated that miR-205, miR-210, miR-492, and miR-1427 are all significantly elevated in PDAC when compared to controls; however, this statistical significance does not exist when compared to patients with CP^[60]. Additionally, by using ROC curves, they determined that combining these 4 miRNAs with serum CA19-9 the sensitivity and specificity of PDAC detection is 91% and 100% respectively, though this analysis was limited by a sample size of 6. Other groups have evaluated the pancreatic juice of patients with PDAC pre-operatively *via* ERCP and from post-operative specimens^[61,62]. Sadakari *et al.*^[61] analyzed the expression

of miR-155 and miR-21 in pancreatic juice sampled *via* ERCP and found that these miRNAs were significantly elevated when compared to patients with CP and healthy controls, though the levels did not correlate with pancreatic juice cytology^[61]. Again these findings are consistent with those from pancreatic tissue and serum. Hong *et al.*^[62] evaluated 158 miRNAs in post-operative fine needle aspiration specimens and found by qRT-PCR that miR-21, miR-27a, miR-146a, and miR-186a were significantly over-expressed in PDAC tissue and miR-217, miR-20a, and miR-96 were significantly down-regulated in PDAC tissue when compared to normal controls^[62]. These two studies have demonstrated the feasibility of detecting miRNA from pancreatic juice, thus indicating the potential for using pancreatic juice biomarkers to detect early lesions given the higher concentration of miRNA in this fluid sample.

miRNA in stool specimens

Frozen stool specimens may serve as potential non-invasive biomarker samples for PDAC. Over-expressed miRNAs from gastrointestinal cancers are shed from the exfoliative cells of the gastrointestinal tract. Intraluminal release of pancreatic juice also allows for detection of miRNAs in the stool^[63-69]. Previous studies have largely focused on genetic markers of tumorigenesis and not miRNA^[70]. Yang *et al.*^[63] performed a feasibility study of using stool miRNAs as a potential screening tool for detection of PDAC. They evaluated expression of 5 miRNAs that had been previously shown to be over-expressed in PDAC and found that miRNA-21 and miR-155 were over-expressed and miR-216 was under-expressed in all PDAC stool specimens when compared to normal controls and CP patients. These findings are consistent with what has been found in whole pancreas, pancreatic cyst fluid, and serum specimens. Additionally, with ROC analysis they demonstrated that combining miR-21 and miR-155 in stool samples there was a sensitivity of 93.33% and specificity of 66.67%. When they combined all 3 miRNAs (miR-21, miR-155, and miR-216), the sensitivity and specificity were 83.33% each. Link *et al.*^[71] selected 7 miRNAs (miR-21, miR-210, miR-143, miR-155, miR-196a, miR-216a, miR-375) and determined that like Yang's group miR-216a was found in lower concentrations in the stool of patients with PDAC. However, unlike Yang's group, they found that miR-155 was down-regulated in this population and miR-21 was unchanged in the stool of controls compared to CP or PDAC^[71].

Ren *et al.*^[72] also evaluated the expression of miR-21, miR-155, miR-181a, miR-196a, and miR-210 and found that miR-181b, miR-196a, and miR-210 were significantly over-expressed in PDAC patients when compared to controls, but only miR-181b and miR-210 were elevated in CP patients, though these elevations were not significant. Ren *et al.*^[72] established a positive correlation between miR-196a levels and tumor size, which had not been previously described in studies of

the serum or stool. Overall, while studies of fecal miRNA have demonstrated feasibility, conflicting data have emerged on which miRNAs are differentially expressed in the stool of PDAC, benign pancreatic disease, and normal controls.

Salivary miRNA

The field of salivonomics has been developing since blood molecules have been found in saliva^[73]. As with stool miRNA, salivary miRNA may serve as a non-invasive biomarker for PDAC. Xie *et al.*^[74] is the only group to have evaluated salivary miRNA in PDAC. They conducted a microarray of 2006 miRNAs and noted that 10, including miR-4433-5p, miR-4665-3p, miR-940, miR-1273g-3p, miR-3676-5p, miR-3679-5p, miR-3940-5p, miR-4327, miR-4442, and miR-5100, were up-regulated or down-regulated in salivary samples. Of these, only miR-940 was significantly up-regulated and miR-3679-5p was significantly down-regulated in the PDAC specimens during the validation phase of the study. Until now, neither has been implicated in PDAC in the serum, stool, whole pancreas, or pancreatic juice. More studies are needed in this area.

ROLE OF MIRNA IN DETECTION OF PRECURSOR LESIONS

The absence of symptoms in early disease makes PDAC a cancer that is detected at very late stages when mortality approaches 100%. Much research has been dedicated to detecting miRNA in patients with PDAC as a novel biomarker for the presence of disease. Given the aggressive nature of PDAC, detection of precursor lesions with malignant potential would be critical to increasing the survival of these patients. PanIN lesions are microscopic PDAC precursor lesions that are graded 1-3 and are categorized based on the level of architectural and cytological atypia that is present^[75,76]. Grade 1a is early intraepithelial proliferative lesions that have flat architecture, while grade 1b lesions have papillary architecture. PanIN-2 lesions have moderate abnormalities and PanIN-3 lesions have severe abnormalities, though none of these lesions invade the basement membrane^[75,76]. IPMN lesions are mucin-producing cystic tumors, which arise from the epithelium of the pancreatic ducts and have the potential for malignant transformation^[77,78]. They are categorized by main duct type (MD) or branch duct type (BD) and histologically are classified as having low-, intermediate-, and high-grade dysplasia^[3]. Their malignant potential differs based on their location within the pancreatic ducts, and MD-IPMN carry a 44%-48% risk compared to BD-IPMNs, which only carry a 11%-17% risk of malignant transformation^[79-82]. MCN are also mucin-producing epithelial neoplasms with ovarian-type stroma occur primarily in middle-aged females and are located in the body and tail of the pancreas and carry a 12% chance of tumor progression^[83-86]. Cystic fluid is analyzed

Table 2 MiRNA in precursor lesions

MiRNA	IPMN	PanIN-1	PanIN-2	PanIN-3
miR-10b			↑[101]	↑[101]
miR-21	↑[23,32,87,88]	↑[100,101]	↑[98,99]	↑[98,99]
miR-92a	↑[93]			
miR-99a	↓[91,93]			
miR-99b	↓[91]			
miR-100	↓[91,93]			
miR-101	↓[32]			
miR-125b	↑[93]			
miR-126	↓[91]			
miR-130a	↓[91]			
miR-145	↑[93]			
miR-148			↓[101]	↓[101]
miR-155	↑[23,32]	↑[101]	↑[98]	↑[98]
miR-182		↑[101]		
miR-196a	↑[90]		↑[97]	↑[97]
miR-196b			↑[97]	↑[97]
miR-200a		↑[101]		
miR-200b		↑[101]		
miR-212	↓[93]			
miR-217			↓[101]	↓[101]
miR-221	↑[87]			
miR-296-5p		↑[101]		
miR-342-3p	↓[91]			
miR-483-3p	↑[88,93]			

miR: MicroRNA; ↑: Up-regulated; ↓: Down-regulated; PanIN: Pancreatic intraepithelial lesions; IPMN: Intraductal papillary mucinous neoplasm.

for CEA and amylase as other tumor markers have not demonstrated reliability in detecting malignant lesions.

As cystic neoplasms of the pancreas carry the risk of malignant transformation, determining a way to accurately predict which will progress to invasive carcinomas may guide surgical management and treatment decisions. MiRNA has been examined in PanIN lesions and IPMN as a potential candidate for early detection and the likelihood of progression to cancer. Understanding which miRNAs become deregulated early in the disease process may lead to advances for treatment decisions (Table 2).

MiRNA in IPMN lesions

Given the increased use of abdominal imaging, more pancreatic cystic lesions are being detected. There are guidelines in place to help guide management based on cystic characteristics that are consistent with malignancy^[77]. The first study looking at miRNA expression levels in precursor lesions of the pancreas was performed by Habbe *et al.*^[23] who determined that miR-155 and miR-21 were over-expressed in the IPMN neoplastic epithelium, specifically those with carcinoma-in-situ^[23,87]. MiR-155 was also significantly elevated in the pancreatic juice of these patients. While the levels of up-regulation of miRNA-21 and miR-155 correlated with the degree of cellular atypia found in the IPMN lesions, the study lacked long-term outcome data, highlighting the need for large and more longitudinal studies. Caponi *et al.*^[32] established a relationship between expression of miR-21 in invasive IPMNs and

clinical outcome and observed that higher levels of miR-21 were correlated with worse overall and disease-free survival. Furthermore, they demonstrated that miR-155 and miR-21 had higher expression levels in invasive IPMN lesions when compared to non-invasive lesions, suggesting that these miRNAs may serve as early markers of malignant transformation^[32,88]. MiR-101 has been shown to be down-regulated in invasive IPMNs when compared to non-invasive IPMNs and normal tissue. This deregulation of MiR-101 has not been described in PDAC samples, which may indicate that miR-101 plays a role in tumor invasion^[32,89]. As with studies of miRNA in the serum and tissue of PDAC patients, miR-196a was found to be up-regulated in the pancreatic juice of intestinal-type IPMN^[90]. In a recent study miR-100, miR-99b, miR-99a, miR-342-3p, miR-126, miR-130a were found to be down-regulated were up-regulated in high-risk vs low-risk IPMN lesions^[91]. Furthermore, low miR-99b in IPMN fluid was associated with MD involvement, which is associated with a greater risk for transformation into a malignant neoplasm. Abue *et al.*^[88] found that miR-483-3p was up-regulated in PDAC cells and plasma when compared to IPMN lesions and may also serve as a useful biomarker in differentiating IPMN lesions with malignant potential from normal tissue and PDAC. The down-regulated miRNAs correlated with high-risk IPMNs and may be involved in cyst invasion and progression. Lee *et al.*^[92] found that miRNA expression varied amongst pancreatic cystic neoplasm. Specifically, miR-31-5p, miR-4830-5p, miR-99a-5p, and miR-375 were characteristic of serous cyst adenomas (SCA), whereas miR-10-5p, miR-202-3p, miR-210, and miR-375 differentiated MCN from SCA, IPMN, and PDAC^[92]. It is unclear why this overlap in miR-375 occurs^[92]. Henry *et al.*^[93] found that miR-92a, miR-99a, miR-100, miR-125b, miR-145, miR-212 and miR-483 were differentially expressed between benign and pre-malignant and malignant lesions of the pancreas and they suggested that a high amount of RNA present in the cystic fluid may suggest the presence of malignant transformation^[93]. As previously described miR-21, miR-155, miR-196a have been implicated in both PDAC and IPMN and given these widely replicated results, studies aimed at detecting these biomarkers in serum, saliva, and stool could help to determine, in a non-invasive way, if they are increased in pre-malignant lesions.

MiRNA in panin lesions

Currently, PanIN lesions are found in the neighboring pancreatic tissue of patients with PDAC; however, there is no consistent way to detect the presence of these lesions^[10,94-96]. Identifying a biomarker to detect PanIN lesions may be critical in early detection of PDAC. Slater *et al.*^[97] demonstrated that miRNA-196a and miR-196b were elevated in PDAC and PanIN-2/3 lesions in both animal models of PDAC and humans with PDAC. Ryu *et al.*^[98] demonstrated that miR-155

is up-regulated in PanIN-2 and PanIN-3 lesions when compared to neighboring healthy pancreatic tissue, but not in PanIN-1 lesions. Furthermore, miR-21 has been shown to be over-expressed in PanIN-2^[98,99] and PanIN-3^[98,99], but not PanIN-1 lesions suggesting that this is a marker for later disease. These are significant findings as miR-155 and miR-21 have been shown to be up-regulated in IPMN lesions and PDAC suggesting that they are early markers for cells with malignant potential. Yu *et al.*^[100] found that miR-196b was up-regulated in PanIN-3 lesions, which correlates with previous studies that have found that miR-196b is up-regulated in PDAC lesions^[100,101]. Importantly, miR-21, miR-155, miR200a, miR-200b, miR-182, and miR-296-5p were deregulated as early as PanIN-1 lesions and remained deregulated until progression to PDAC, with the exception of miR-200c that normalized in PanIN-3 lesions. A recent publication describing miRNA expression in PanIN lesions found that miRNA-148a and miR-217 were down-regulated while miR-10b was up-regulated in PanIN-2 and PanIN-3 lesions^[101]. While miR-21 has been shown repeatedly to be over-expressed in PDAC, there are conflicting studies on its deregulation in early PanIN lesions suggesting that it may represent a later and more aggressive dysregulation in the progression to PDAC. A non-invasive method to detect advanced PanIN lesions would represent a significant advance in the field.

DISCUSSION

While PDAC is the fourth most common cause of cancer-related deaths, there is still no reliable way to detect early disease and patients present with late-stage disease with a nearly 100% mortality. Research in the field of biomarkers shows a great deal of promise as current research aims to understand the molecular mechanisms and stromal microenvironment of this deadly tumor. MiRNA are small nucleotides that control the genetic expression in all cells and importantly in an organ-specific manner. Abberant miRNA expression has been identified in various cancers^[102-106], and factors such as transcriptional deregulation, epigenetic alterations, mutations, DNA copy number abnormalities, and defects in the miRNA biogenesis pathway may account for these differences in expression^[107,108]. C-myc and p53 are two transcriptional factors that have been associated transcriptional deregulation of miRNA^[109-111]. Epigenetic regulation of miRNAs by DNA methylation and histone tail modification play a role in miRNA expression through chromatin remodeling^[105,112-114]. Both germ-line and somatic mutations are responsible for miRNA expression levels in various types of cancers^[115-117]. It has been described by Calin *et al.*^[118], that miRNAs are located a fragile sites on the chromosome, minimal regions of loss of heterozygosity, minimal regions of amplifications, and common breakpoints, thus increasing the risk for DNA copy abnormalities. DNA copy abnormalities have been found in melanoma, breast cancer, ovarian cancer,

leukemia, colorectal cancer^[119-122]. Lastly, defects in miRNA biogenesis pathway may contribute to varying expression levels and cancer phenotype as miRNA undergoes complex processing intracellularly prior to reaching its mature form^[123-127]. In addition to the aforementioned mechanisms, dietary components, such as folate, retinoids, curcumin, and Vitamin D have been implicated in the modulation of miRNA expression^[128-130]. Some miRNAs have been shown to increase muscle loss in cancer cachexia and specifically, increased miR-21 levels have been shown to increase muscle breakdown in pancreatic cancer^[131,132]. Deeper understanding of the regulatory mechanisms of miRNA expression will hopefully give new insight to the factors responsible for miRNA deregulation and lead to miRNA-based diagnostic testing and miRNA-directed therapy for PDAC.

Some limitations that exist with the current miRNA research at this time include standardization of extraction, reproducibility of testing, diagnostic yield in the various sample methods, and small sample sizes. Additionally, despite finding biomarkers for this disease, there is limited evidence that miRNA will impact PDAC-related mortality. Dysregulation of miRNA affects the cell cycle, proliferation, apoptosis, epigenetics, oncogenesis, tumor differentiation, tumor invasion, tumor metastasis and migration, prognosis, and chemoresistance in numerous cancers^[133]. Increased efforts to understand the biological function of miRNA expression and its effects on cancer development are needed.

Despite these limitations, great advances have been made in this field and now miRNA expression is being analyzed not just in pancreatic tissue and cystic fluid, but also in stool, saliva, and serum; which would lead to non-invasive ways by which to analyze the expression levels of miRNA in patients at high risk. There have been great efforts to identify which of the greater than 2000 miRNAs are deregulated in PDAC and its precursor lesions and miRNA-21, miR-155, and miR-196b seem to be dysregulated in both early lesions and advanced cancer and show promise as potential screening tools in the future.

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Antitumor effects of the benzophenanthridine alkaloid sanguinarine: Evidence and perspectives

Roberta Gaziano, Gabriella Moroni, Cristina Buè, Martino Tony Miele, Paola Sinibaldi-Vallebona, Francesca Pica

Roberta Gaziano, Gabriella Moroni, Cristina Buè, Martino Tony Miele, Paola Sinibaldi-Vallebona, Francesca Pica, Department of Experimental Medicine and Surgery, University of Rome Tor Vergata, 00133 Rome, Italy

Author contributions: Gaziano R, Moroni G, Buè C, Miele MT, Sinibaldi-Vallebona P and Pica F contributed to this paper.

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Correspondence to: Francesca Pica, MD, PhD, Department of Experimental Medicine and Surgery, University of Rome Tor Vergata, Via Montpellier, 1, 00133 Rome, Italy. pica@uniroma2.it
Telephone: +39-6-72596462
Fax: +39-6-72596550

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Abstract

Historically, natural products have represented a significant source of anticancer agents, with plant-derived drugs becoming increasingly explored. In particular, sanguinarine is a benzophenanthridine alkaloid obtained

from the root of *Sanguinaria canadensis*, and from other poppy *Fumaria* species, with recognized anti-microbial, anti-oxidant and anti-inflammatory properties. Recently, increasing evidence that sanguinarine exhibits anticancer potential through its capability of inducing apoptosis and/or antiproliferative effects on tumor cells, has been proved. Moreover, its antitumor seems to be due not only to its pro-apoptotic and inhibitory effects on tumor growth, but also to its antiangiogenic and anti-invasive properties. Although the precise mechanisms underlying the antitumor activity of this compound remain not fully understood, in this review we will focus on the most recent findings about the cellular and molecular pathways affected by sanguinarine, together with the rationale of its potential application in clinic. The complex of data currently available suggest the potential application of sanguinarine as an adjuvant in the therapy of cancer, but further pre-clinical studies are needed before such an antitumor strategy can be effectively translated in the clinical practice.

Key words: Sanguinarine; Cancer; Apoptosis; Cell-cycle; Chemotherapy

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Core tip: Sanguinarine is a benzophenanthridine alkaloid isolated from the root of *Sanguinaria canadensis*, and other poppy *Fumaria* species, which exhibits a clear-cut anticancer potential by inducing apoptosis and/or antiproliferative effects on tumor cells. Sanguinarine also shows antiangiogenic and anti-invasive properties, as demonstrated *in vitro* and *in vivo*. In consideration of the multiple biological effects of sanguinarine, which suggest its possible use in cancer therapy, further detailed pharmacokinetic and toxicologic studies are required to assess both the efficacy and safety of the compound before proposing a possible translation into the clinic.

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INTRODUCTION

Tumor initiation is the result of multiple genetic and epigenetic events. Transformed cells are characterized by indefinite proliferation, apoptosis-resistance and the capability to metastasize and support angiogenesis^[1].

Chemotherapy, irradiation and/or immunotherapy represent the gold standard approach for the treatment of cancer worldwide. The increased frequency of tumor relapse and the toxicity of the anticancer drugs, however, often reduce the therapeutical effectiveness of several antitumor therapy protocols. Therefore, the identification of more effective therapeutic protocols is needed and, in this direction, phytochemicals may represent an attractive alternative because of their low toxicity and low cost^[2]. In this scenario, sanguinarine (Figure 1) and chelerythrine are the principal members of quaternary benzo[c]phenanthridine alkaloids (QBAs)^[3] obtained from *Sanguinaria canadensis*, *Chelidonium majus*, and *Macleaya cordata*. Alkaloids include a large group of secondary metabolites (SMs) that differ in relation to structure, function and biodistribution^[4]. In the past, QBAs have attracted the attention of many pharmacologists because of their own low toxicity^[5,6] and their multiple biological activities, such as the antitumor^[7], antimicrobial^[8,9], anti-inflammatory^[10], anti-HIV^[11], anti-platelet^[12], anti-angiogenesis^[13], and antiparasitic activities^[14-16]. The influence of QBAs on the activity of various important biological enzymatic pathways has been also demonstrated^[7]. For long times, sanguinarine-containing herbs were believed to possess anticancer activity but only recently evidence that sanguinarine possesses a strong anti-neoplastic activity, which is mediated mainly by the induction of tumor cell apoptosis has been proved.

This review summarizes the most recent findings on the molecular mechanisms underlying the antitumor activity of sanguinarine both *in vitro*, in a variety of human tumor cells, and *in vivo* in selected experimental tumor models, together with the rationale of its potential application in clinical practice.

SANGUINARINE INDUCES APOPTOSIS IN TUMOR CELLS

Physiologically, the human body controls homeostasis by eliminating damaged and aged cells by means of a genetically programmed process named apoptosis^[17,18]. Tumor cells evade apoptosis and grow indefinitely. Several proteins, among which are caspases, pro-

apoptotic Bax and anti-apoptotic B cell lymphoma (Bcl)-2, cytochrome c, and apoptotic protease activating factor -1, carry out the apoptotic programme either by intrinsic or extrinsic pathways. The first one is dependent on mitochondria, whereas the second one is initiated by the so-called death receptors (DRs). Selected anti-apoptotic proteins, among which Bcl-2, have been found over-expressed in different types of cancers. The down-regulation of anti-apoptotic proteins in cancer cells represents a promising therapeutic strategy of intervention in cancer therapy.

A number of plant-derived agents, have been shown to be capable of hampering disease progression by inducing cell apoptosis in multiple types of human and experimental cancers. Recently QBAs, and particularly sanguinarine, have been indicated as potential anti-cancer compounds. In detail, it has been reported that micromolar concentrations of sanguinarine are capable of inhibiting tumor cell growth, and this inhibitory effect is associated with cell cycle arrest and induction of apoptosis^[19-22]. The anti-proliferative and/or pro-apoptotic activities of sanguinarine have been demonstrated in *in vitro* studies on several cancer cell types including epidermal^[23], keratinocyte^[24,25], prostate^[26-28], cervical^[29], breast^[20,30-33], leukaemia^[34,35], lymphoma^[36], melanoma^[37-39], colon^[40,41], colorectal^[21], gastric^[42], pancreatic^[19], lung^[22], neuroendocrine^[43], osteosarcoma^[44], and human neuroblastoma cells^[45]. By contrast, there are few studies on the *in vivo* effectiveness of sanguinarine administration *per os*^[46,47] in animal tumor models^[33,48].

It has been reported that sanguinarine exerts an antiproliferative activity on murine melanoma cells both *in vitro* and *in vivo* (B16 melanoma 4AS in the syngeneic host C57BL/mice), as well as in A375 human melanoma xenografts in athymic nude mice^[48]. We also have conducted a study aimed at evaluating the anti-tumor effect of sanguinarine both *in vitro* and *in vivo* in a rat colorectal cancer model (DHD/K12/TRb cell line)^[49]. We found that the *in vitro* addition of sanguinarine has a dose-dependent inhibitory effect on the proliferation of DHD/K12/TRb cells and induces tumor cell apoptosis. Sanguinarine also showed a clear-cut *in vivo* anti-tumor activity, leading to an inhibition of tumor growth higher than 70%^[49]. The sanguinarine-induced inhibition of tumor growth was associated with its pro-apoptotic effect on tumor cells, as confirmed by the *ex-vivo* histopathological examinations performed on experimental tumor sections and by TUNEL assay^[49].

It is known that sanguinarine-induced apoptosis occurs through multiple pathways, including the activation of nuclear factor- κ B (NF- κ B)^[50], the mitochondrial damage resulting in activation of the caspase machinery^[24] and the cell cycle arrest^[27]. In detail, the sanguinarine-induced apoptosis occur either *via* a mitochondrial pathway dependent on caspase-9 or by the DR pathways, with the activation of caspase 8. The activation of caspase 3, which represents a key factor for apoptosis execution in both pathways, and the following

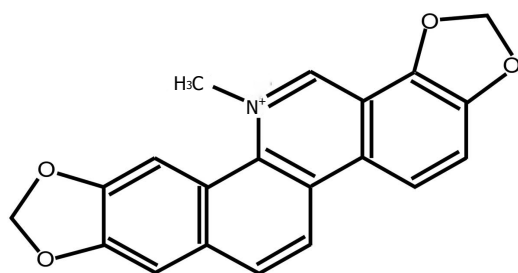


Figure 1 Chemical structure of sanguinarine.

cleavage of PARP together with the down-regulation of Bcl-2 and c-FLIP, may play a very important role in the apoptosis induced by sanguinarine^[26,51,52]. Studies performed in human neuroblastoma cells SH-SY5Y have shown that sanguinarine reduces the expression of anti-apoptotic genes, particularly of NOL3, BCL2, and HRK genes^[45]. A down-regulation of pro-caspase 3, Bcl-2, cIAP2, XIAP, and c-FLIPs^[20,52] has been also observed in basal cell-like MDA-MB-231 human breast carcinoma cells treated with sanguinarine. The effect of sanguinarine treatment has been evaluated also on the expression levels of Bax and Bcl-2 proteins in immortalized human keratinocytes (HaCaT)^[24,25], human leukaemia JM1 and K562 cells^[35] and in HeLa and SiHa human cervical tumor cells^[53]. These findings indicate that sanguinarine, depending on the dose employed, down-regulates the expression levels of Bcl-2 protein while increasing those of Bax protein, which is a key regulator of mitochondrial damage. Notably, Bax expression has been associated with an increased sensitivity of cancer cells to chemotherapy^[54], whereas an increase of Bcl-2 has been associated with the occurrence of drug-resistance phenomena^[55].

It has been proved that sanguinarine is capable of inducing DNA damage, acting as an intercalating agent^[56,57], and also a very rapid cell apoptosis which does not seem to be mediated by a p53-dependent DNA damage signalling in human colon cancer^[41] and in malignant melanoma cells^[38].

The concentration of sanguinarine plays a key role in the induction of cell death. Consistently, both apoptotic and non-apoptotic cell death pathways have been observed in response to sanguinarine. Thus, a sanguinarine-related and bimodal cell death effect, which consists of two different types of cell death, *i.e.*, by apoptosis (induced by low SA concentration; characterized by caspase 3 and PARP positivity) and oncosis (induced by high SA concentration; characterized by caspase 3 and PARP negativity), has been demonstrated in various cancer cells types^[52].

SANGUINARINE INDUCES ALTERATIONS IN CELL CYCLE

Tumor cells are characterized by deregulated proliferation. Conversely, normal cells proliferation is the

results of the action of selected growth signals [cyclins and cyclin-dependent kinases (CDKs)] and anti-growth signals (p21 and p27 proteins). Cyclins and CDKs cooperate in G1 for the initiation of the S phase and in G2 for inducing mitosis, whereas p21 and p27 selectively block the catalytic activity of CDK. Following addition of anti-mitogenic compounds or DNA injury, p21 and p27 bind to cyclin-CDK complex blocking their catalytic activity and consequently the cell cycle progression.

Actually a number of inhibitors and/or regulators of the cell cycle, among which sanguinarine, are suggested as potential antitumor agents. Sanguinarine treatment (0.2-2 mol/L for 24 h) blocks cell cycle by enhancing the expression of CDK inhibitors and by reducing not only cyclin D1, D2 and E, but also CDK2, 4 and 6 in human prostate cancer cells^[27]. This alkaloid also up-regulates p27 and down-regulates cyclin D1, while inhibiting the activation of STAT3, as demonstrated *in vitro* in basal cell-like MDA-MB-231 human breast cancer cells and *in vivo* in a murine breast cancer model^[33]. Holy *et al.*^[31] studied the effects of sanguinarine (5-10 µmol/L) on the cell cycle regulatory molecules, by immunocytochemistry, that visualized the cyclin D1 and topoisomerase II in MCF-7 breast cancer cells. They reported that sanguinarine-mediated cellular events induce cell cycle arrest in G0/G1 and inhibit cell proliferation, which is associated with a striking re-localization of cyclin D1 and topoisomerase II from the nucleus to the cytoplasm.

SANGUINARINE-INDUCED APOPTOSIS THROUGH THE GENERATION OF REACTIVE OXYGEN SPECIES

Apoptosis induced by sanguinarine has been associated also with the production of reactive oxygen species (ROS)^[20,36,52,58]. ROS are a group of highly reactive molecules, among which are superoxide anion radical, hydrogen peroxide, singlet oxygen, and hydroxyl radical. ROS are the products of the oxygen metabolism within the cell. ROS are known as key regulators of normal cell proliferation and differentiation, however, high levels of ROS have also been associated with damage of DNA and proteins and thus with the occurrence of apoptosis^[59,60]. Moreover, an overdone oxidative stress has been shown capable of inducing a reduction of the normal mitochondrial membrane potential, which in turn leads to apoptosis^[21,61-63]. It has been shown that ROS generation, is crucial for the apoptosis induced by sanguinarine in human breast cancer^[52], SK-Mel-2 human melanoma^[37], human prostate cancer^[25] and in both HCT-116^[21] and HT-29 human colon cancer cells^[40]. Consistently, pre-treatment of tumor cells with antioxidants such as *N-acetylcysteine* or glutathione counteracts the apoptosis induced by sanguinarine^[21,32,37,52]. Moreover, the over-expression of cyclooxygenase-2 (COX-2) also rescues prostate cancer cells from sanguinarine-induced apoptosis by

inhibiting the activity of NO synthase, thus suggesting the possibility to use a combination of COX-2 inhibitors and sanguinarine in the treatment of human prostate cancer^[28].

SANGUINARINE-MEDIATED INHIBITION OF NF- κ B

The molecular pathways associated with carcinogenesis are linked also with chronic inflammation, which emerges as an important co-factor in tumor development. The NF- κ B controls the inflammatory gene expression and recently it has been suspected to be involved also in the control of tumor development^[64]. Resting NF- κ B localizes within the cell cytoplasm in the form of a heterodimer composed by p50, p65, and the inhibitory subunit I κ B α ^[65]. Following activation, the I κ B α protein is phosphorylated, ubiquitinated and finally degraded. Then, the p50 and p65 reach the nucleus of cell, where they interact with selected DNA sequences localized in the promoter region of various genes, leading to their transcription. Consistently, the NF- κ B signalling pathway has been indicated as a key-target for the development of new chemotherapeutic approaches in cancer.

Sanguinarine has been suggested as a potential actor in the control of NF- κ B-dependent pathological responses by blocking phosphorylation and degradation of I κ B α . Studies by Chaturvedi *et al.*^[50] showed that in human myeloid ML-1a cells, the treatment with sanguinarine is capable of abrogating, dose- and time-dependently, the activation of NF- κ B induced by tumor necrosis factor.

INHIBITION OF TUMOR ANGIOGENESIS BY SANGUINARINE

Many reports indicate that sanguinarine exerts antitumor activity not only by inhibiting tumor cells migration and/or invasion, but also by repressing angiogenesis^[22,66]. Since solid tumors require active angiogenesis, the inhibition of endothelial cell proliferation result in the inhibition of tumor growth and progression. The best known angiogenic growth factor is represented by VEGF. Several studies have explored the relationship existing among sanguinarine, angiogenesis and metastatization. In particular, Eun and Koh^[13] showed that sanguinarine inhibits the VEGF-induced endothelial cell migration, sprouting and survival *in vitro*, and blocks blood vessel formation *in vivo* in different experimental models. Furthermore, Basini *et al.*^[67] showed that sanguinarine is capable of blocking the VEGF-induced blood vessel growth. Depending on the concentration used, sanguinarine also inhibits VEGF secretion in human microvascular endothelial cells HMVEC as well as in A549 lung cancer cells^[68]. This inhibitory effect has been associated with the suppression of the phosphorylation of Akt, p38 and VE-cadherin, which are well known modulators of the VEGF signal transduction pathway^[67,69]. Moreover,

sanguinarine enhances apoptosis in human mammary adenocarcinoma MCF-7 through the inhibition of VEGF release, induced by generation of ROS^[32]. Sanguinarine also inhibits angiogenesis in preclinical experimental tumor models, such as mouse melanoma^[48] and rat colorectal cancer, as we reported previously^[49]. In both the experimental studies, the therapeutic efficacy of sanguinarine could not be attributed only to a direct anti-proliferative activity but also to the inhibition of tumor angiogenesis induced by this alkaloid.

The rationale of using VEGF-targeted therapies in the treatment of cancer lies in the possibility they offer to counteract the over-expression of VEGF provoked by chemotherapeutic drugs and radiation^[70]. Consistently, dacarbazine, which is used in the therapy of human melanoma, induces increased VEGF-A production^[71], and dacarbazine-resistant melanoma cells show an increased *in vivo* growth together with an increased microvessel density^[72]. These studies suggest the potential application of sanguinarine, alone or in association with other VEGF inhibitors, in the control of both angiogenesis and metastatization of solid tumors.

INHIBITION OF TUMOR CELL INVASION BY SANGUINARINE

In solid tumors, neoplastic cells can penetrate the basement membrane by proteolysis and initiate metastatization, which accounts for the majority of cancer deaths. Metastatization is the result of the cooperation between cancer cells and a sort of "inflamed" micro-environment^[73]. Consistently, inflammatory cells are an important source of proteases capable of causing a degradation of extracellular matrix, which represents a crucial event in the initiation of cancer cell invasion. Matrix metalloproteinases (MMPs) are an example of agents capable to degrade the extracellular matrix^[74,75] and an over-production of these enzymes has been detected in various metastatic cancers^[76-78]. Indeed, there is a strong evidence that increased expression and activation of MMP-2 and MMP-9 is present in tumor tissues but not in normal tissues in patients with breast cancer^[79] and that MMP-2 induces cancer cell migration by means of its interaction with collagen^[80].

Recent findings show that sanguinarine inhibits the tetradecanoylphorbolmyristate acetate (TPA)-induced breast cancer cell migration and invasion while inhibiting the expression of MMP-9, NF- κ B and AP-1 signaling pathways^[81]. Moreover, previous studies by Sun *et al.*^[66] have showed that sanguinarine reduces prostate cancer cell growth and invasion by the inhibition of STAT3 activation. STAT3 is constitutively active in human prostate cancer metastases and has a key role in the phenomena of tumor cell migration and invasion in different types of cancer^[82-84]. Since the invasivity and/or metastatic potential of a tumor parallel its malignancy, the above findings indicate that sanguinarine may play a crucial role as a therapeutic agent in anticancer therapy

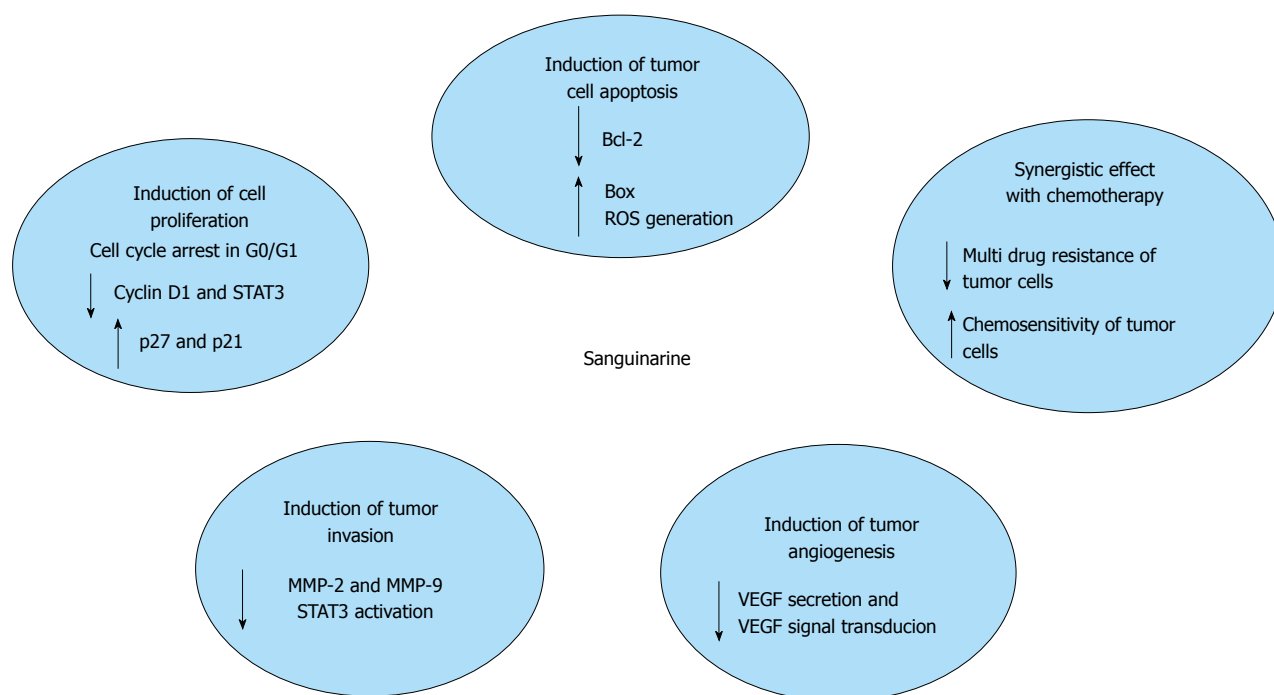


Figure 2 Cellular and molecular mechanisms underlying the antitumor activity of sanguinarine, as assessed by means of *in vitro* and *in vivo* experimental studies. ROS: Reactive oxygen species; Bcl: B cell lymphoma; VEGF: Vascular endothelial growth factor; MMP: Matrix metalloproteinase; STAT: Signal transducer and activator of transcription.

not only for its ability to induce apoptosis but also for its own “anti-invasive” properties.

SYNERGISTIC INTERACTION OF SANGUINARINE WITH CHEMOTHERAPEUTIC AGENTS

Several plant SMs are capable of influencing effectively the multidrug resistance phenomenon in tumor cells and are able also to “chemo-sensitize” them^[85-89]. Some clinical studies have explored the possible advantage of combining natural products with classical chemotherapeutic regimens^[90-92]. Phytotherapy, which employs plants extracts, is still used worldwide for the treatment of various human diseases. However, evidence has been proved that combinations of individual SM in an extract may exert synergistic effects. As an example, a recent study demonstrates that the combined use of non-toxic concentrations of sanguinarine and digitonin with doxorubicin, synergistically sensitizes Caco-2 (human colorectal adenocarcinoma) and CEM/ADR5000 adriamycin-resistant leukemia cells and increases the cytotoxicity of the chemotherapeutic agent doxorubicin^[93]. In this regard, it is worth mentioning that the main advantage of combination therapies is represented by the possibility of reducing the doses and thus the toxicity of chemotherapy, while retaining its own efficacy. Thus, because of its potential synergistic interaction with chemotherapeutic agents, the therapeutic use of sanguinarine as an adjuvant, in association with chemotherapy, might be considered as a theoretical option in cancer therapy.

CONCLUSION

A successful resolution to the design of antitumor drugs relies, at least in part, on the possibility to overcome the intrinsic resistance to undergo apoptosis detected in many transformed cells. Findings from the studies above mentioned show that sanguinarine is capable of inhibiting tumor growth through different molecular pathways (Figure 2). A summary of the results is shown in Table 1. In conclusion, despite sanguinarine has been extensively studied, the precise mechanisms responsible for its antitumor effects still have not been completely elucidated and are strictly dependent on the cell type studied. According to the results obtained so far, it can be said that the anti-tumor action of this alkaloid is the result of a combined effect both on proliferation and invasiveness of tumor cells, that on regulation of the complex phenomena of tumor angiogenesis. In particular, owing to its pro-apoptotic potential, sanguinarine is a good candidate for the development of new anticancer therapies either when used alone or in combination with other chemotherapeutic regimens. More extensive investigation and greater caution are needed, however, to clarify the following important issues. First of all, most of the studies above mentioned have been performed *in vitro* using cancer cell lines, whereas there are only a few *in vivo* studies validating the efficacy and safety of sanguinarine administration in animal tumor models. The results of our *in vivo* studies confirm the effectiveness and safety of using oral sanguinarine administration to control tumor growth in rats^[49]. Similar results had been previously reported in a murine melanoma model^[48]. In that study, and

Table 1 The antitumor activity of sanguinarine

Sanguinarine induces apoptosis in tumor cells through multiple pathways, including the activation of NF- κ B, the mitochondrial damage and cell cycle arrest
Sanguinarine-induced apoptosis is associated with the decrease of Bcl-2 and the increase of Bax proteins and the generation of reactive oxygen species
Sanguinarine causes cell cycle arrest by increasing the expression of p27 and decreasing cyclin D1, D2 and E, and CDK2, 4 and 6
Sanguinarine inhibits tumor progression associated with chronic inflammation <i>via</i> the inhibition of NF- κ B
Sanguinarine inhibits tumor angiogenesis through the inhibition of VEGF secretion and VEGF signal transduction (Akt, p38 and VE-cadherin)
Sanguinarine has an inhibitory effect on tumor cell migration by the inhibition of MMP-9 and STAT3 activation
Sanguinarine exerts a synergistic effect with chemotherapeutic agents and enhances the chemosensitivity of Caco 2 and CEM/ADR5000 adriamycin-resistant leukemia cells

NF- κ B: Nuclear factor- κ B; CDK: Cyclin-dependent kinase; Bcl: B cell lymphoma; VEGF: Vascular endothelial growth factor; STAT: Signal transducer and activator of transcription.

in agreement with our findings, the anti-proliferative and anti-angiogenic effects of the oral sanguinarine administration were observed at a dosage, *i.e.*, 5 mg/kg, devoid of apparent toxicity. On the other hand, an increase of serum levels of transaminases and LDH, hepatic vacuolization, lipid accumulation and peroxidation in the liver and a reduction of triglycerides, were observed in mice treated with high-dose sanguinarine (10 mg/kg), suggesting liver injury^[94]. Previous studies showed that sanguinarine can cause physiological dysfunction in skeletal, smooth and cardiac muscles^[95-97]. More recent studies clearly indicate that sanguinarine acts as a pro-apoptotic factor and alters mouse normal embryonic development at a physiological dosage, *i.e.*, 0.5-2 μ mol/L, which are obtained *via* dietary intake^[98]. These experimental results need further confirmation in view of the possible administration of the compound in pregnancy, although at present no teratogenic effects have been reported in humans.

Most of the studies actually known have reported that sanguinarine exerts cytotoxic activity selectively on cancer cells. Consistently, sanguinarine is a negative regulator of human epidermoid carcinoma cells (A431) but not of normal epidermal keratinocytes^[23]. Evidence of this differential activity have been reported recently, showing that mouse lymphocytic leukemic cells are more sensitive to sanguinarine than normal splenocytes^[99].

It is a matter of fact, however, that sanguinarine has been listed as responsible for the toxicity of *Argemone mexicana* seed oil^[100-102]. Das *et al.*^[103] reported that topical use of argemone oil (0.15-0.3 mL) or sanguinarine (4.5-18 μ mol/L) followed by application of TPA induces tumor development in a murine experimental model. Ansari *et al.*^[104] also reported that intraperitoneal administration of sanguinarine induces DNA damage in Swiss albino mice. Sanguinarine in argemone oil, is suspected to cause glaucoma^[101,102]. Argemone oil increases incidence of bladder cancer in animal models^[103] and of gall bladder cancer in humans^[104]. Furthermore, sanguinarine extract from bloodroot (*Sanguinaria canadensis*), previously used in oral hygiene products, was discontinued until a link between product administration and occurrence of leukoplakia was established^[105,106]. Hepatic microsomes transform sanguinarine in a mutagenic epoxide and the same sanguinarine is capable of activating polycyclic aromatic

hydrocarbon signaling^[107]. However, related to this topic, the results available in literature are not univocal^[3]. So that is still not clear if sanguinarine may act as a carcinogenic without the cooperation of other risk factors or it is capable of acting in concert with various co-carcinogens. In light of the above facts, the possibility of obtaining beneficial effects in humans by using sanguinarine remains largely unpredictable.

Finally, since at present there is increasing interest in nanotechnology application in cancer therapy and in order to prevent the potential toxic and/or side effects induced by sanguinarine administration, *in vivo* studies might be performed in experimental tumor models by encapsulating the alkaloid in tumor-targeted nanoparticles^[108], which accumulate preferentially in tumors recognizing single cancer cells for diagnosis and treatment. Actually, the administration of sanguinarine (10 mg/kg) *per os* and encapsulated by lipid nanoparticles (SG-SLNs), has been shown to induce an anti-inflammatory effect in an LPS-induced endotoxin shock murine model, and the pharmacokinetic studies have proved that the AUC₀₋₂₄ and C_{max} of SG-SLNs were significantly increased when compared to those of sanguinarine alone^[109].

In conclusion, several studies indicate the potential application of sanguinarine as an adjuvant in the therapy of cancer, but further detailed pharmacokinetic and toxicology studies, which have to be conducted in appropriate experimental tumor models, are absolutely required to assess the efficacy and safety of this compound before such an antitumor strategy can be translated in clinical trials.

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***Helicobacter pylori* infection and gastric carcinoma: Not all the strains and patients are alike**

Natale Figura, Luigi Marano, Elena Moretti, Antonio Ponzetto

Natale Figura, Department of Medical, Surgical and Neurological Sciences, University of Siena and Policlinico S. Maria alle Scotte, 53100 Siena, Italy

Luigi Marano, General, Minimally Invasive and Robotic Surgery, Department of Surgery, Hospital San Matteo degli Infermi, 06049 Spoleto, Perugia, Italy

Elena Moretti, Department of Molecular and Developmental Medicine, University of Siena, 53100 Siena, Italy

Antonio Ponzetto, Department of Medical Sciences, University of Torino, 10126 Torino, Italy

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Correspondence to: Natale Figura, Professor, Department of Medical, Surgical and Neurological Sciences, University of Siena and Policlinico S. Maria alle Scotte, Viale Bracci, 53100 Siena, Italy. natale.figura@unisi.it
Telephone: +39-5-77585463
Fax: +39-5-77233446

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Abstract

Gastric carcinoma (GC) develops in only 1%-3% of *Helicobacter pylori* (*H. pylori*) infected people. The role in GC formation of the bacterial genotypes, gene polymorphisms and host's factors may therefore be important. The risk of GC is enhanced when individuals are infected by strains expressing the oncoprotein CagA, in particular if CagA has a high number of repeats containing the EPIYA sequence in its C-terminal variable region or particular amino acid sequences flank the EPIYA motifs. *H. pylori* infection triggers an inflammatory response characterised by an increased secretion of some chemokines by immunocytes and colonised gastric epithelial cells; these molecules are especially constituted by proteins composing the interleukin-1beta (IL-1 β) group and tumour necrosis factor-alpha (TNF- α). Polymorphisms in the promoter regions of genes encoding these molecules, could account for high concentrations of IL-1 β and TNF- α in the gastric mucosa, which may cause hypochlorhydria and eventually GC. Inconsistent results have been attained with other haplotypes of inflammatory and anti-inflammatory cytokines. Genomic mechanisms of GC development are mainly based on chromosomal or microsatellite instability (MSI) and deregulation of signalling transduction pathways. *H. pylori* infection may induce DNA instability and breaks of double-strand DNA in gastric mucocytes. Different *H. pylori* strains seem to differently increase the risk of cancer development run by the host. Certain *H. pylori* genotypes (such as the *cagA* positive) induce high degrees of chronic inflammation and determine an increase of mutagenesis rate, oxidative-stress, mismatch repair mechanisms, down-regulation of base excision and genetic instability, as well as generation of reactive oxygen species that modulate apoptosis; these phenomena may end to trigger or concur to GC development.

Key words: *Helicobacter pylori* infection; CagA; CagA gene polymorphism; Haplotype; Human gene mutation;

Gene methylation; Gastric carcinoma; Inflammatory cytokine

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Core tip: CagA and the *cagA* types may play different roles in the intestinal and diffuse histotypes of gastric carcinoma (GC); The current criteria of *Helicobacter pylori* (*H. pylori*) strain classification based on their carcinogenic potential gave rise to confusion and should be unified. The possible role of inflammatory cytokine haplotypes in GC development should be reassessed taking into account some host's factors, the most important being different ethnic origin. Infection by the *cagA* positive *H. pylori* genotype may determine an increased inflammatory response and a consequent enhancement of mutagenesis rate, oxidative-stress, reactive oxygen species generation, dysfunction of DNA repair mechanisms, genetic instability and resultant high risk of GC development.

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INTRODUCTION

Gastric carcinoma (GC) is the second most frequent cause of death from cancer worldwide and the most common example of a neoplasia developing on a ground of a chronically inflamed mucosa. GC has also another record: It is the only known malignant tumour that can develop as a consequence of a chronic bacterial infection^[1]. In 1994, the International Agency for Research on Cancer classified the organism responsible for the infection, *Helicobacter pylori* (*H. pylori*) - a Gram negative, microaerophilic and spiral-shaped species that finds its *habitat* in human stomachs - as a definite carcinogen to humans (Group 1): The connection of *H. pylori* with gastric cancer was considered similar to that existing between the cigarette smoke and lung cancer^[2].

The bacterium *H. pylori*: Not all the strains are alike

It soon became clear, however, that such comparison was reductive and too simplistic, especially because the ability of these bacteria to trigger a neoplasm is not limited to the inflammatory and immune response to the infection that they cause, but it also resides in a series of bacterial factors capable of prompting and modulating the carcinogenic process^[3].

As is the case for all diseases, also GC develops from the concomitance of three factors: The etiological agent, the host and the environment. Of course, many other factors may occur; for example, the cancer histological

variant, the degree of differentiation of the neoplasia *etc.*^[4]. Regarding the etiological agent, *H. pylori*, there are many indications that not all strains are equivalent in their carcinogenic potential and that those expressing an immunodominant peptide determinant called CagA (cytotoxin associated gene A), are endowed with an increased inflammatory and carcinogenic potential^[5-9]. A first point has therefore been established: Strain genomic diversity corresponds to different ability to promote cancer. The possibility that a bacterial factor (CagA) could trigger or concur to the development of GC is one of the most important scientific achievements following the isolation of *H. pylori*.

The importance of being called CagA positive

It is worthwhile mentioning the steps that paved the way to the discovery of CagA. At the end of the 80ies, Leunk *et al.*^[10] first proposed that *H. pylori* should not be considered a clonal pathogen, as a relevant proportion of isolates produce a vacuolating toxin, which could account in part for the gastric mucosa damage observed in infected individuals. Afterward, our group suggested that infection by cytotoxic strains increased the risk of developing peptic ulceration^[11] and that virtually all cytotoxic isolates also secreted a 120 kDa highly immunogenic protein, later called CagA^[12]. In 1992, Crabtree *et al.*^[13] demonstrated, through *ex vivo* experiments, that such a protein was produced either by the bacteria isolated in culture and also by the organisms colonizing the gastric epithelium: Gastric antral explants of patients with GC and other pathologies were cultured *in vitro* for a few days; the bacteria that colonized the mucosa kept on secreting this peptide, which could lastly be detected in the culture medium by using immunological methods^[13]. In 1993, the same team established, for the first time, the existence of a relationship between infection by strains expressing the 120 kDa protein and GC development^[14]. Their observations were important also because these researchers found anti-120 kDa protein mucosal IgA antibodies even in the absence of systemic IgG to this protein and, in some patients, also in cases with urease negative biopsies (false negatives). In the same year (1993), the gene encoding for the 120 kDa protein was cloned, sequenced and called *cagA* due to the strict association of protein expression with cytotoxin production^[5]. As a result of these findings, the number of studies dealing with the characterisation of CagA and its potential carcinogenicity increased exponentially and results lead to the common conclusion that such peptide is a major factor in gastric carcinogenesis.

CagA is the product of the homonymous gene placed at the end of the so-called pathogenicity island (PAI) *cag*, a fragment of DNA encompassing an approximately 40 kb cluster of genes involved in virulence. In the field of bacteriology there are numerous examples of PAIs harboured by diverse bacterial species or their virulent variants, whether they are human (*Bordetella*

pertussis, *Escherichia coli*, *Salmonella enterica*, etc.) or plant pathogens (*Agrobacterium tumefaciens*). In some species, PAI genes cooperate to translate effectors (mainly proteins) endowed with carcinogenic potential inside colonised cells. In *H. pylori*, such determinant is CagA. Similarly, *A. tumefaciens* exploits the Type IV secretion system *vir* to translate a single-stranded form of T-region (T-strand) coated by the ssDNA-binding protein VirE2 (T-Complex) into the host's vegetal cell nuclei. Once inside the nucleus, the T-strand can be converted in a double-stranded form (T-DNA), whose expression causes an uncontrolled host cell proliferation and tumour development^[15].

Epidemiological and genomic studies suggest that the development of GC is a possible consequence of infection by strains expressing CagA^[1,8,9,14,16]. In effects, using Mongolian gerbils infected experimentally, it was shown that only CagA positive (CagA+) *H. pylori* strains were able to induce stomach tumours^[17]. In addition, a study of our group revealed that, while virtually all patients with intestinal histotype of GC had serum antibodies to CagA, the prevalence of anti-CagA antibodies in patients with the diffuse GC variety was similar to that observed in infected controls without neoplasia^[16]. These data were confirmed by the results of an epidemiological study: The overall GC risk in infected people lacking anti-CagA antibodies (CagA-) was increased, but in non-significant way; in any case, CagA- *H. pylori* infection was associated with the growth of the diffuse variety of GC (with an OR of 9.0)^[8].

It therefore seems that infections by CagA+ strains expose people to an increased risk of GC respect to infections by CagA- *H. pylori* strains, which can only be associated with the diffuse histotype. In effect, things work slightly differently. In a recent study, we examined for the presence of *cagA* up to 25 distinct, well separated colonies per patient with GC; even though only individuals with diffuse histotype GC harboured *cagA* negative (*cagA*-) organisms, in all cases patients were also infected by at least one *cagA* positive (*cagA*+) strain^[18]. These observations do not corroborate the supposed propensity of strains lacking *cag* PAI to concur to diffuse GC development and suggest that, for a better comprehension of the role played by *cagA* in the histological variety of GC, many colonies per patient should be examined genomically.

CagA phosphorylation by mucocytes: Like shooting oneself in the foot

H. pylori organisms expressing CagA differ in their carcinogenic potential. Let us have a look at the mechanisms that may influence the ability of such a protein to trigger and/or concur to GC formation. CagA, following colonisation, is translated into the gastric epithelial cells through a conjugative apparatus encoded by the *cag* PAI genes upstream *cagA*; then, a portion of intracellular CagA is phosphorylated by numerous kinases, members of the host cell Src family (such as Yes, Lyn, Fyn and

c-Src,) at the EPIYA C'-terminal site (Glu-Pro-Ile-Tyr-Ala) motif of tyrosine^[19]. Phosphorylated CagA physically interacts with the oncogenic tyrosine phosphatase SHP-2 (Src homology phosphatase 2), modifying cellular functions and altering mammalian signal transduction machineries. SHP-2, in fact, is implicated in the regulation of cell adhesion, spreading and migration. In this manner, phosphorylated CagA causes deregulation of SHP-2 and induces abnormal proliferation, as well as movement of cells of the gastric epithelial layer, activates mitogenic signalling and disturbs host-signalling routes^[20]. All these events may also predispose cells to accumulate multiple genetic and epigenetic alterations involved in gastric tumorigenesis^[21].

Unphosphorylated CagA, on the other hand, interacts with the tumour suppressor protein of p53 (ASPP2), which also exert an apoptosis-stimulating activity^[22]. In normal conditions, following genotoxic and oncogenic stimuli, ASPP2 associates with tumour suppressor p53, activates it and induces apoptosis. After interaction with CagA, cytosolic p53 is recruited by ASPP2 and subsequently is degraded by an enzyme complex that control cell-cycle and apoptosis, the proteasome. As a consequence, the apoptotic response of host cells is inhibited. In other words, unphosphorylated CagA takes control of ASPP2 and subverts the tumour suppressor pathway of apoptosis-stimulating protein p53 with consequent promotion of cell survival and cell transformation^[20-22]. The resultant abnormal proliferation of gastric epithelial cells may contribute to GC development.

Individual CagA proteins have different biological activity and tumorigenic potential: When size matters

H. pylori strains secreting CagA protein endowed with increased biological activity can be considered more virulent and even more closely associated with gastric cancer. At the end of 90's, the group of Graham discovered the ability of distinct CagA proteins to perturb cellular functions might vary in different isolates^[23]. The CagA C'-terminal region contains one or more repeats of the same amino acids in sequence (Glu-Pro-Ile-Tyr-Ala, or EPIYA)^[5]; the teleonomic significance of such phenomenon reflects the bacterial strategy to generate antigenic diversity, which may protect the organisms from the immune response. The number of EPIYA motifs correlates with the size of the *cagA* variable region. Yamaoka *et al.*^[23] basing of the amplicon sizes obtained with primers encompassing the entire *cagA* variable region, classified *H. pylori* isolates in *cagA* structural types A, B, C and D (amplicons characterising types B and D have the same size and can be differentiated by sequencing). Strains with the *cagA* structural type C have the highest number of EPIYA phosphorylation motifs and were isolated significantly more often from patients with GC^[23], confirming a previous observation that individuals with GC are infected by strains expressing CagA proteins with higher mass^[24]. The incr-

eased carcinogenic potential of the *cagA* structural type C was also confirmed by another study of the same group, which showed that patients infected by *H. pylori* with this *cagA* genotype run a higher risk of developing gastric mucosa atrophy, a precancerous condition^[25].

Now, it should be highlighted that the primers used in these studies to amplify the *cagA* variable region were designed on oriental (Japanese) strains, which may differ from western strains in the nucleotide sequence encoding the C'-terminal variable region^[26]. Probably for this reason not all surveys on the same subject have confirmed the Yamaoka *et al.*^[23,25]'s findings. In an initial study of our group, in which we used the Yamaoka's primers to amplify the *cagA* variable region of Italian strains^[27], we sometime obtained amplicons shorter than the PCR product that characterises the *cagA* structural type A; strains producing such amplicons, which presented a deletion of about 100 bp respect to *cagA* type A, were named type A(I), with (I) standing for Italy, because, as far as we know, similar *cagA* structural variety had not previously been described. In a more recent investigation, we observed similar proportions of the various *cagA* structural types in Italian *H. pylori* organisms isolated from GC cases and from controls (patients without neoplasia, with chronic gastritis only)^[28]. In addition, in the control subjects, we frequently detected strains with the *cagA* structural type A(I). The increased prevalence of such a *cagA* type in individuals without GC prompted us to hypothesise that the reduced dimensions of the encoded CagA may decrease the ability of these bacteria to trigger a neoplastic process. As a matter of fact, the capability of CagA of binding SHP-2, and therefore of disturbing the various cellular functions, is regulated by the amounts of tyrosine phosphorylation site sequences, *i.e.*, the CagA size^[20]; therefore, the shorter the protein, the less numerous are the EPIYA repeats that undergo phosphorylation and the lower is the carcinogenic potential of strains.

The employment of primers proposed by Yamaoka *et al.*^[23] to amplify the *cagA* variable region of western strains has sometimes led to results similar to those of the Japanese researchers: South African researchers, for instance, have confirmed that patients with GC have an increased prevalence of strains with type C CagA^[29]. Investigations dealing with this important subject, however, are not numerous; in addition, sometimes they attained different conclusions and have even contributed to create confusion in this subject. Malaysian authors, for instance, using the primers designed by Yamaoka *et al.*^[23], determined the presence and distribution of *cagA* variants among different ethnic groups and various gastroduodenal diseases^[30]. They obtained three types of amplicons and named the *cagA* subtypes with capital letters, A, B and C (like did Yamaoka *et al.*^[23]), but, just to complicate this topic further, they used a different criterion (respect to that of the previous study) and called subtype C the strains with the smallest amplicon

size and subtype B those with the greatest one. Specific *cagA* subtype A strains (those yielding amplicons of intermediate size) were predominantly isolated from Chinese compared to Malays and Indians patients. Since Chinese patients have the highest risk of GC disease respect to the other ethnic groups, these investigators concluded that *cagA* subtyping could be used as a clinical biomarker for severe outcome of infection. Such statement, however, was based on indirect observations because they did not examine strains from GC cases^[30].

In 2002, Higashi *et al.*^[20] observed that the ability of CagA secreted by different strains to disturb host-cell functions can be influenced by the strength of SHP-2 binding activity, which was increased in *H. pylori* strains obtained from patients living in East Asian areas (Japan, Chorea and China) respect to those isolated from patients of Western countries (Europe, America, and Australia). In addition to the number of EPIYA repeats, it was also found that polymorphism in the nucleotide sequence flanking the regions that encode EPIYA could affect the potential of different *H. pylori* strains to promote gastric carcinogenesis^[31]. According to the geographic regions in which strains are isolated, it is possible to characterise the *cagA* variable region on the basis of the number and the type of sequences. The Western *H. pylori* CagA has two segments of 32 and 40 amino acids flanking EPIYA (EPIYA-A and EPIYA-B types) and one to three 34 amino acid EPIYA-C segments (A-B-C type CagA)^[21]. Eastern CagA presents EPIYA-A and EPIYA-B segments, but none of the EPIYA-C fragment; it has instead one copy alone of a segment called EPIYA-D, which represents the main tyrosine phosphorylation site^[20,21]. In western strains, the major site of tyrosine phosphorylation of CagA is EPIYA-C; the tyrosine residues that characterize EPIYA-A and EPIYA-B segments are phosphorylated only very weakly. Such a difference in phosphorylation degrees resides in the diverse consensus high-affinity binding sequence for the SH2 domains of SHP-2. Unlike what happens for EPIYA-D type, the western EPIYA-C type of CagA differs by a single amino acid from the consensus SHP-2 binding sequence^[21].

In conclusion, the carcinogenic potential of *H. pylori* varies according to the number and the *cagA* structural types of the EPIYA flanking regions. East Asian CagA has an increased virulence and a strong ability to trigger GC, while, among western isolates, more carcinogenic are the helicobacters with two or three CagA EPIYA-C sites. This was also demonstrated by a study in which it was observed that 83.3% of GC strains possessed multiple EPIYA-C sites, vs only 5.2% of strains isolated from patients with chronic gastritis only (controls)^[32].

Often, *cagA*+ strains are also isolated from patients with duodenal ulcer (NF personal observation). This finding may create confusion, because is common knowledge that patients with duodenal ulcer are like protected from GC development; however, the results of a recent study^[33] showed that the increased virulence

of strains with CagA EPIYA-C type augmented the risk of gastric cancer and not peptic ulceration. Some studies diverge from these conclusions: Findings of an investigation carried out in Colombia suggest that polymorphic CagA proteins, based on sequences flanking the EPIYA motifs, are not clearly associated with the outcome of the infection^[34]. The absence of association between the CagA polymorphisms and pathogenesis of gastroduodenal diseases could be due to geographic factors and/or the host's genetic features and environmental determinants.

In conclusion, the results of this kind of investigations are potentially useful, but the confusion existing in this field ought to be rectified and the different researchers should use the same criteria for classification. The various groups, however, have reached a common conclusion: Not all the strains are alike in their carcinogenic potential.

NOT ALL PATIENTS ARE ALIKE: THE ROLE OF THE HOST'S INFLAMMATORY CYTOKINE HAPLOTYPES IN GC DEVELOPMENT

Background

The hypothesis that human genetic polymorphisms may affect predisposition to GC has recently been explored. GC develops in only 1%-3% of *H. pylori* infected individuals, which suggests that the host background matters in this neoplasia. Several pro-inflammatory cytokines are produced by the immune system against *H. pylori*; among them, IL-1 β is of paramount importance; a second one is tumor necrosis factor-alpha (TNF- α); both of them are closely related to epithelial injury and gastric hypochlorhydria^[35,36]. At low concentrations, TNF- α enhances the protective inflammatory response; at high concentrations, it can injure the gastric mucosa and cause severe pathology^[37]. IL-1 β increases the surface molecule expression on endothelial cells, causing leukocytes to adhere; IL-1 β also induces the production of macrophage chemokines leading to neutrophil activation. Recent investigations have revealed that there is a genetic regulation of the host cytokine response to inflammatory stimuli. Genomic variants of *IL-1 β* and *TNF- α* were shown to correlate with the clinical outcomes of tumors, including GC^[38,39]. The *IL-1 β* gene cluster is polymorphic, with some alleles present at relatively high frequencies. Particular *IL-1 β* haplotypes enhance the risk of GC because they induce an over expression of its product in the stomach, causing chronic hypochlorhydria, which in turn may produce gastric atrophy and, eventually and in the presence of other risk factors, GC^[40]. In addition, patients with a particular haplotype of the gene that encodes IL-1RA (receptor antagonist) have an elevated risk of developing GC. IL-1RA is an anti-inflammatory cytokine, which is a competitor for IL-1 β receptors, thus regulating the possible

harmful effects of IL-1 β receptors.

The role of the host in GC development could be important because the inflammatory response to infections varies from patient to patient due to the gene polymorphism of inflammatory and anti-inflammatory cytokines. Many studies have been performed in the last 15 years, with the scope of identifying a genetic marker that can determine whether or not people carrying the infection might be at risk of developing GC in *H. pylori* infected patients^[41-59]. Such a marker is still lacking, and we shall try to explain some of the reasons underlying this problem.

The association between chronic inflammation and cancer has been known since Virchow, in 1864, wrote that cancer would arise from sites of inflammation: "Chronic irritation which is manifested by a chronic inflammation is a key promoter of cancer" (Quoted by Balkwill and Mantovani^[60]). Individual cytokines were specifically examined, in particular the proinflammatory ones. The most well known cytokine is IL-1, along with its receptor (IL-1R) and the antagonist of this receptor (IL-1RA); all of them share the chromosomal location of the *IL-1* gene family (namely 2q13-14). IL-1 β has thus been established as an important regulator of carcinogenesis, characteristic of interactions between the host and environment^[54].

IL-1 β haplotypes

The *IL-1 β* gene displays considerable polymorphism^[54]; the presence of C to T transition was frequently found either in the promoter region at positions -511 (CT; dbSNP: rs16944), at position -31 (TC; dbSNP: rs1143627) or in the coding region at position +3954 (CT; dbSNP: rs1143634) base pairs from the origin of transcription. The two single nucleotide polymorphisms (SNPs) within promoter region are in linkage disequilibrium. The *IL-1 β* -31 TC substitution disrupts a TATA-box motif; this leads to several transcription factors having altered binding affinities, resulting in modified IL-1 β transcription. The *IL-1 β* +3954 CT substitution is a synonymous SNP. It was demonstrated *in vitro* that the C to T transition at positions -511 and +3954 correlated with elevated IL-1 β levels as a result of lipopolysaccharide (LPS)-stimulated IL-1 β protein secretion^[54].

The paradigm of all subsequent studies regarding GC with respect to different haplotypes in cytokines was the focus of the paper by El-Omar *et al.*^[38], who noted for the first time that the presence of two polymorphisms (rs16944 and rs1143627) in the promoter region of the *IL-1 β* gene, identified an increased risk of hypochlorhydria, as a result of *H. pylori* infection and GC^[38]. These polymorphisms determine an increased secretion of IL-1 β ^[61], a conclusion confirmed and generalized by later reports^[62]. The discrepancy of results reached in different papers was clarified only after several years, once taking into account the origin of the population and the type of GC. Overall, more than 90 publications dealt with this issue, over half of them from Asia, and a single one

from North America, a clear indication of the perceived relevance of this neoplasia in the different populations.

Since the single studies are extremely inconsistent and, if taken alone, contribute little to the general overview, we opted for reporting from selected meta-analyses in this paper. Meta-analyses accrued from time to time in a number of studies originating from different countries, which is one of the main causes of contradictory results, as well as from different histological variety of stomach malignancies, another cause of strong difference in results^[41-59].

The overall findings from the large amount of efforts can be summarized as follows:

(1) *IL-1 β receptor antagonist (IL-1 β RA)* polymorphism: The most credible and consistent association of peculiar genetic variation with GC was found for *IL-1 β RA* haplotypes. Four alleles, numbered 1 to 4, are widely present in the general populations. People carrying the homozygous allele 2/2 (*IL-1 β RN2*) were found to be at higher risk of developing cancer among non-Asian populations. Moreover, the analysis of GC patients altogether, without stratifying according to histological type, anatomic site or country of origin, showed that patients carrying homozygous allele 2, or *IL-1 RN2* had an increased risk of developing cancer, which was statistically significant. The risk was found both in cardia and non-cardia types of neoplasia. A possible explanation for the risk stems from the high *IL-1 β* levels circulating among *IL-RA* allele 2/2 carriers^[63].

(2) *IL-1 β -31 CT* polymorphism: A second plausible association was the decreased risk of GC in Asians carrying the haplotypes in the *IL-1 β -31 CC* promoter region. A decreased risk of GC among *IL-1 β -31C* carriers was confirmed, but solely for Asian patients.

(3) *IL-1 β -511 CT* polymorphism in populations of different ethnic origin: Sub-analysis of various populations revealed a statistically significant association of stomach cancer with the *IL-1 β* polymorphism at promoter region -511 CT in case-control studies based on populations (OR = 1.20, 95%CI: 1.00-1.43)^[54]. The association is more consistent if only Caucasian populations are analyzed. Nevertheless, if taken together, the studies failed to show the association when stratified by ethnicities; *IL-1 β -511 CT* polymorphism according to tumor site: A significant association of *IL-1 β -511 CT* promoter region polymorphism was observed for stomach cancers when the tumor site (cardia vs non-cardia) was taken into account, as well as for histology subtypes (intestinal or diffuse/mixed). The association was present both in the case of non-cardia GC (OR = 1.57, 95%CI: 1.06-2.31) as well as intestinal GC (respectively OR = 1.57, 95%CI: 1.06-2.31 and OR = 1.24, 95%CI: 1.04-1.49)^[54]; *IL-1 β +3954 CT* polymorphism: Recently, Xu *et al*^[54] performed a meta-analysis that was confirmed by that one by Xue *et al*^[49]: There is a lack of association between *IL-1 β +3954 CT* and GC risk.

(4) *IL-10* haplotypes: *IL-10* - regarded as the ma-

jor anti-inflammatory cytokine - will bind in form of homodimer its complex receptor, comprising four *IL-10* receptor molecules, namely 2 *IL-10 R1* and 2 *IL-10 R2*. The binding induces *STAT3* signaling *via* the phosphorylation of the cytoplasmic tails of *IL-10* receptor 1. *IL-10* can inhibit the synthesis of pro-inflammatory cytokines; moreover it can block the function of nuclear factor-kappa B (*NF- κ B*), and has other regulatory properties, *e.g.*, *JAK-STAT* signaling^[64]. The *IL-10* gene is known to possess several SNPs, some in the distal region upstream of the coding gene (-1082 A/G, -819 T/C) and a proximal one (the -592 A/C). Again, the complex signaling and polymorphism of *IL-10* can explain the contradictory results of the investigations.

(5) *IL-10 -1082 AG* polymorphism: A clear and curious dichotomy is evident, that is, when the studies were stratified according to Asian and non-Asian populations the observations reached opposite results. The Asian populations had greater risk of GC among *IL-10 -1082 G* carriers; conversely, there was a decreased risk among the non-Asian populations. Meta-analysis specific for *IL-10* confirmed for Asian population the increased risk for intestinal type of gastric neoplasia in *IL-10 -1082 GG* or *GA* haplotypes^[53]; *IL-10 -592 AC* polymorphism: The -592 AC polymorphism failed to show any association, as the odd ratios for GC were 0.93 and 0.94 for homozygous and heterozygous population^[55,65]; *IL-10 -819 TC* polymorphism: Little data is available for this polymorphism, confirming a protective effect in Asian populations. Nevertheless, it was not found to be associated with the reduced susceptibility to GC in individuals infected with *H. pylori* compared to uninfected controls. The *IL-10 -819 TT* genotype was found to be inversely correlated with the risk of the diffuse subtype, but not the intestinal subtype GC^[51].

(6) *IL-8 - 251* polymorphisms: Continuous expression of human *IL-8* in transgenic mice (whereby *IL-8* is under the control of its own regulatory elements) increased tumorigenesis. Therefore, *IL-8* may play an important role in gastrointestinal cancers. Elevated *IL-8* levels could be linked to a poor prognosis of neoplasia, henceforth its levels may be indicative of more aggressive GCs.

Early data seemed to provide a possible association in GC as well^[66]. A recent meta-analysis showed that the *IL-8 -251 AA* genotype in the Han population correlates with augmented risk of developing GC and *AA* genotype carriers appear to be more likely to develop GC in Asian populations. In addition, the *IL-8 -251 AA* genotype tended to be related to intestinal GC, but not with *H. pylori* infectious status^[52]. There was no link between *IL-8* polymorphisms and *H. pylori*-related gastric malignancies in non-Asian populations in all the meta-analyses examined^[48,67].

(7) *TNF- α* polymorphism: Experimental studies have implicated *TNF- α* in processes that are involved in cancer progression, including promotion of metastatic behaviour and cancer associated cachexia^[68,69]. The lack of *TNF- α*

in mice makes them resistant to carcinogenesis^[70]. Clearly, such observation highlighted the link between genetic haplotypes for *TNF-α* and GC.

***TNF-α* -308 AG polymorphism:** It was surprising to find a lack of association of this polymorphism with increased risk of GC, with only one exception: Non-Asian patients with distal cancer and homozygous for -308 AA alleles; the association, moreover, appeared to exist for cancer of diffuse type only. However, this association was not confirmed when only good quality studies were taken into account, according to Persson *et al.*^[48]. Opposite conclusions were obtained by Zhu *et al.*^[59]; they recently analyzed all studies and concluded that, in the Caucasian populations, *TNF-α* rs1800629 (-308 AG) polymorphism indeed posed increased risk of GC. They used several genetic comparison models, *i.e.*, A vs G, AA vs GG and AA vs GG/GA that gave OR respectively of 1.32, 1.76 and 1.62, all highly significant (A vs G: OR = 1.32, 95%CI: 1.12-1.56, *P* = 0.001; AA vs GG: OR = 1.76, 95%CI: 1.37-2.26, *P* < 0.001; AA vs GG/GA: OR = 1.62, 95%CI: 1.27-2.07, *P* < 0.001)^[59].

***TNF-α* -238 polymorphism** did not correlate with an increased cancer risk^[48,58].

***TNF-α* 857 CT polymorphism:** Reports on this topic are quite controversial. Cen *et al.*^[57] recently published his analysis of nine studies (all the reported ones); overall, they confirm that the *TNF-α* 857 CT polymorphism posed an elevated risk of GC solely among Asians; all four genetic models considered T vs C, TT vs CC, CT vs CC and TT vs CT gave consistent data, respectively with OR of 1.19, 1.44, 1.19 and 1.21 (their statistical significance being *P* = 0.002, *P* = 0.032, *P* = 0.008, *P* = 0.003 respectively).

***TNF-β* 252 AG polymorphism:** A weak association with stomach malignancy was present in Asian populations, according to Xu *et al.*^[71]. Analysis by ethnicity revealed that the *TNF-β* 252 AG polymorphism correlated with a minor risk of GC (G vs A: OR = 1.10, 95%CI: 1.02-1.19, *P* = 0.015) exclusively in Asians, not in Caucasians.

Dutch patients were analyzed for their polymorphism of IL-1β; they were found to carry lower risk of GC when heterozygous for either the IL-1B -511 and for the IL1β -31 TATA-box (genotype T/C)^[72]. The EBV status of the patients did not affect this correlation and there could therefore be an early shared molecular mechanism in the progression of EBV-positive and negative GCs^[72].

IL-6 polymorphism was not studied in relation to GC.

IL-6 knockout mice develop cancer less frequently^[73]. It is therefore plausible that high IL-6 levels will promote tumorigenesis. Today, IL-6 is considered to be a relevant tumor-promoting factor also in humans. Indeed, it was correlated with glioma, lymphoma and melanoma at first, then with solid cancers such as breast and colorectal neoplasia (also ovarian and pancreatic), prostate, renal and colorectal cancers.

IL-6 is a critical factor during chronic inflammation, since it is required for the induction of effector Th17

cells and inhibits the differentiation of regulatory T cells.

Stomach cells, however, lack IL-6 receptors; hence it cannot dimerize with the second receptor (gp 130) and the "classic signaling" is restricted to cells bearing both mIL-6R and gp130 on their surface. The latter is widely expressed, whilst mIL-6R expression is limited to some leukocytes, hepatocytes and cancer cells. It is therefore quite understandable that, as recently reported, a large meta-analysis on 105000 people established the lack of association of cancer risk with *IL-6* polymorphism in Caucasians^[74], despite the association which holds true for Africans.

Other cytokines

Gene polymorphism concerning cytokine different from those we have dealt with was recently considered in relation to the risk of developing GC. The studies are not sufficiently large so far; however it is worth reporting that a haplotypes of *IL-17*, *IL-17F* rs763780 TC, was significantly associated with GC development in Asian population^[75].

IL-11 was taken into consideration in a single study^[76]. A reduced risk for developing cancer at the gastric site was found for a polymorphism in the *IL-4* -590 CT gene in Caucasian but not in Asian populations. *H. pylori* status was not taken into consideration in these studies^[77].

Different results in different studies: The origin of the problem

IL-1β, *TNF-α* and the remaining dozens of cytokines are not the final executor of immune signaling or the resulting consequences in cancer promotion and spread. IL-1, *TNF-α*, together with bacterial antigens, LPS and several other signaling molecules bind their respective receptors on the cell membrane; a cascade of signals ensues upon receptor activation, which depends on the levels of Mg-ATP availability (in turn on Mg²⁺ concentration in cells). Numerous different proteins are involved and regulate the signaling pathway, which finally results in the activation of a large family of DNA-binding proteins, the NF-κB family^[78], which is a complex that regulates DNA transcription. NF-κB dimers are formed upon activation, stimulating the transcription of genes that encode cytokines, growth factors, chemokines, and anti-apoptotic factors^[79]. However, some NF-κB dimers act by repressing, whilst others activate specific genes.

Cytokine polymorphism and Epstein-Barr virus-associated GC

Worldwide, it was noted that Epstein-Barr virus (EBV) is present in a relevant proportion of malignant tumors of the stomach, with an incidence that is inversely proportional to that of GC. In the USA 16% to 18% of all stomach tumors were found EBV-associated (EBVaGC), in Southern China only 4.3%^[80]; a survey of 101 published papers reported that EBVaGC was evident in 7.08% of intestinal type GC, while diffuse type GC had

an incidence of 9.82%^[80]. Western and Central Asian countries had significantly more EBV positive cases than South-Eastern countries; in Europe, the frequency of EBV infection ranged from 1.7% in the United Kingdom to 40% in Poland^[80].

An *in vitro* model of EBVaGC was used to demonstrate that gastric cells, following EBV infection, have a high IL-1 β expression, compared to EBV-negative gastric tumour cells. EBV-positive clones rapidly proliferated and were shown to be anchorage-independent in colony-forming assays^[81].

Since EBV infection is highly prevalent in all populations, whilst EBVaGC is quite rare, there were attempts to identify people who run an increased risk of developing GC. Polymorphisms of proinflammatory, as well as anti-inflammatory cytokines were studied, in particular in the promoter regions of *IL-10* and *TNF- α* . For the latter, the allele -308 A (linked to high levels of *TNF- α*) had significantly higher frequency among EBVaGC individuals (23.3%) when compared to control subjects (12.0%, $P < 0.05$). The opposite was found in the case of the anti-inflammatory *IL-10*: The high-producer allele (-1082 G) was found to be less frequent in EBVaGC patients in comparison to controls (6.3% vs 3.0%, $P < 0.05$)^[39,72].

The extreme complexities of all these interactions can explain the great variability in data when investigating the possible correlation between cytokine haplotypes and GC.

Gleanings on the usefulness of characterising *H. pylori* infected individuals for inflammatory haplotype

In addition to the complexity of this subject, the expectations created by the assertion that the host's factors could contribute to the development of GC are disappointing, at least as far as the host's inflammatory response to *H. pylori* infection is concerned. Once we get into details, we realize that in fact, the only determinant that really matters is the infection. The examination of the scientific literature on the cytokine subject has led to contradictory results: For each cytokine, the observations made by studying Caucasian people cannot be applied tout course to Asians; in certain cases, we get opposite results. In the different surveys, one can find association of determined haplotypes of inflammatory cytokines with an increased risk of GC, the opposite, or nil. Even the results of meta-analyses do not agree one another, according to whether studies are carried out by Chines or researchers from other nations. What does it mean? Is it because cytokines are not the final effectors, as they principally work on the long and winding road paved by the broad NF- κ B family, which leads to GC (which means that the final response to inflammatory stimuli is far from hitting its target)? And what about the observations that people suffering from diseases far more inflammatory than chronic gastritis, such as rheumatoid arthritis, are likely protected from developing GC^[82].

These observations may suggest that, if host factors are important in GC development, they probably have to be sought outside of the genes encoding the inflammatory cytokines.

NOT ALL PATIENTS ARE ALIKE:

MOLECULAR BIOLOGY

The recent advances of molecular biological techniques allowed researchers to reach important insights into the oncogenesis mechanisms in gastric cancer. Besides the well-known pathogenic factor, *H. pylori*, several oncogenes and tumour suppressor genes, including cell cycle regulation genes involved in the growth and signal transduction pathways, have been identified^[83-85]. In particular, alterations of genes involved in signalling pathways deregulation, patterns of aberrant DNA methylation, and chromosomal imbalances have been evidenced^[86,87].

CHROMOSOMAL INSTABILITY

Chromosomal instability (CIN) represents one of the main type of genomic instability observed in several neoplasms and it has been observed in a large cohort of patients with gastric cancer^[88]. In particular, it is commonly detected in gastric malignant tumours and has been shown in up to 84% of gastrointestinal cancers^[89].

CIN is characterized by chromosomal anomalies, including gain or loss of the complete chromosome (aneuploidy) and segments of chromosomes (loss of heterozygosity, amplifications and translocations)^[90]. These abnormalities can impact on the oncogenes expression, tumour suppressor genes and other genes, as well as those involved in digestion, DNA repair, growth regulation, and control of cell cycle checkpoint^[91-93]. The genetic mechanisms leading to CIN are not entirely known; *H. pylori* infection, smoking habit and some chemical substances such as nitrates and nitrites probably have an effect on inducing CIN; anyway their influence is actually uncertain^[94]. On the other side, defects of chromosome segregation (CS), imperfect DNA damage response (DDR), anomalies in cell cycle regulators and telomere dysfunction have been identified as factors leading to numerical and structural chromosome alterations^[95,96]. These carcinogens may alter chromosomes and the cytoskeleton promoting malignant modification^[97].

CS alterations

CS represents an important cellular process inducing the gastric epithelial cells division. Alterations of CS regulating mechanisms can cause DNA alterations or mitotic failures, leading to unfixable mutations as well as chromosomal number alterations^[98]. In particular, the three recently proposed ways producing CIN are: Altered expression, polymorphisms and/or mutations of mitotic genes implicated in CS and the carcinogen activity upon

susceptible genetic background of individuals^[99,100]. Many authors showed an aberrant expression of mitotic genes in CS. Moreover, the altered expression of BUB1 protein (involved in controlling the spindle assembly checkpoint), was significantly increased in patients with diffuse type gastric adenocarcinoma, but not related to DNA ploidy^[89]. Furthermore, in another study, BubR1 and AURKB (proteins involved in the mitotic spindle assembly) expression resulted in association with a low risk of GC progression^[101-103]. Aurora kinase A (AURKA/STK15), a cell-cycle-regulated kinase with important role in microtubule formation and stabilization during CS, is often overexpressed in adenocarcinomas of the stomach, showing a suggestive new oncogenic pathway in GC^[104].

Defective DDR

The mucosa of the stomach is continually subject to several environmental and intracellular mutagens, like ROS, *H. pylori* infection, nitrates, sodium, nitrites, and other water and food contaminants, able to induce DNA damage through different mechanisms^[105,106]. Failure of the most important mechanisms of repair [nucleotide excision repair, base excision repair, mismatch repair (MMR) and recombination and/or DDR] may conduce to CIN and genetic aberrations, favouring carcinogenic process^[107,108]. Several studies revealed differential mRNA expression of genes implicated in DNA repair process: *ATM* and *HMGB1* (implicated in base excision repair), *RAD23B* (involved in nucleotide excision repair), *UBE2V2*, *MUS81* [involved in resolving Holliday junctions (a branched DNA structure that contains four double-stranded arms joined together, considered the central intermediate in homologous recombination)], *REV3L* (involved in replication post-DNA damage), *TP53*, *hHR23A* and *DDB1* (implicated in nucleotide excision repair), and *XRCC1* (implicated in single-strand breaks repair) and *MUTYH* (implicated in base excision repair)^[109-113].

H. pylori

H. pylori has been shown to be able to induce DDR and double-strand breaks in gastric cancer with a mechanism of adhesion of bacteria that takes place between Lewis epitopes of the host and BabA adhesin^[114]. Anyway, gastric mucosa cells can repair the DNA lesions induced by short-term infections. On the other side, prolonged infections induce saturation of repair mechanisms with a consequent ineffective DNA repair and malignant process begin. Moreover, continued infections lead to chronic inflammation, with resulting increase of mutagenesis rate, oxidative-stress, down-regulation of MMR mechanisms, instability of genes and modulation of apoptosis by means of ROS formation^[115-119]. Gastric inflammation represents an important host response able to induce *H. pylori*-related carcinogenesis^[120]. In fact, in infected patients with *IL-1 β* , *TNF- α* , *IL-10* and *IL-8* polymorphisms, has been observed an in-

creased risk of distal gastric cancer progression^[120,121]. Furthermore, different *H. pylori* strains seem to differently increase cancer risk by means of host genotypes^[122] as these bacteria are able to communicate with their hosts. The equilibrium is determined both by host and bacterial features and may explain the reason why some *H. pylori* strains augment the carcinogenesis risk. For example, CagA positive strains promote severe gastritis and increase the pro-inflammatory cytokines' level. This may lead to an environment favourable to the growth of other bacteria that can support inflammation and continually induce oxidative stress, increasing the risk for GC^[1].

MICROSATELLITE INSTABILITY

Microsatellite instability (MSI) represents a genomic instability commonly detected in almost half of patients with GC. It is often observed in the Lynch syndrome (hereditary non-polyposis colorectal cancer) and in several sporadic cancers^[123]. MSI phenotype is characterized by a high replication mistake rate leading to insertions and/or deletions of nucleotides within microsatellite repeats in neoplastic areas^[123]. The MMR proteins are able to detect and repair these alterations, causing the dysfunction in MMR genes (*MLH1* and *MSH2*) a MSI phenotype's establishment, with a consequently power off of cancer suppressor genes' and loss of heterozygosity^[124,125]. To this address, genes that are frequently modified induce cell cycle regulation and apoptosis (*TGF β RII*, *RIZ*, *IGFIR*, *TCF4*, *BAX*, *FAS*, *CASPASE5*, *BCL10* and *APAF1*) or are involved in the maintenance of genomic integrity (*MSH6*, *MED1*, *MSH3*, *BLM*, *RAD50*, *ATR*, and *MRE11*)^[126].

DEREGULATION OF SIGNALLING TRASDUCTION PATHWAYS

The effects of genomic destabilization consist of aneuploidy and gain or loss of the chromosome tracts involved in mRNA transcription. Genomic alterations can modify the normal cellular biology with a consequent neoplastic switch^[127]. The clearly explored pathways that probably are involved in gastric pathogenesis are Wnt/betacatenin, extracellular signal-regulated MAPK, Hedgehog, Notch, NF- κ B, TGF- β /BMP pathways, COX2/PGE₂, and tyrosine kinase signalling^[128-143].

Finally, several studies evidenced that pathway deregulation involved in systemic inflammatory response, such as IL-11/STAT1/gp130/STAT3, can induce a carcinogenic transformation too^[144,145].

CONCLUSION

GC is a multifactorial disease. The main determinant, *H. pylori* infection, can be considered a *sine qua non* for GC development; however, despite almost all individuals who get GC are currently, or have been infected, it

is neither a necessary nor a sufficient condition. The intricacy of this topic resides in the proportion of infected people that will never get GC: 97% to 99%, according to the ethnic groups and geographic areas. Other remarks are unraveling the tangle: Almost all *H. pylori* strains from Japan and East Asia, the areas with the highest incidence of GC, are *cagA*+ and can be considered highly carcinogenic; in addition, the infection by certain *cagA* genotypes in western countries increases by far the risk of GC.

At this point, one may wonder why these strains keep on infecting people. Following along with the evolution, only the characteristics that provide a selective advantage continue to be transmitted; this is a basic rule in eukaryotic and prokaryotic worlds. What is the benefit of being infected by carcinogenic strains? Why do they not disappear? People infected by strains that multiply the risk of GC by many times over, cannot be considered advantaged. Possible answers could reside in the following observations: (1) 97% to 99% of people never acquire GC; (2) the development of the sequence gastritis-metaplasia-dysplasia-cancer takes 40 years, or more, after the infection; which means that all women, as well as men, are fertile before the age at which GC occurs (many men also in old age, but they are less important, statistically speaking); hence, fertility is not affected by cancer development; and (3) women, who develop GC far less frequently than men, are the necessary genetic traits holders. Could these answers satisfy the laws of evolution (or distract them)? And how can the occurrence of GC in younger and younger ages be explained?

Apart from the complexity of this subject, the prospect created by the assertion that the host's factors, such as the way the host reacts to infectious stimuli, may be important in the development of GC is discouraging. Despite cytokines involved in the inflammatory response to infection, there are more than 30, just over half a dozen that have been examined in the relationship of *H. pylori* infection with GC and only haplotypes of *IL-1* and *TNF- α* genes were found to possibly increase the GC risk, but only if the ethnicity of patients is not considered. Pursuing this line of inquiry makes us run the risk of sounding racist.

The hypothesis that the dissection of oncogenes and tumour suppressing genes could provide us with an answer to the question whether host factors are important in GC development has only been partly proved. However, the conclusions have led us to a starting point, that is, they ended to indirectly confirm the pathogenic role of strains expressing CagA. The local and systemic levels of substances endowed with an increased mutagenic potential, ROS, generated by immunocytes, and the consequent DNA damage are far higher when the infecting organisms harbour the *cag* PAI.

In conclusion, as regards the development of GC, not all the *H. pylori* strains and patients are alike and not all share the same responsibility, but the only deter-

minant that really matters is the infection.

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State of the art biological therapies in pancreatic cancer

Mariacristina Di Marco, Elisa Grassi, Sandra Durante, Silvia Vecchiarelli, Andrea Palloni, Marina Macchini, Riccardo Casadei, Claudio Ricci, Riccardo Panzacchi, Donatella Santini, Guido Biasco

Mariacristina Di Marco, Elisa Grassi, Sandra Durante, Silvia Vecchiarelli, Andrea Palloni, Marina Macchini, Guido Biasco, Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Sant'Orsola-Malpighi Hospital, 40138 Bologna, Italy

Riccardo Casadei, Claudio Ricci, Department of Medical and Surgical Sciences, University of Bologna, Sant'Orsola-Malpighi Hospital, 40138 Bologna, Italy

Riccardo Panzacchi, Donatella Santini, Pathology Unit, Sant'Orsola-Malpighi Hospital, 40138 Bologna, Italy

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Correspondence to: Elisa Grassi, MD, Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Sant'Orsola-Malpighi Hospital, Massarenti Street 11, 40138 Bologna, Italy. elisa.grax@gmail.com

Telephone: +39-051-2143812

Fax: +39-051-6364037

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal malignancies with a five-year survival rate of approximately 5%. Several target agents have been tested in PDAC, but almost all have failed to demonstrate efficacy in late phase clinical trials, despite the better understanding of PDAC molecular biology generated by large cancer sequencing initiatives in the past decade. Erlotinib (a small-molecule tyrosine-kinase inhibitor of epidermal growth factor receptor) plus gemcitabine is the only schedule with a biological agent approved for advanced pancreatic cancer, but it has resulted in a very modest survival benefit in unselected patients. In our work, we report a summary of the main clinical trials (closed and ongoing) that refer to biological therapy evaluation in pancreatic cancer treatment.

Key words: Pancreatic cancer; Molecular characterization; Targeted therapy; Epidermal growth factor receptor inhibitors; Embryonic pathway inhibitors; Antiangiogenic therapies

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Core tip: Our study aims to give an overview of the progress made in molecular targeted therapy for pancreatic cancer in recent years and the current status of clinical trials in the field. Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal malignancies with a five-year survival rate of approximately 5%. Several target agents have been tested in PDAC but almost all have failed to demonstrate efficacy in late phase clinical trials, even with a better understanding of PDAC molecular biology generated by large cancer sequencing initiatives in the past decade. Erlotinib (small-molecule tyrosine-kinase inhibitor of epidermal growth factor receptor) plus gemcitabine is actually the only schedule

with a biological agent approved for advanced pancreatic cancer, but it resulted in a very modest survival benefit in unselected patients. In our work, we reported a summary of the main clinical trials (close and ongoing) that refer to biological therapy evaluation in pancreatic cancer treatment.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal malignancies, representing the fourth leading cause of cancer death. The five-year survival rate is approximately 5%, and surgery remains the most effective treatment^[1].

Unfortunately, only 20% of patients are suitable for radical resection, and recurrence of disease occurs in 80% of patients who undergo resection^[2].

The most important improvement concerns the conventional chemotherapy, represented by FOLFIRINOX and gemcitabine plus nab-paclitaxel regimens, but it results in a modest outcome advantage^[3,4].

No significant progress has been made in the field of targeted therapy. Erlotinib [a small-molecule tyrosine-kinase inhibitor of epidermal growth factor receptor (EGFR)] plus gemcitabine is actually the only schedule with a biological agent approved for pancreatic cancer, but it results in a very modest survival benefit in unselected patients^[5].

In recent decades, several combinations of classic chemotherapy and novel biological agents have been studied, but they have not improved overall survival, and furthermore, those trials did not use biomarkers to select responder patients^[6].

Our study aims to give an overview of the progress made in molecularly targeted therapy for pancreatic cancer in recent years and the current status of clinical trials in the field, as summarized in Tables 1-3.

MOLECULAR CHARACTERIZATION OF PDAC: HAS A BETTER UNDERSTANDING OF THE TUMOUR'S MOLECULAR BIOLOGY REALLY IMPROVED TARGETED THERAPY APPLICATIONS?

Large cancer sequencing initiatives generated a large quantity of data in the past decades. Those findings showed a complex genomic landscape characterized particularly by inter-tumoural and intra-tumoural hetero-

geneity involving genomic aberration^[7].

With the exception of the well-known KRAS, TP53, CDKN2A and SMAD4 alterations occurring at respective frequencies of 71%, 49%, 22% and 20%, a large number of genomic rearrangements with mutational frequencies less than 2% were found^[8,9].

The majority of single gene mutations in pancreatic cancer can be grouped into common cellular pathways. Jones *et al.*^[10] identified 69 mutated gene sets in most of the 24 samples analysed in their pioneering sequencing study, of which 31 could be grouped into 12 core signalling pathways. These pathways included KRAS signalling, the transforming growth factor β (TGF- β) pathway, DNA damage control, apoptosis, regulation of G1/S cell cycle transition, Hedgehog signalling, the homophilic cell adhesion pathway, integrin signalling, TGF- β signalling, Wnt/Notch signalling, and the invasion pathway^[10].

Genomic heterogeneity, a characteristic of PDAC, implies genomic instability, which is due to the acquisition of telomere dysfunction and abnormal cell-cycle control occurring predominantly in early cancer stages, but it persists after cancer dissemination, resulting in parallel evolution among different metastases. Cell clones arranging metastasis may require other driver mutations compared with primary tumour cells implementing genetic variation in pancreatic cancer^[11,12].

Given this molecular complexity, it is very difficult to separate passenger from driver mutations, to identify molecular mutations with a crucial role in pancreatic carcinogenesis that can be developed into actionable molecular targets of novel biological agents or to identify patients potentially responsive to existing agents already approved for human use in other cancers (Figure 1), and currently no predictive or prognostic biological factors are employed in clinical practice.

TARGETED THERAPY IN PDAC

EGFR pathway inhibitors

EGFR is a transmembrane receptor member of the ErbB family with a tyrosine kinase domain that is activated by many ligands including epidermal growth factor (EGF), TGF- α , heparin-binding EGF, amphiregulin, epiregulin, betacellulin and neuregulin (an epidermal growth factor). EGFR is involved in cell cycle regulation, cell survival, adhesion and differentiation through activation of the Ras/MAP kinase, phosphatidylinositol 3'-kinase (PI3K)/Akt, Janus kinase/Stat and phospholipase C/protein kinase C pathways. Several trials showed that EGFR is overexpressed in up to 90% of pancreatic cancer samples. Therefore, inhibitors targeting EGFR have been considered a promising therapeutic agent^[13].

Erlotinib is a tyrosine kinase inhibitor (TKI) molecule that competes with ATP for binding to the kinase domain, thereby blocking downstream signal transduction. A possible therapeutic role was evaluated in a large phase III trial, enrolling 569 chemotherapy naïve patients with locally advanced or metastatic pancreatic

Table 1 Principal phase III clinical trials involving targeted therapy in pancreatic cancer

Agent	Target pathway	Treatment	Setting	n	mOS (mo)	PFS (mo)	FDA approval	Ref.
Erlotinib	EGFR signaling	GEM plus erlotinib vs GEM plus P	M/LA	569	6.24 vs 5.91 (<i>P</i> = 0.038)	3.75 vs 3.55 (<i>P</i> = 0.004)	Yes	[5]
Cetuximab	EGFR signaling	GEM plus cetuximab vs GEM	M/LA	766	6.5 vs 6 (<i>P</i> = 0.14)	3.5 vs 3 (<i>P</i> = 0.058)	No	[17]
Tipifarnib	KRAS pathway	GEM plus tipifarnib vs GEM	M/LA	688	6.3 vs 6 (<i>P</i> = 0.75)	3.7 vs 3.6 (<i>P</i> = 0.72)	No	[30]
Ganitumab	IGFR pathway	GEM plus ganitumab (12 mg/kg or 20 mg/kg) vs GEM plus P	M	800	12 mg/kg arm 7.0 vs 7.2 (<i>P</i> = 0.494) 60 mg/kg arm 7.1 vs 7.2 (<i>P</i> = 0.397)	12 mg/kg arm 3.7 vs 3.6 (<i>P</i> = 0.520) 60 mg/kg arm 3.7 vs 3.7 (<i>P</i> = 0.403)	No	[35]
Bevacizumab	Angiogenesis	GEM plus bevacizumab vs GEM plus P	M/LA	602	5.7 vs 6.0 (<i>P</i> = 0.40)	4.8 vs 4.3 (<i>P</i> = 0.99)	No	[36]
Aflibercept	Angiogenesis	GEM plus aflibercept vs GEM plus P	M/LA	546	6.5 vs 7.8 (<i>P</i> = 0.203)	3.7 vs 3.7 (<i>P</i> = 0.864)	No	[38]
Axitinib	Angiogenesis	GEM plus axitinib vs GEM plus P	M/LA	632	8.5 vs 8.2 (<i>P</i> = 0.543)	4.4 vs 4.4 (<i>P</i> = 0.520)	No	[41]
Marimastat	Tumor stroma	GEM plus marimastat vs GEM	M/LA	239	5.4 vs 5.4 (NA)	3 vs 3.1 (NA)	No	[75]

GEM: Gemcitabine; P: Placebo; mOS: Median overall survival; PFS: Progression free survival; n: Number of patients enrolled; LA: Locally advanced cancer; M: Metastatic cancer; NA: Not available.

Table 2 Principal phase II clinical trials involving targeted therapy in pancreatic cancer

Agent	Target pathway	Treatment	Setting	n	Ref.
Cetuximab	EGFR signaling	GEM plus cisplatin plus cetuximab vs	M/LA	84	[16]
Gefitinib	EGFR signaling	GEM plus cisplatin GEM plus gefitinib (single arm)	M/LA	57	[18]
Trastuzumab	EGFR signaling	GEM plus trastuzumab (single arm)	M/LA	34	[20]
Trastuzumab	EGFR signaling	Capecitabine plus trastuzumab (single arm)	M/LA 2+/3+ HER-2 expression 3+ HER-2 expression or gene amplification	17 (212 screened)	[21]
Nimotuzumab	EGFR signaling	GEM plus nimotuzumab (single arm)	M/LA	18	[23]
Nimotuzumab	EGFR signaling	Nimotuzumab monotherapy (single arm)	Refractory to first line standard chemotherapy M/LA	56	[24]
Selumetinib	KRAS/MEK pathway	Capecitabine plus selumetinib vs Capecitabine	Refractory to first line standard chemotherapy M/LA	70	[31]
Trametinib	KRAS/MEK pathway	GEM plus trametinib vs GEM plus P	M/LA	160	[32]
Sorafenib	Angiogenesis	GEM plus sorafenib (single arm)	M/LA	70	[40]
RO4929097	Hedgehog signaling	RO4929097 monotherapy (single arm)	Refractory to first line standard chemotherapy M	18	[57]
Everolimus	mTOR pathway	Everolimus plus capecitabine (single arm)	M/LA	31	[67]

GEM: Gemcitabine; P: Placebo; n: Number of patients enrolled; LA: Locally advanced cancer; M: Metastatic cancer.

adenocarcinoma randomized to receive gemcitabine plus placebo or gemcitabine plus erlotinib 100-150 mg daily. The median overall survival (mOS) and progression free survival (PFS) were modestly, but statistically significantly, improved in the combination arm, 6.24 mo vs 5.91 mo (*P* = 0.038) and 3.75 mo vs 3.55 mo (*P* = 0.004), respectively^[5].

Neither EGFR status nor KRAS status analysed in the

subgroup of patients treated with erlotinib was shown to be predictive of a survival benefit in patients receiving the combination schedule^[14].

Erlotinib has been approved by the FDA in combination with gemcitabine as a first-line treatment for advanced pancreatic adenocarcinoma.

Cetuximab is a monoclonal antibody binding the extracellular domain of EGFR. After encouraging results

Table 3 Principal ongoing trials involving targeted therapy in pancreatic cancer

ClinicalTrials.gov identifier	Agent	Target	Status
NCT01728818	Afatinib	EGFR signaling	Recruiting
NCT01659502	TL-118	Angiogenesis	Not yet recruiting
NCT01621243	Necuparab	Angiogenesis	Recruiting
NCT01088815	Vismodegib	Hedgehog signaling	Recruiting
NCT01096732	Vismodegib	Hedgehog signaling	Terminated
NCT01431794	LDE-225	Hedgehog signaling	Recruiting
NCT00515866	KU-0059436	PARP inhibitor	Completed
NCT01585805	Veliparib	PARP inhibitor	Recruiting
NCT01571024	BKM120	mTOR and PI3K/ Akt pathway	Recruiting
NCT01028495	RX-0201	mTOR and PI3K/ Akt pathway	Completed
NCT01337765	BEZ235 + MEK162	mTOR and PI3K/ Akt pathway	Completed
NCT00560963	Everolimus	mTOR pathway	Completed
NCT00075647	Temsirolimus	mTOR pathway	Completed
NCT01839487	PEGPH20	Tumor stroma	Recruiting

EGFR: Epidermal growth factor receptor; mTOR: Mammalian target of rapamycin.

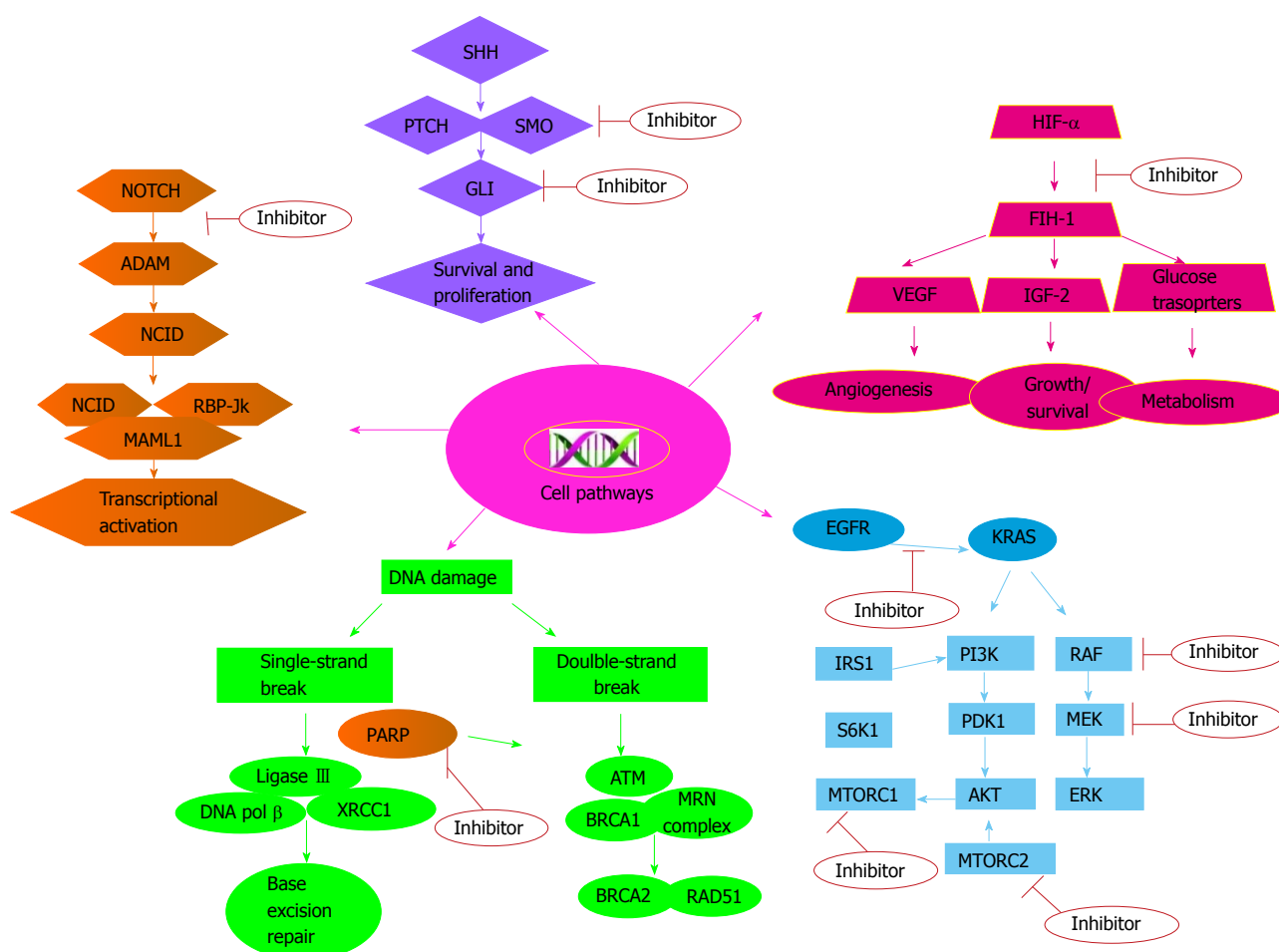


Figure 1 Principal cell signaling pathways involved in pancreatic ductal adenocarcinoma carcinogenesis and actionable molecular targets. SHH: Sonic hedgehog; PARP: Poly ADP-ribose polymerase; VEGF: Vascular endothelial growth factor; IGF: Insulin like growth factor; EGFR: Epidermal growth factor receptor; PI3K: Phosphatidylinositol 3'-kinase.

in a phase I trial, subsequent studies in association with gemcitabine-based chemotherapy have failed to demonstrate any survival benefit^[15,16].

A phase II study has evaluated the possible therapeutic role of gefitinib, a competitive inhibitor of ATP binding to the intracellular kinase domain of EGFR, in

combination with gemcitabine in inoperable or metastatic pancreatic cancer patients. The combination demonstrated promising activity with a mOS and PFS in the combination arm of 7.3 and 4.1 mo, respectively, but other evidence supporting a role of gefitinib in PDAC treatment is lacking^[17].

Another ErbB family of transmembrane tyrosine kinase receptors is HER-2, which is overexpressed in 11% of pancreatic adenocarcinoma cases. HER2-positive status has also been correlated with shorter survival^[18].

Trastuzumab plus gemcitabine was tested in 34 metastatic pancreatic cancer patients with HER-2 overexpression as determined by immunohistochemistry, and partial responses were observed in 6% of cases^[19]. Harder *et al.*^[20] in a multicentre phase II study, investigated the efficacy and toxicity of the HER2 antibody, trastuzumab, plus capecitabine in patients with pancreatic cancer and HER2 overexpression, but this treatment did not perform favourably with respect to either PFS or OS compared with standard chemotherapy.

After FDA approval of lapatinib, clinical trials have been initiated to test the effect of this HER-2 inhibitor combined with chemotherapy in pancreatic carcinoma. In particular, lapatinib was tested in combination with capecitabine as a second-line treatment in advanced pancreatic cancer with promising preliminary results. Further studies are needed to evaluate the real effectiveness and role of this molecule in the treatment of PADC^[21].

Nimotuzumab, another anti-EGFR monoclonal antibody, showed promising results^[22]. In a phase II trial where advanced pancreatic cancer patients were randomized to receive second-line monotherapy with nimotuzumab, Strumberg *et al.*^[23] showed PFS after 1 year of 10.3% and median overall survival of 18.1 wk with a tolerable toxicity profile.

Based on preclinical evidence, afatinib, an inhibitor of EGFR, HER2 and HER4, is under evaluation in an ongoing phase II trial^[24,25].

The KRAS pathway and downstream signalling cascade inhibitors

KRAS activating mutations are present in 70% to 90% of cases of pancreatic cancer. K-Ras is a GTPase protein belonging to the Ras protein family, which has oncogenic activity, and gain-of-function mutations resulting in constitutive activation promote proliferation and inhibit apoptosis through the RAF/MEK/ERK and PIK3/AKT pathways. K-Ras is very difficult to target, and no inhibitors are actually available to use in clinical practice^[26].

Preclinical study has shown that farnesylation is an important post-translational modification required for Ras activation, allowing the protein to be attached to the plasma membrane for signal transduction^[27].

After promising results in terms of anti-proliferative activity in pancreatic tumour cell lines, farnesyl-transferase inhibitors, particularly tipifarnib, failed to improve overall survival either as a single agent or in combination with gemcitabine in a phase III trial^[28,29].

Due to the difficulty of targeting Ras directly, a possible solution could be to block targets downstream of KRAS, such as the protein kinase MEK. Selumetinib is an oral small molecule that inhibits MEK1/2. In a phase II trial, patients were randomized to receive single-agent

capecitabine or selumetinib as a second-line treatment for advanced pancreatic cancer. The selumetinib arm showed a median overall survival of 5.4 mo vs 5.0 mo in the capecitabine arm, but this result was not statistically significant^[30].

Another MEK1/2 inhibitor, trametinib, was tested in pancreatic cancer in combination with gemcitabine against a regimen of gemcitabine plus placebo in a phase II randomized multicentre study. Nevertheless, no significant advantages were demonstrated in terms of overall survival or PFS^[31].

Rigosertib, a first-in-class Ras mimetic and small molecule inhibitor of multiple signalling pathways, including polo-like kinase 1 and phosphoinositide 3-kinase (PI3K), was assessed in combination with gemcitabine in patients with treatment-naïve metastatic pancreatic adenocarcinoma in a phase II/III randomized study, but the combination regimen did not improve survival or response, as recently presented at the 2015 ASCO Annual Meeting^[32].

Research in this field is in development, but the available trials have failed to show any survival benefit.

IGFR pathway inhibitors

Another possible target in ductal pancreatic cancer is represented by insulin like growth factor 1 receptor, which is highly expressed in pancreatic cells, and upon ligand binding activates several pathways involved in cell proliferation and cell survival such as the PIK3/AKT pathway^[33].

Monoclonal antibodies against IGFR (cixutumumab, ganitumab) were evaluated in PDAC treatment, but unfortunately, they failed to show a statically significant survival benefit^[34].

In particular, the phase III trial assessing ganitumab in combination with gemcitabine was closed early based on a pre-planned futility analysis: The median overall survival was 7.1 mo in the maximum dose ganitumab arm vs 7.2 mo in the placebo arm (HR, 0.97, $P = 0.397$)^[35].

Angiogenesis pathway inhibitors

Neoangiogenesis is essential for tumour progression and metastatization mechanisms. Vascular endothelial growth factor (VEGF) stimulates the proliferation of endothelial cells and is overexpressed in human pancreatic cancer. Nevertheless, neoangiogenesis inhibitors, particularly VEGF inhibitors, failed to improve overall survival in combination with gemcitabine in advanced pancreatic cancer. After encouraging results, phase III trials that tested the efficacy of bevacizumab in association with gemcitabine alone, or gemcitabine plus erlotinib, did not confirm previous findings^[36,37].

Aflibercept, a new recombinant fusion protein with extracellular portions of VEGFR-1 and VEGFR-2, which binds VEGF-A, VEGF-B and placental growth factors 1 and 2 thereby inhibiting VEGF-ligand-dependent signalling processes, suppresses tumour growth in pancreatic cell lines and xenografts. Nevertheless, a

phase III study aiming to investigate OS in metastatic pancreatic cancer patients receiving standard gemcitabine and either aflibercept or placebo demonstrated that adding aflibercept to gemcitabine did not improve OS in metastatic pancreatic cancer patients^[38].

Similarly sorafenib, an oral multikinase inhibitor of Raf-kinase, VEGF-R2/-R3 and PDGFR- β , tested alone or in combination with gemcitabine in small phase I and II trials, and axitinib, an anti-angiogenesis agent assessed in combination with gemcitabine, showed no statistically significant efficacy in a phase III trial in advanced PDAC^[39-41].

Phase II studies combining chemotherapy with promising new anti-angiogenic molecular agents, such as TL-118, a nonsteroidal anti-inflammatory oral medication, or necuparanib, which is re-engineered from heparin with possible anti-tumour activity, are underway^[42,43].

Embryonic pathway inhibitors

Hedgehog signalling has a critical role in cell proliferation and survival during embryonic development. Normal pancreatic cells silence this pathway, but pathological activation is observed in many solid tumours, particularly in PADC. Hedgehog binds to the extracellular receptor Patched, which, in the absence of Hedgehog, suppresses activation of the G-protein-coupled receptor Smoothened and upregulates glioma associated oncogene homolog1 transcriptional activity. Cancer cell lines show both Hedgehog ligand-dependent and -independent mechanisms of aberrant signalling^[44].

Bailey *et al.*^[45] showed how Sonic hedgehog (SHH) and other proteins downstream of the Hedgehog pathway, detected in precursor lesions and in PDAC primary tumour samples, contribute to the formation of the desmoplastic reaction, an important characteristic of pancreatic cancer that limits the effective delivery of anticancer agents to pancreatic cancer cells. Genetically engineered mouse models demonstrated a depletion of tumour matrix from SHH pathway inhibition, which could be a promising strategy in pancreatic cancer therapy^[46].

Vismodegib (GDC-0449), an oral small-molecule inhibitor targeting Smoothened^[47], is under assessment in open phase II trials in combination with gemcitabine in advanced cancer, in combination with gemcitabine and nab-paclitaxel in metastatic settings with promising preliminary data^[48], and as a single agent in neoadjuvant settings followed by surgery^[49-51].

The Smoothened inhibitor saridegib (IPI-926) was tested in association with gemcitabine against gemcitabine plus placebo in a randomized, double-blind, placebo-controlled phase II trial enrolling patients with metastatic disease. Unfortunately, this study was closed ahead of time due to evidence of decreased patient survival in the saridegib arm^[52].

Hedgehog inhibitors are an active research field, and several clinical trials are ongoing^[53]. Notch signalling is another embryonic pathway crucial for pancreatic organogenesis, but after pancreas development, it is

active only in a stem cell subgroup. This pathway is upregulated in PDAC and promotes tumourigenesis. Binding of Notch ligand to its receptor promotes a cascade of proteolytic cleavages, mediated by γ -secretase (presenilin). The activated form ICN (intra cellular notch) forms part of a transcription complex that, after translocating to the nucleus, regulates transcription of several genes involved in proliferation and differentiation of cells, interacting with other pathways such as Hedgehog, KRAS and NF- κ B signalling^[54,55].

RO4929097 is a selective inhibitor of the γ -secretase enzyme with anti-tumour activity in preclinical studies^[56].

A recent phase II single-arm trial assessed the possible role of RO4929097, enrolling 18 previously treated advanced PDAC patients. The treatment was well tolerated; the median survival was 4.1 mo, and the median progression-free survival was 1.5 mo^[57].

Encouraging clinical results were observed testing demcizumab, an anti- Delta-like ligand 4 antibody, plus gemcitabine and nab-paclitaxel in advanced PDAC in a phase I b trial. Further evidence is needed to confirm these preliminary data^[58].

PARP inhibitors

Mutations affecting BRCA pathway components, especially the tumour suppressor gene BRCA2, which is associated with hereditary predisposition to breast, ovarian and pancreatic cancer, promote deficiency in DNA damage repair mechanisms and genomic instability^[11].

Poly ADP-ribose polymerase (PARP) is a nuclear enzyme recruited to repair cell DNA damage, and as recent evidence showed, patients with defects in the homologous DNA recombination pathway may benefit from the use of PARP inhibitors as monotherapy or in combination with radiation or other chemotherapeutic agents. Clinical trials testing those new agents in selected patients are currently in the development phase^[59-61].

mTOR and PI3K/Akt pathway inhibitors

After activation, Ras can phosphorylate PI3K, which activates Akt, a serine/threonine kinase. Signal transduction by activated PI3K/Akt plays a role in tumour cell proliferation, survival and metabolism, usually through several downstream targets, including the mammalian target of rapamycin (mTOR)^[62].

Several trials testing PI3K/AKT axis inhibitors are currently ongoing in advanced pancreatic cancer patients after encouraging preclinical model results^[63]. These trials included the following PI3K/AKT axis inhibitors: BKM120, a PI3K inhibitor tested in combination with the mFOLFOX-6 schedule; RX-0201, an Akt antisense oligonucleotide tested in a phase II study plus gemcitabine; and BEZ235, a combined inhibitor of PI3K and mTOR assessed in a phase study in combination with the MEK inhibitor MEK162^[64-66].

Wolpin *et al.*^[67] evaluated a possible role of everolimus, an oral mTOR inhibitor, as monotherapy in 33

gemcitabine-refractory pancreatic cancer patients. The PFS and OS were 1.8 and 4.5 mo, respectively.

Recently, the results of a single arm phase II study where everolimus was tested in combination with capecitabine were published. The median OS was 8.9 mo and PFS was 3.6 mo^[68].

The results of a phase I / II study testing everolimus in combination with gemcitabine in advanced settings and the results of a phase II trial testing temsirolimus, another mTOR inhibitor, in locally advanced or metastatic settings are anticipated^[69,70].

Tumour stroma inhibitors

The stroma is a dynamic compartment of pancreatic tumours that is critically involved in tumour formation, progression and the metastasis process. Therefore, targeting stromal microenvironment elements could be an efficient therapeutic strategy in addition to previously described trials evaluating Hedgehog signalling inhibitors^[71].

After promising data derived from a preliminary clinical study on the possible role of PEGPH20, a pegylated formulation of recombinant hyaluronidase, a phase II trial is currently in the recruitment phase. The purpose of that study is to enrol untreated patients with metastatic disease to receive a combination of PEGPH20, nab-nab-paclitaxel and gemcitabine or a combination of nab-paclitaxel and gemcitabine^[72,73].

Additionally, inhibition of PDGFR, a receptor expressed in stromal cells with a critical role in recruiting pericytes and in interstitial fluid pressure in the tumour stroma, could be an interesting molecular target, as suggested by preclinical studies using an orthotopic pancreatic tumour mouse model^[74].

TKI258, a PDGFR inhibitor, is under evaluation in a phase I dose assessment for advanced pancreatic cancer patients^[75].

In the past, matrix metalloproteinase inhibitors such as marimastat were tested. Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes responsible for the degradation of connective tissue proteins, and aberrant MMP expression is observed in PDAC. Nevertheless, the results of a phase III trial provided no evidence to support a combination of marimastat with gemcitabine in patients with advanced pancreatic cancer^[76].

CONCLUSION

Knowledge of the molecular biology of PDAC has important potential clinical relevance, but current efforts to improve understanding of the mutational profile of this tumour have not provided any significant advantage in the use of targeted therapy. Several agents have been tested in PDAC, but almost all have failed to demonstrate efficacy in late phase clinical trials. Only erlotinib has been approved by the FDA for advanced pancreatic cancer treatment, but the improvement of overall survival is barely 2 wk compared with gemcit-

abine alone^[5].

There could be many reasons for those unsatisfying results. First of all, the extreme genomic heterogeneity of PDAC is an important block to identifying new candidate actionable molecular targets or to testing existing biological therapies already approved for human use for other cancers. In addition, no significant results have been observed by matching targeted agents with patients harbouring the cognate molecular abnormality, such as, for example, the use of trastuzumab in HER2 overexpression cases. Due to poor results derived from targeting a single molecule, new strategies using multitargeted agents or molecular agent combinations are in the development phase in order to block more than one driving genomic aberration and to prevent or evade resistance.

Additionally, the type of chemotherapy used in combination could be a failure factor. Indeed, the majority of trials have combined target agents with gemcitabine, but actually, the first-line schedules are represented by FOLFIRINOX or gemcitabine plus Nab-paclitaxel. Therefore, greater efficacy may be obtained from the combination of target agents with those chemotherapeutic drugs.

Furthermore, most studies in which molecular or chemotherapeutic agents in pancreatic cancer were tested enrolled an unselected population of patients to treat. In the last 3 years, approximately 116 trials specific for PDAC systemic therapy were registered of which only about 8% applied criteria to select a patient subset based upon molecular biomarkers^[77].

FUTURE CHALLENGES

Most studies in which molecular or chemotherapeutic agents in pancreatic cancer were tested enrolled an unselected population of patients to treat. In the last 3 years, approximately 116 trials specific for PDAC systemic therapy were registered of which only about the 8% applied criteria to select a patient subset based upon molecular biomarkers^[77].

To stratify patients, the Australian Pancreatic Cancer Genome Initiative has started a pilot study to evaluate the feasibility of assessing a more stratified approach in the management of pancreatic cancer through predefined actionable molecular phenotypes. Patients are enrolled in this trial, called IMPaCT (Individualised Molecular Pancreatic Cancer Therapy), after a preliminary phenotype screening in order to compare the use of gemcitabine in an unselected population to a stratified approach. The aim of the study is to create a tailored approach to pancreatic cancer treatment, which seems to be one of the major challenges for the future^[78].

Finally, thanks to biotechnology advancement, biological agents can find application in cancer treatment by tumour-targeted delivery of cytotoxic drugs. Particularly, Ahn *et al.*^[79] developed antibody fragment-installed polymeric micelles *via* maleimide-thiol conjugation

for selective delivery of platinum drugs to pancreatic tumours. This antibody-drug conjugate significantly suppressed the growth of pancreatic tumour xenografts. This technology, with potential activity *in vitro* and in a mouse model, could be a promising future strategy in pancreatic cancer therapy^[79].

In conclusion, the lack of efficacy of targeted therapy in PDAC represents a challenge for the future, and more efforts are needed in order to make pancreatic cancer a curable disease.

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Multimodality treatment strategies have changed prognosis of peritoneal metastases

Corneliu Lungoci, Aurel Ion Mironiuc, Valentin Muntean, Traian Oniu, Hubert Leebmann, Max Mayr, Pompiliu Piso

Corneliu Lungoci, Aurel Ion Mironiuc, Valentin Muntean, Traian Oniu, Department of Surgery, "Iuliu Htieganu" University of Medicine and Pharmacy Cluj-Napoca, 400015 Cluj-Napoca, Cluj Province, Romania

Hubert Leebmann, Max Mayr, Pompiliu Piso, Clinic of Visceral and General Surgery, Krankenhaus Barmherzige Brueder, Academic Teaching Hospitals of the University Regensburg, 93049 Regensburg, Bavaria Province, Germany

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Correspondence to: Dr. Pompiliu Piso, Professor of Surgery, Dr. Honoris Causa, Clinic of Visceral and General Surgery, Krankenhaus Barmherzige Brueder, Academic Teaching Hospitals of the University Regensburg, 86 Profeninger Street, 93049 Regensburg, Bavaria Province, Germany. pompiliu.piso@barmherzige-regensburg.de
Telephone: +49-941-3692201
Fax: +49-941-3692206

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Abstract

For a long time, treatment of peritoneal metastases (PM) was mostly palliative and thus, this status was link with "terminal status/despair". The current multimodal treatment strategy, consisting of cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC), has been strenuously achieved over time, but seems to be the best treatment option for PM patients. As we reviewed the literature data, we could emphasize some milestones and also, controversies in the history of proposed multimodal treatment and thus, outline the philosophy of this approach, which seems to be an unusual one indeed. Initially marked by nihilism and fear, but benefiting from a remarkable joint effort of human and material resources (multi-center and -institutional research), over a period of 30 years, CRS and HIPEC found their place in the treatment of PM. The next 4 years were dedicated to the refinement of the multimodal treatment, by launching research pathways. In selected patients, with requires training, it demonstrated a significant survival results (similar to the Hepatic Metastases treatment), with acceptable risks and costs. The main debates regarding CRS and HIPEC treatment were based on the oncologists' perspective and the small number of randomized clinical trials. It is important to statement the PM patient has the right to be informed of the existence of CRS and HIPEC, as a real treatment resource, the decision being made by multidisciplinary teams.

Key words: Hyperthermic intraperitoneal chemotherapy; Peritonectomy procedures; Systemic chemotherapy; Peritoneal metastases; Survival

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Core tip: The multimodal treatment of peritoneal metastases (PM), involving cytoreductive surgery and hyperthermic intraperitoneal chemotherapy, has been strenuously achieved over time, but seems to be the best treatment option, for selected cases. This paper addresses data about the multimodal treatment strategy, focused to patient's survival, the key indicator for assessing results, in the case of PM. Also, it were highlighted the treatment key aspects and the controversies, high in the 35 years of treatment implementing. By understanding the philosophy of multimodal treatment, physicians will be able to offer an alternative to the routine systemic chemotherapy.

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INTRODUCTION

Peritoneal metastases (PM) were described by Sampson *et al.*^[1] (1931) in an ovarian cancer patient. For a long time since then, treatment was mostly palliative and thus, PM was linked to "terminal status/despair". The current multimodal treatment consisting of cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC) has been strenuously achieved over time, but seems to be the best treatment option for selected PM cases.

As we reviewed the literature data, we could emphasize some milestones and also, controversies in the history of PM treatment and thus, outline the philosophy of proposed multimodal radical treatment, which seems to be an unusual one indeed. To understand this radical treatment, we start with the natural evolution of PM and "conventional" systemic chemotherapy approach, in fact the treatment of choice for stage IV cancer, regardless of the dissemination type and location. Four periods must be considered in the evolution of "dedicated" PM treatment: Before 1980; 1980-2000; 2000-2010 and 2010-present. The first time period, before 1980, was a period of palliative intraperitoneal treatment of ascites. In 1980-2000 were proposed the methods and define the foundation laying for a new multimodal approach of PM by intraperitoneal chemotherapy and CRS. The next periods, until 2010, were those of the progressive development of dedicated multimodal treatment strategy, concluding with the actual CRS-HIPEC. From 2010, the studies were focused about new research pathways. The PM treatment approach related survival was the main issue considered in this review.

NATURAL EVOLUTION OF PM

In the literature concerned with PM, the EVOCAPE I study^[2] is classical and it reflected the natural evolution of patients with non-gynecological PM. The mean survival (mS) was 6 mo, significantly correlated with the PM stage (Figure 1), according Gilly system^[3] (nodules/lumps < 5 mm: 9.8 mo; > 2 cm: 3.1 mo). PM of pancreatic origin had the lowest mS (2.9 mo), followed by PM of gastric origin (6.5 mo) and of colorectal origin (6.9 mo). The degree of differentiation had no influence on survival.

Several other "historical"^[4-6] studies confirmed the unfavorable prognosis of PM. Ascites is a negative prognostic factor: In pancreatic cancer, median survival (MS) was < 1 mo, with an important negative impact on the quality of life. Surgery was aimed at palliating gastrointestinal complications, as it was contraindicated in patients with gastric, pancreatic tumors or ascites^[4]. In colorectal cancer (marked by a favorable biological pattern), MS was significantly less for synchronous PM than that in metachronous (7 mo vs 28 mo, $P < 0.001$)^[6].

"CONVENTIONAL" SYSTEMIC CHEMOTHERAPY OF PM

Recent studies on colorectal cancer compared patients having only PM, as a distant metastatic location, with patients having other systemic dissemination. Franko *et al.*^[7] (2012) revealed a significantly lower ($P < 0.001$) global MS (12.7 mo vs 17.6 mo) and disease-free survival (5.8 mo vs 7.2 mo) for patients with PM vs other metastatic locations. Also, the poor global MS of PM metastasis patient was unchanged by various chemotherapy regimens (Figure 2).

Systemic chemotherapy carried MS significant benefit ($P = 0.026$) in colorectal PM patients only compared to those patients who did not receive chemotherapy (Figure 3): Increasing from 5 mo without chemotherapy (95%CI: 3-7 mo) to 11 mo with the fluorouracil-leucovorin protocol (95%CI: 6-9 mo), and to 12 mo with the oxaliplatin-irinotecan protocol (95%CI: 4-20 mo)^[8].

Despite the progressive development of systemic chemotherapy, in a population-based study, Lemmens *et al.*^[9] confirmed the poor MS in PM patients (1995-2001: 7 mo; 2002-2008: 8 mo), unlike that of patients with liver metastases, which underwent improvement (1995-2001: 8 mo; 2002-2008: 12 mo).

"DEDICATED" INTRAPERITONEAL TREATMENT OF PM

Palliative treatment (< 1980)

The first attempts for a treatment approach of peritoneal malignancies began in 1950, with the sporadic use of intraperitoneal chemotherapy for malignant ascites. For this intraperitoneal treatment, hemisulphur mustard^[10],

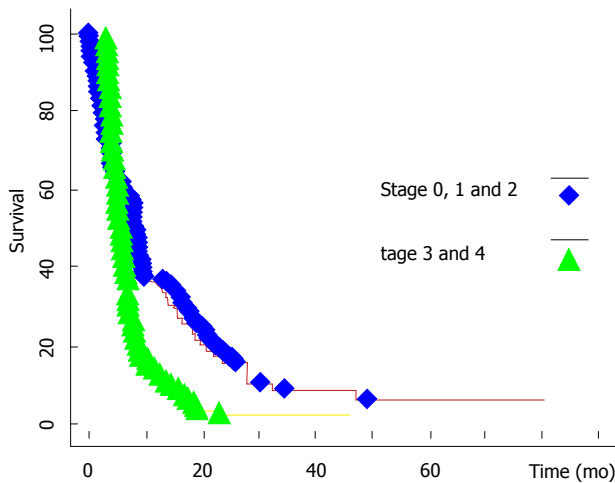


Figure 1 Kaplan-Meier survival curve of non-gynecological peritoneal carcinomatosis, stratified according to the Gilly staging system (Stage 0, 1 and 2 vs Stage 3 and 4)^[2] (with permission). Kaplan-Meier survival curve according to PM staging^[2]. Available from: URL: <http://click.info.copyright.com/?qs=e232cb87f594dcec17211f4d00b2929249713825e6b912c104538a1695c335dde91d3fd5f03fa97>. PM: Peritoneal metastases.

thiotepa^[11], nitrogen mustard^[12], quinacrine^[13] and bleomycin^[14] were used. Nitrogen mustard had a high digestive toxicity, so it was replaced by thiotepa (considered elective for ascites control in 1964), but even at that time it was foreseen that it would be replaced by 5-fluorouracil in digestive cancer palliation^[15]. Nevertheless, nitrogen mustard was the basis for developing further drugs: Cyclophosphamide, chlorambucil, uramustine, ifosfamide, melphalan, and bendamustine.

Multimodal treatment - methods and foundation-laying (1980-2000)

In 1978-1980, the first documented data became available, at first referring to the clearance of intraperitoneal cytostatic drugs^[16], then to circulating intraperitoneal cytostatic solutions, all thanks to the contributions of Speyer *et al*^[17] and Spratt *et al*^[18]. They were “the fathers” of regional intraperitoneal chemotherapy. Spratt augmented the cytostatic effect by hyperthermia using a specially designed device (Thermal Infusion Filtration System). Thus, the foundation was laid for a multimodal treatment of PM by normothermic and HIPEC.

The mechanism by which hyperthermia cytotoxic effects associating with and increasing the cytostatic drug effect is due to certain particularities of these drugs; studies regarding this aspect are exhaustive^[19-25].

The key merit in developing and implementing this multimodal treatment strategy belongs to Sugarbaker, who outlined and detailed the premises substantiating it. The starting model was that of PM in appendiceal cancer^[26]. The most important phase was the adjustment in the existing pathophysiological concept of PM, as a systemic disease and, consecutively, its treatment with systemic chemotherapy. In the new approach, the peritoneum was regarded as an organ (similar to the liver), the pathogeny of PM implying, first and foremost, peritoneal dissemination. Thus, it appears natural to use

a regional treatment in PM^[27-31]. Sugarbaker’s research was regarded mistrustfully, and 25 years had to pass before the “European contributions to the Sugarbaker protocol”^[32] appeared: One multicenter retrospective study^[33], two randomized prospective phase III studies^[34,35] and the use of oxaliplatin and irinotecan as new cytostatic drugs in the protocols for intraperitoneal chemotherapy^[36,37].

Sugarbaker also has the merit of being the first to have described and implemented the surgical procedures associated with regional chemotherapy, generically named “Peritonectomy”^[38]. So, the road lay open for the PM multimodal treatment by CRS and HIPEC.

He then brought in other special aspects regarding the surgical technique of Peritonectomy procedures: Required electrocautery, circumferential skin traction, dissection of subpyloric space and falciform ligament^[39-42]. He also described a method for staging PM and assessing the result of CRS, which were subsequently used in the majority of studies: The “Peritoneal Cancer Index” (PCI), based on the extension of peritoneal injury and the size of peritoneal deposits, respectively, the “Completeness of Cytoreduction Score” (CCRS), based on the size of the remaining peritoneal nodules/lumps^[43].

PM MULTIMODAL TREATMENT - CONFIRMATION, ASPECTS, PATIENT SELECTION, CONTROVERSIES (2000-2010)

“Confirmation”

Although the number of patients progressively and significantly increased and the so-called “long-term survivors” were identified, it took about 20 years before the “confirmations of a multimodal treatment option for PM” appeared. The initiator was Verwaal (2003)^[35], who carried out a randomized clinical trial for patients with colorectal PM. He showed that, during a mean follow-up of 21.6 mo, the MS of patients treated with CRS-HIPEC (22.3 mo) was significantly ($P < 0.032$) improved compared to patients treated with palliative surgery and systemic chemotherapy with fluorouracil-leucovorin (12.6 mo) (Figure 4A).

Verwaal’s research was confirmed by two other reference-worthy studies, which had the merit of comparing CRS-HIPEC with modern systemic chemotherapy treatment, in colorectal PM. Elias *et al*^[44] (2009) compared systemic irinotecan-oxaliplatin chemotherapy with CRS-HIPEC. Global survival in the CRS-HIPEC group (2 years: 81%, 5 years: 51%) was significantly ($P < 0.05$) improved compared to the systemic chemotherapy (2 years: 65%, 5 years: 13%). Franko *et al*^[45] (2010) analyzed systemic chemotherapy with irinotecan, oxaliplatin, bevacizumab, and cetuximab. MS in the CRS-HIPEC treatment group (34.7 mo) was significantly ($P < 0.01$) longer than systemic chemotherapy (16.8 mo) (Figure 4B). It was emphasized that the best results

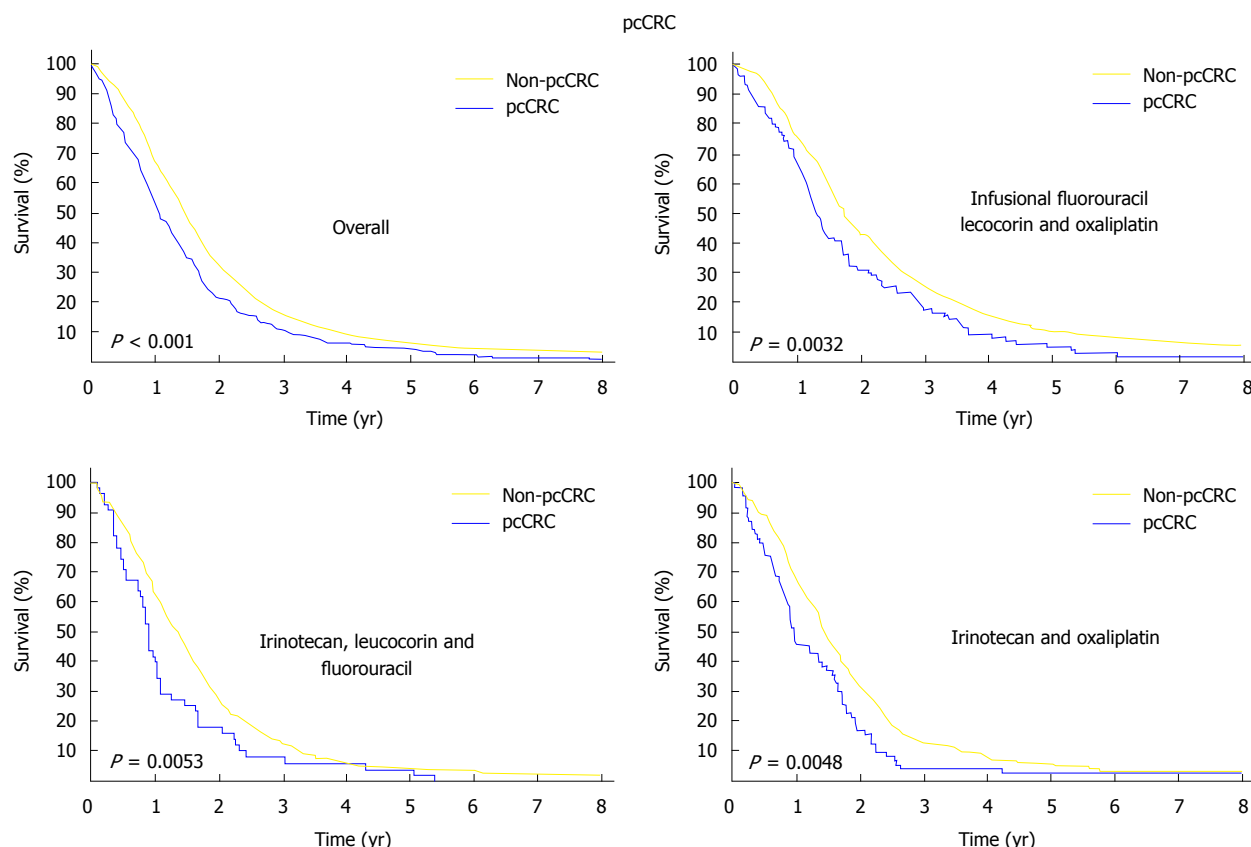


Figure 2 Kaplan-Meier survival curve of metastatic colorectal cancer stratified according to the metastatic locations (peritoneal carcinomatosis vs non peritoneal carcinomatosis) and chemotherapy protocols (fluorouracil, leucovorin and oxaliplatin; fluorouracil, leucovorin and irinotecan; irinotecan and oxaliplatin)^[7] (with permission). Kaplan-Meier survival curve of Metastatic Colorectal Cancer stratified by peritoneal metastatic status and chemotherapy protocols^[7]. Available from: URL: <http://click.info.copyright.com/?qs=da4305023350a474912bb7bdbc04210a63a294c209e715b6497fdff613ea3697d486bdb9bffe0470>. pcCRC: Peritoneal carcinomatosis from colorectal cancer.

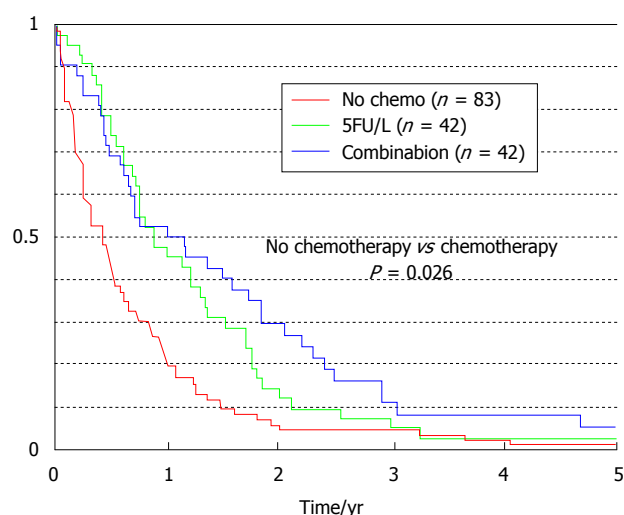


Figure 3 Kaplan-meier survival curve of colorectal peritoneal carcinomatosis stratified by chemotherapy protocols^[8] (with permission). Kaplan-Meier survival curve of Colorectal PM stratified by chemotherapy protocols^[8]. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (Available from: URL: <http://creativecommons.org/licenses/by/2.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. PM: Peritoneal metastases. 5FU: 5-fluorouracil.

systemic chemotherapy.

For gastric PM, studies also showed a benefit in terms of survival. The prospective randomized clinical trial GYMSSA^[46] compared survival in patients treated with CRS-HIPEC and systemic chemotherapy vs systemic chemotherapy treatment alone. Within the limitation of a small number of patients, it showed a longer MS (11.3 mo vs 4.3 mo) for CRS-HIPEC treatment trial arm.

Likewise, Yang *et al.*^[47] showed in a phase III randomized clinical trial the importance of connecting CRS with HIPEC, in the treatment of PM of gastric cancer origin. The CRS-HIPEC association vs CRS alone significantly ($P = 0.046$) increased MS: 11 mo (95%CI: 10-11.9 mo) vs 6.5 mo (95%CI: 4.8-8.2 mo) (Figure 5).

A meta-analysis of randomized clinical trials performed by Yan *et al.*^[48] showed that in advanced gastric cancer, HIPEC associated with surgery led to a significant increase in survival, compared with patients benefiting from surgery alone.

In addition to randomized clinical trials (the gold standard in the treatment implementation), there are a series of multi-center studies showing survival results for patients treated with CRS-HIPEC.

Thus, several multi-center studies, focused on pseudomyxoma peritonei, colorectal and ovarian cancers, were conducted in France (Figure 6). For the treatment of

were achieved by associating CRS-HIPEC treatment with

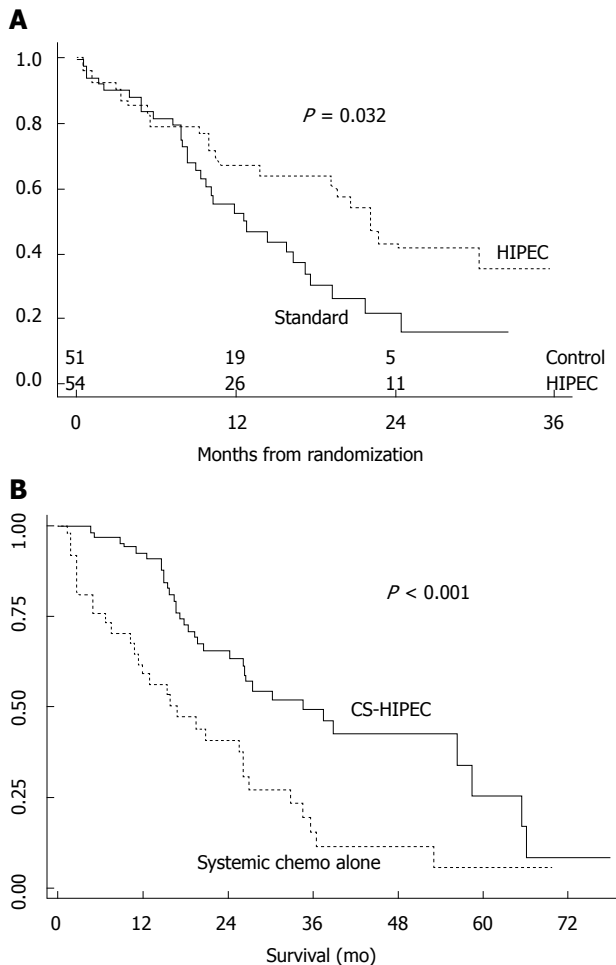


Figure 4 Kaplan-Meier survival curve comparing hyperthermic intraperitoneal chemotherapy to standard treatment, for colorectal peritoneal carcinomatosis^[35] (with permission) (A) and comparing cytoreductive surgery and hyperthermic intraperitoneal chemotherapy treatment to systemic chemotherapy alone, for colorectal peritoneal carcinomatosis^[45] (with permission) (B). A: Kaplan-Meier survival curve comparing HIPEC to standard treatment, for Colorectal PM^[35]. Available from: URL: <http://click.info.copyright.com/?qs=2b7046b10dd0c5471bbb88bac0096e00e895f442c0326423c4ec2ccdd7b4701b99fd4bd5b32f0283>; B: Kaplan-Meier survival curves comparing CRS-HIPEC treatment to systemic chemotherapy alone, for Colorectal PM^[45]. Available from: URL: <http://click.info.copyright.com/?qs=2b7046b10dd0c5471bbb88bac0096e00e895f442c03264235cab52a23497a2d7e22ca0949c16bf1f>. CRS-HIPEC: Cytoreductive surgery and hyperthermic intraperitoneal chemotherapy; PM: Peritoneal metastases.

pseudomyxoma peritonei, CRS-HIPEC was designated the “gold standard” due to the yielded results (at 5 years: 73% global survival, 56% disease-free survival)^[49]. Favorable results were shown for colorectal cancer (at 5 years: 27% global survival, 10% disease-free survival)^[50], and also for ovarian cancer (global survival: Advanced forms 35.4 mo, recurrent forms 45.7 mo)^[51].

The results were also confirmed by a long-term data analysis in the Netherlands, after the implementation of the CRS-HIPEC treatment: MS was 33 mo (95%CI: 28-38 mo) in colorectal cancer and 130 mo (95%CI: 98-162 mo) in pseudomyxoma peritonei (Figure 6)^[52].

The experience of a reference center for PM treatment (St. George's Hospital, Sydney) sustains the higher results obtained by the use of CRS-HIPEC treatment in

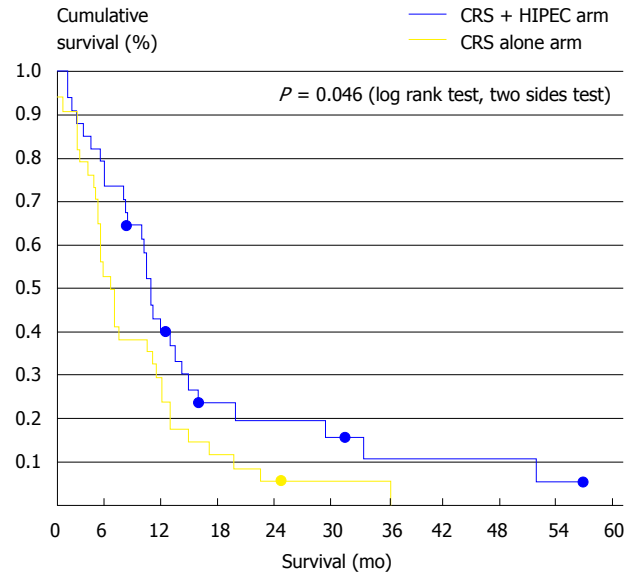


Figure 5 Kaplan-Meier survival curves comparing cytoreductive surgery and hyperthermic intraperitoneal chemotherapy treatment to cytoreductive surgery alone, for gastric peritoneal carcinomatosis^[47] (with permission). Kaplan-Meier survival curves comparing CRS-HIPEC treatment to CRS alone, for Gastric PM^[47]. This is an open access article distributed under the terms of the Creative Commons Attribution Noncommercial License, which permits any noncommercial use, distribution, and reproduction in any medium, provided the original author(s) and source are credited. CRS-HIPEC: Cytoreductive surgery and hyperthermic intraperitoneal chemotherapy; PM: Peritoneal metastases.

pseudomyxoma peritonei (MS 104 mo; 5-year survival 75%) and colorectal cancer (MS 33 mo; 3-year survival 46%)^[53].

A series of systematic reviews were conducted under the leadership of Sugarbaker, demonstrating a better survival for CRS-HIPEC treatment compared to conventional systemic chemotherapy. PMs of different origins were analyzed: Colorectal^[54], gastric^[48], ovarian^[55] cancers, and malignant peritoneal mesothelioma^[56]. Other systematic review studies also reported higher results for CRS-HIPEC in colorectal^[57] and gastric^[58] cancers.

All these studies (numerous and enrolling an increased number of patients) shows joint international efforts to identify the role of CRS-HIPEC in multimodal PM treatment. They have allowed the development of an important medical database which, by confirming the higher results in terms of survival and disease-free survival, upholds this treatment strategy. This is also confirmed by the evidence-based medicine approach studies^[59].

The MS, as a result of CRS-HIPEC treatment, related to the tumor entities and study type, were presented in Table 1.

As a recognition of the foundation-laying treatment of PM with CRS-HIPEC, this was included in the treatment guidelines in France^[60], Germany^[61], United Kingdom (<http://www.nice.org.uk/guidance/IPG331/PublicInfo>), the Netherlands^[52,62]. There are a number of ongoing symposia focused on Peritoneal Surface Malignancies, under the patronage of the European Society of Surgical

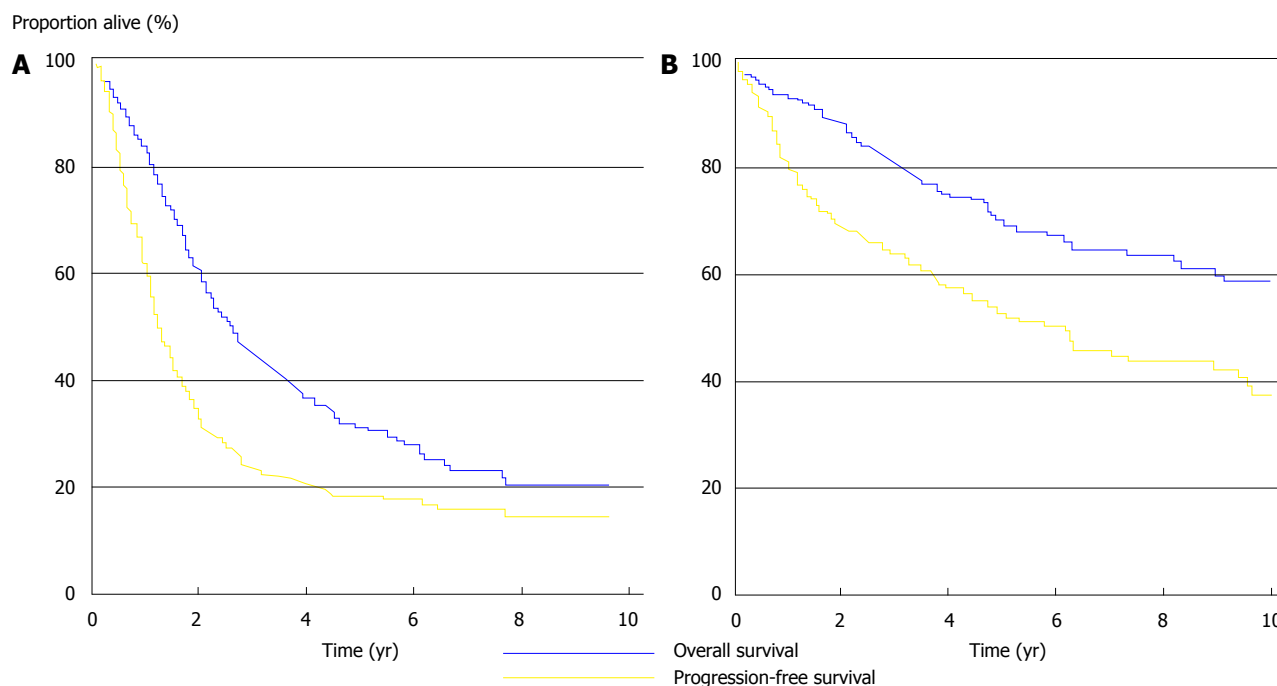


Figure 6 Overall survival compared with disease-free survival for pseudomyxoma peritonei (A) and colorectal peritoneal carcinomatosis (B), treated with cytoreductive surgery and hyperthermic intraperitoneal chemotherapy, from the Netherlands^[52] (with permission). Overall and disease-free survival of Pseudomyxoma Peritonei (A) and Colorectal PM (B), treated with CRS-HIPEC, from the Netherlands^[52]. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. CRS-HIPEC: Cytoreductive surgery and hyperthermic intraperitoneal chemotherapy; PM: Peritoneal metastases.

Table 1 Cytoreductive surgery and hyperthermic intraperitoneal chemotherapy median survival, related to the tumor entities and study type

Primitive tumor origin	Study type	Median survival (mo)
Colorectal	Randomized clinical trials	22.3 ^[35]
	Single center experience	33 ^[33] , 34.7 ^[45]
	Systematic reviews	13-29 ^[54]
	Multi-institutional studies	33 ^[52]
Gastric	Randomized clinical trials	11.3 ^[46] , 11 ^[47]
	Systematic reviews	10.4 ^[53]
Pseudomyxoma peritonei	Single center experience	104 ^[53]
Ovarian	Multi-institutional studies	130 ^[52]
	Systematic reviews	35.4-45.7 ^[51]
	Systematic reviews	22-54 ^[55]
Malignant peritoneal mesothelioma	Systematic reviews	34-92 ^[56]

Oncology, as well as a world congress.

The view of those who consider that multimodal PM treatment is "more of an experimental kind, based on common-sense evidence rather than on solid data"^[63,64] is already obsolete. Even so, the confirmation of CRS-HIPEC, as an effective treatment approach by medical studies, such as randomized clinical trials, is not mandatory^[65]. And more than that, the life of patients could be endangered by denying them a treatment resource, since the validation process may take as long as 40 years^[66].

Aspects

Unfortunately, there is no single perfect treatment

option, valid in any setting, so the problems raised were concerned with treatment risk (morbidity, mortality, medical team risk), costs and with the indication of CRS-HIPEC, in terms of prognostic factors.

In the beginning, morbidity and mortality were the key factors initiating distrust among patients and physicians^[67]. The causes of morbidity and mortality may well be suspected to belong to either CRS (surgery proper), or HIPEC (thermal effects of circulating fluid and toxic cytostatic drug effects). In a systematic-review study, morbidity was 21.5% and mortality 4.8%^[68], but literature reports data within a large range: Morbidity 12%-67% and mortality 0%-9%. The main complications include digestive fistulae, postoperative bleeding, pleural-pulmonary complications, bone marrow suppression, hemodynamic instability and renal failure. Protective ileostomy, chest drain and postoperative thoracic imaging are routinely used.

However, it was shown that morbidity and mortality were not significantly increased, compared to extensive organ resection surgery (e.g., Whipple's operation)^[69]. Treatment complications were significantly correlated with the number of Peritonectomy procedures, left diaphragmatic Peritonectomy, duration of surgery and the number of large bowel anastomoses^[70]. The global incidence for the 1st to 4th degree of gastrointestinal toxicities (according to the Common Terminology Criteria for Adverse Events) was 17%, and for symptomatic surgical site infections incidence reached 35.85% (with a global morbidity of 45%)^[71].

The learning curve must be respected by any medi-

cal procedure, even more so when it implies new skills for the surgeon. The “breaking points” of the learning curve in achieving complete CRS, of a morbidity less than 3th degree and an absence of treatment-related mortality are evaluated to be 141, 158 and 144 cases, respectively (the Milan experience), or 126, 134 and 60 cases, respectively (the Bentivoglio experience)^[72].

As the Dutch model of CRS multimodal treatment implementation was analyzed, it was proven that, unlike in the initial stage (experience gathering), in the stage of treatment becoming standard, the percentage of radical surgeries increased significantly (66% vs 86%, $P < 0.001$) and major morbidity (3th-5th degree) decreased significantly (64% vs 32%, $P < 0.001$)^[73].

If there existed accreditation centers and HIPEC registers, coursing through the learning curve could be faster^[74]. Suggestions were made for training in CRS-HIPEC to begin during the residency programmer^[75].

As for the risks the medical team are exposed to during HIPEC, it was shown in several pharmacological studies that, in relation to the HIPEC method (closed/open), with required training, these risks may be reduced to a minimum^[76-78].

The costs implied by CRS-HIPEC treatment are definitely high, but financial calculations show that it is a better solution in terms of treatment results^[53,79-81]. This is why in Germany, HIPEC is adopted, considered a surgical procedure and coded as such^[61].

Patient selection

One of today's challenges in treating PM is patient selection. This is why literature studies are focused on factors/variables correlating with survival.

The randomized clinical trial performed by Verwaal *et al.*^[35] showed that in colorectal cancer, the variables with the highest impact are extension of PM and the radical feature of CRS ($P < 0.0001$) (Figure 7). The GYMSSA randomized clinical trial^[46] also showed that essential conditions for a significant increase in survival in gastric cancer are complete CRS and a PCI ≤ 15 .

A single-center experience, the most comprehensive in terms of the number of patients (1000 patients), shows that the prognostic factors significantly correlated with survival ($P < 0.001$) are: The performance index, the location of the primary tumor, the CCRS, and the center experience^[82]. Another single-center experience (109 patients) correlated survival to the following factors: Histology of non-adenocarcinoma ($P = 0.001$), appendiceal location ($P = 0.001$), absence of liver metastases ($P = 0.01$), and complete resection of all gross disease ($P < 0.001$)^[83].

A multi-center French study shows that in colorectal cancer, CCRS is the most important prognostic factor for MS: 32.4 mo for complete vs 8.4 mo for incomplete CCRS ($P < 0.001$) (Figure 8)^[33]. The multi-center SITILO study^[84] also reveals in colorectal cancer the following independent prognostic variables correlated with survival: PCI, CCRS, and presence of hepatic metastases.

The consensus conference on PM treatment in

colon cancer statutes that the indication of CRS-HIPEC treatment should be based on complete CRS^[85].

All those prognostic factors are in dynamics, the trend being towards broadening the indication range, except for the radical feature of CRS: It is absolutely necessary that this should be complete, or at least optimal. Traditionally, the treatment approach in PM was reserved for colon or appendicular cancer, pseudomyxoma peritonei and peritoneal malignant mesothelioma, but now the range of indications includes rectal, gastric and ovarian cancers. The importance of PCI is not an absolute one, it must be correlated with primary tumor location (colonic PM origin vs gastric), histological grading (well and moderate differentiated vs poorly differentiated and undifferentiated), and the anatomical sites involved (Treitz ligament, porta hepatis and suprahepatic veins, coelic axis and mesenteric vessels). The presence of other systemic dissemination (abdominal or extra-abdominal) was a contraindication for the treatment approach in PM, but this is no longer (Figure 9) the case provided that complete CRS can be obtained^[86-89].

Except for the patient status (evaluated by the performance index), the main CRS-HIPEC treatment contraindication is small bowel involvement^[90], which is regarded as an independent prognostic factor^[91-93].

A series of studies have confirmed the prognostic value of tumor grading in colorectal cancer. The “signet-ring cell” vs other types of differentiation has a significantly poorer MS (14.1 mo vs 35.1 mo; $P < 0.01$) and an increased relapse rate (68.8% vs 43.7%; $P = 0.05$)^[94]. CRS-HIPEC treatment carries no survival benefit in colorectal PM with signet-ring cell histology, unless complete CRS is obtained^[95]. Also, for the aggressive type of pseudomyxoma peritonei, systemic chemotherapy is indicated instead of CRS-HIPEC treatment^[96].

In some studies, the prognostic factors were grouped into scores. The Peritoneal Surface Disease Severity Score may be used to stratify patients in clinical trials^[97]. Different scores used in PM of colorectal origin are: The Peritoneal Surface Disease Severity Score, the Prognostic Score, and the Colorectal Peritoneal Score. The Colorectal Peritoneal Score (value ≥ 6) identifies patients with an unfavorable prognosis in terms of survival, and it does so better than PCI (value > 20) or the other two scoring systems^[98].

Controversies

At the same time, there are also reserved attitudes regarding the CRS-HIPEC approach of PM. This is mainly the position of oncologists, who are refractory to this treatment option, using the argument of the risk/benefit ratio. The theoretical premise they use is based on the lack of difference (pathophysiology, evolution, and treatment) between PM and other systemic dissemination. The treatment is not adapted, so different protocols of systemic chemotherapy (from the classic de Gramont chemotherapy to oxaliplatin, irinotecan and biological molecular agents) are given in different clinical

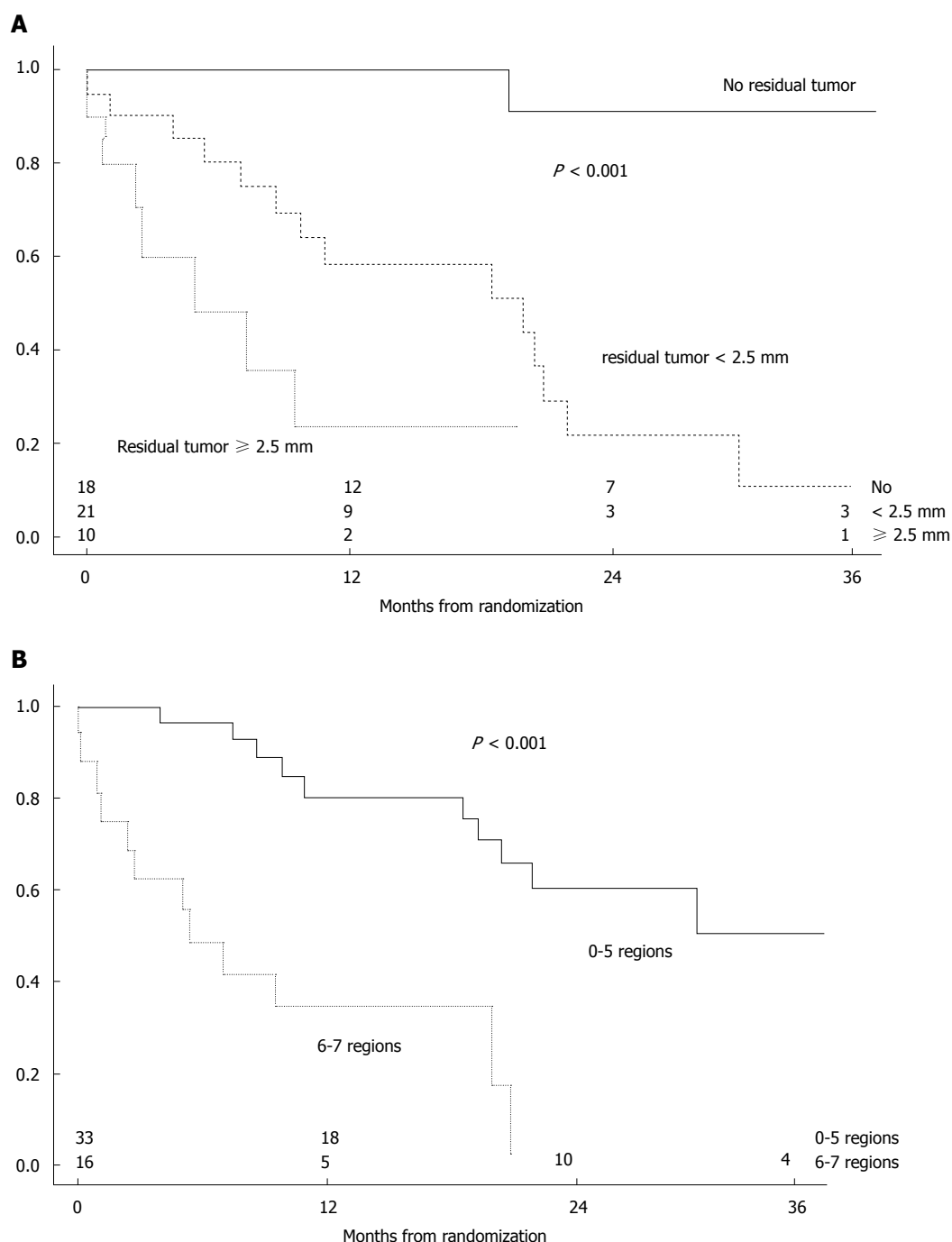


Figure 7 Kaplan-Meier survival curve of colorectal peritoneal carcinomatosis treated by cytoreductive surgery and hyperthermic intraperitoneal chemotherapy, stratified according to the size of residual tumors (A) and the number of regions with residual tumors (B)^[35] (with permission). Kaplan-Meier survival curve of CRS-HIPEC treatment, for Colorectal PM, stratified by the number of regions affected (A) and the number of regions with residual tumors (B)^[35]: <http://click.info.copyright.com/?qs=2b7046b10dd0c5471bbbb8bac0096e00e895f442c0326423c4ec2ccdd7b4701b99fd4bd5b32f0283>. CRS-HIPEC: Cytoreductive surgery and hyperthermic intraperitoneal chemotherapy; PM: Peritoneal metastases.

trials, without considering the variances between the specific dissemination biology^[99-101]. The indispensable condition that oncologists require for inclusion in a clinical trial is the presence of measurable lesions. In the case of PM, this condition is almost impossible to meet. Imaging modalities have a low sensitivity in detecting the peritoneal dissemination, and this is true for computer tomography as well as for magnetic resonance imaging. The only parameters based on which the results of CRS-

HIPEC treatment may be assessed are disease-free survival and global survival.

Furthermore, concerning the oncologists' perspective, there is at least one more important argument supporting the dedicated multimodal treatment of PM: The studies matching hepatic metastases vs PM. These have shown that the pathway of dissemination are different and the treatment results are comparable (Figure 10), if treatment is potentially radical^[102-104].

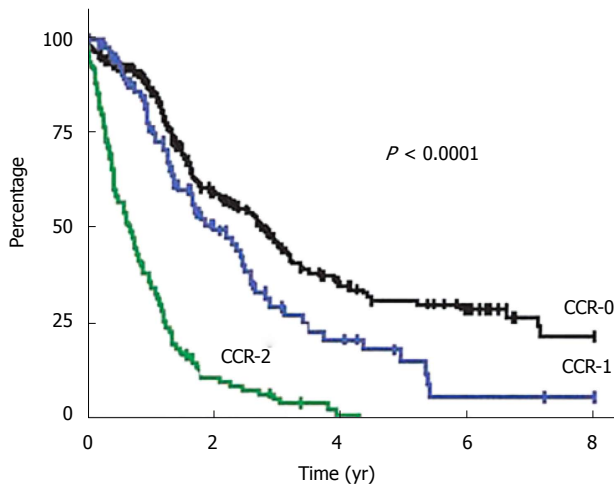


Figure 8 Kaplan-Meier survival curve of cytoreductive surgery and hyperthermic intraperitoneal chemotherapy treatment, for colorectal peritoneal carcinomatosis, stratified by completeness of cytoreduction score^[33] (with permission). Kaplan-Meier survival curve of CRS-HIPEC treatment, for Colorectal PM, stratified by CCRS^[33]. Available from: URL: <http://click.info.copyright.com/?qs=2b7046b10dd0c5471bbb8bac0096e00e895f442c0326423b795894c1892230b84270e7e00c4e014>. CCRS: Completeness of cytoreduction score; CRS-HIPEC: Cytoreductive surgery and hyperthermic intraperitoneal chemotherapy; PM: Peritoneal metastases.

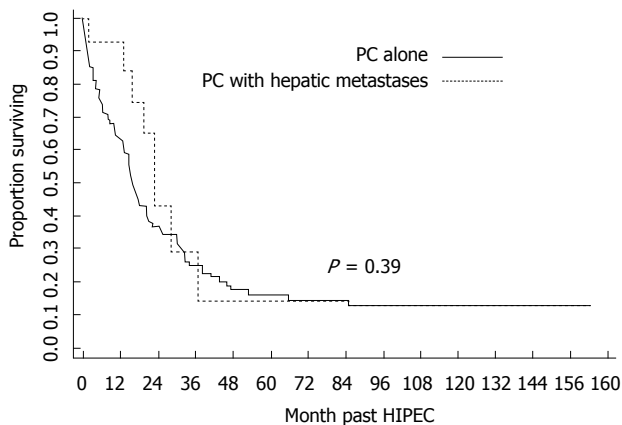


Figure 9 Kaplan-Meier survival curve of colorectal peritoneal carcinomatosis stratified according to hepatic involvement (peritoneal carcinomatosis alone vs peritoneal carcinomatosis with hepatic metastases)^[86] (with permission). Kaplan-Meier survival curve of Colorectal PM alone and PM with Hepatic Metastases^[86]. Available from: URL: <http://click.info.copyright.com/?qs=2b7046b10dd0c5471bbb8bac0096e00e895f442c032642364879a25688a50cc82757e6f8509f6e1>. PM: Peritoneal metastases.

Nowadays, in colorectal cancer, by analogy with Hepatic Metastases, multimodal CRS-HIPEC treatment has been adopted and is the indicated approach for CP, in most institutions in the United States^[31].

PM MULTIMODAL TREATMENT - RESEARCH PATHWAYS (2010-2014)

Currently, there is reason to talk about a higher level, where the problem of timing for CRS-HIPEC is raised. There are studies showing that PM prophylaxis is a valid approach.

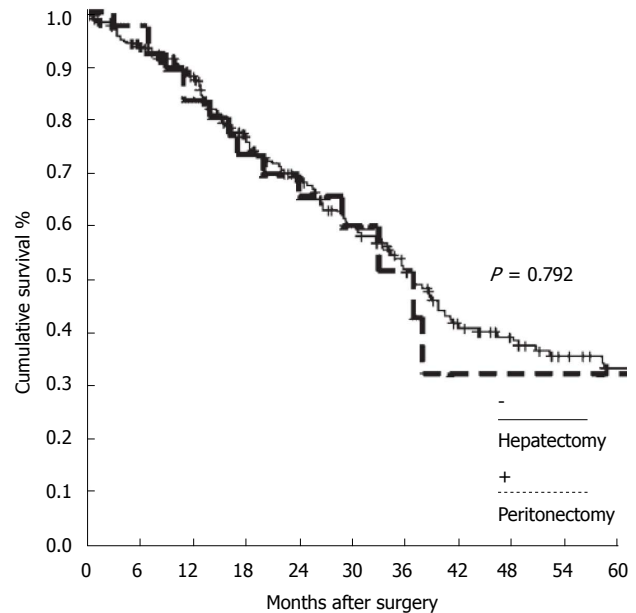


Figure 10 Kaplan-Meier survival curve for colorectal peritoneal metastases and colorectal hepatic metastases, who achieved optimal treatment^[102] (with permission). Kaplan-Meier survival curve for Colorectal PM and Colorectal Hepatic Metastases, with optimal treatment^[102]. Available from: URL: <http://click.info.copyright.com/?qs=751ae7c8f8e03ecb0a4741c79a3b341599b5318899e258b3d458148f31158885e37d98de5cb03229>. PM: Peritoneal metastases.

“Proactive management” defines a treatment concept, targeting patients with a high risk to develop PM, and prescribes surgery with HIPEC. In colorectal cancer, this has brought about a significant results ($P < 0.03$), related to control group (only surgery), in terms of PM developed and local recurrence (4% vs 28%, over a 48-mo follow-up period). Patients had also significant longer MS (59.2 mo vs 52 mo; $P < 0.04$) and disease-free survival ($P < 0.05$)^[105].

There was a hypothesis of second-look surgery at 1 year in patients at high risk for PM, after the first radical surgery for colorectal cancer. PM might be identified and treated at an earlier stage in about 55% of patients^[106]. Such researches were also led for gastric cancer, with promising preliminary results^[107,108].

In the same context of colorectal PM prophylaxis, the HIPEC laparoscopic approach was described and indicated after a mean interval of 6 wk (3-9 wk). This approach showed its feasibility^[109].

The main issue is selecting high-risk patients for developing PM. The debated risk factors are: Invasion of or beyond the serosa (pT3, pT4), perforated tumors, positive peritoneal cytology (augmented by immunohistochemistry), occurrence of Krukenberg tumors and mucinous type of tumor^[110]. Following a systematic review, three situations were identified to be associated with an increased frequency of metachronous PM development: Synchronous PM, ovarian metastases, and perforated tumor^[111].

Despite all human and material efforts dedicated to CRS-HIPEC treatment, there are patients in whom evolving disease occurs. The right question is whether

Table 2 Key aspects and peritoneal metastases model in the evolution of multimodal treatment

Period	PM treatment	Key aspects	PM model
All the period	"Conventional" systemic chemotherapy	Significant lower survival for PM <i>vs</i> other type of metastases	Colo-rectal
1950-1980	"Dedicated" intraperitoneal treatment - Palliative treatment	The basis for developing further cytostatic drugs	Malignant ascites
1980-2000	"Dedicated" intraperitoneal treatment - Multimodal radical treatment	Regional intraperitoneal normothermic and hyperthermic chemotherapy Peritonectomy procedures Define PCI and CCRS	Appendicular
2000-2010	Multimodal radical treatment - confirmation, aspects, patient selection, controversies	Significant higher survival <i>vs</i> palliative surgery and diverse systemic chemotherapy regimes Acceptable morbidity and mortality, no significant risk for medical team Respect de learning curve High costs Define the prognostic factors Position of oncologists Comparison with hepatic metastases	Colo-rectal Appendicular Pseudomyxoma peritonei Malignant peritoneal mesothelioma Gastric Ovarian PM with hepatic metastases
2010-2014	Multimodal treatment - research pathways	PM prophylaxis Laparoscopic HIPEC Integration of chemotherapy with surgery Extension of CRS	High-risk patients for developing PM Recurrent PM

PM: Peritoneal metastases; CCRS: Completeness of cytoreduction score; CRS-HIPEC: Cytoreductive surgery and hyperthermic intraperitoneal chemotherapy; PCI: Peritoneal cancer index.

iterative treatment of PM might be a solution. There are at least three problems: Estimate the morbidity/mortality associated with the iterative treatment; documentation the prognostic factors; estimation the treatment results in terms of survival. The only few related studies define no clear attitude, supported by statistically significant data. Morbidity/mortality does not seem to differ significantly^[112-114], only one single study reporting increased values^[115].

HIPEC remains an important tool in the treatment of recurrent PM. Age ($P = 0.049$), time lapse between surgeries ($P = 0.08$), association of HIPEC ($P = 0.005$), and small bowel resections ($P < 0.001$) are statistically correlated with survival in PM appendiceal origin and malignant peritoneal mesothelioma^[113]. The iterative approach of colorectal PM results in a MS of 22.6 mo, with the following survival percentages: 1 year - 94%; 2 years - 48%; 3 years - 12%^[114].

The iterative approach treatment of the patient with PM and Hepatic Metastases has also promising results^[116].

An important research pathway is the way by which chemotherapy might be integrated into various treatment approaches: Perioperative neoadjuvant; HIPEC; bidirectional intraoperative; early postoperative intraperitoneal. The multidisciplinary approach of PM is based on treatment with complete CRS. Unfortunately, this cannot be achieved in approximately 70% of patients^[31].

The place of systemic chemotherapy in PM mul-

timodal treatment is difficult to assess. There are definite reports regarding the results of perioperative neoadjuvant chemotherapy, demonstrated by histological response in colon cancer^[117] and by increased survival in appendix cancer^[118]. Likewise, in gastric cancer, survival benefits have been shown for perioperative neoadjuvant and early postoperative intraperitoneal chemotherapy^[119].

The complexity of multimodal treatment approach is also certified by the wide range of cytostatic drugs and the occurrence of new principles. It is possible that Mitomycin C, in colorectal PM, in the context of complete CRS, may yield superior survival results compared to oxaliplatin^[120]. For gastric PM, catumaxomab seems to confirm positive results^[119], but in colorectal PM, bevacizumab leads to a double mortality rate after CRS-HIPEC treatment^[121].

Furthermore, as far as the array of CRS treatment is concerned, along with visceral resection and Peritonectomy procedures, surgery on the urinary tract was assessed. Partial cystectomy and ureter segmental resection were the most used. There was no further increase in the recorded morbidity or mortality and survival was comparable to that of patients with CRS-HIPEC without urinary tract surgery^[122,123]. The same statute was dedicated to the hepatobiliary procedures^[124].

At present, there are over 50 clinical trials underway, aimed at assessing multimodal radical PM treatment (<http://clinicaltrialsfeeds.org/clinical-trials/results/?term=HIPEC>). Out of these, some were reported in the literature: GASTRICHIP (D2 gastric resection and HIPEC

for locally advanced gastric cancer)^[125], COMBATAC (multimodal treatment of PM of appendiceal and colorectal origin)^[126], NCT01095523 (second-look and CRS-HIPEC treatment for colorectal cancer at risk for PM)^[127].

Key aspects and PM model in the evolution of multimodal treatment strategies

We summarized the key aspects of the evolution of multimodal treatment strategies in Table 2.

CONCLUSION

Initially marked by nihilism and fear, but benefiting from a remarkable joint effort of human and material resources (multi-center and -institutional research), over a period of 30 years, CRS-HIPEC found its place in the multimodal treatment of PM. The next 4 years were dedicated to the refinement of multimodal treatment, by launching research pathways. In selected patients, with requires training, it demonstrated a significant survival results (similar to the Hepatic Metastases treatment), with acceptable risks and costs. Also, CRS-HIPEC opens a lot of new opportunities with reference to the patients' selection and adopted methodology of this multimodal treatment.

The main debates regarding CRS-HIPEC were based on the oncologists' perspective and the small number of randomized clinical trials. It is hard to find a common view on different challenges, raised by CRS-HIPEC in the treatment of PM. Probably, Dr. Bernard Fisher met the same mistrust as he revolutionized breast cancer treatment and the same could be said about the surgical approach to metastatic melanoma. Indeed, there is a discrepancy between the great number of multi-center, -institutional studies and the small number of randomized clinical trials.

We may say that there are a series of determining factors, for the long-term assessment of multimodal PM treatment and the bias in medical studies type. Treatment complexity, results from the interaction between different therapeutic principles (surgery, chemotherapy, and hyperthermia), are an essential factor. Also, the peritoneal cavity is a complex anatomical space, and the pathogenesis of PM is indeed multifactorial conditioning (loco-regional and systemic dissemination). Not least, the multidisciplinary approach of PM implies the teamwork of specialists with different training, treatment concepts and results assessment, making it difficult to find a common view.

It is important to statement the patients with colorectal, appendicular, gastric, and ovarian peritoneal carcinomatosis, as well as patients with pseudomyxoma peritonei and peritoneal malignant mesothelioma must be informed about CRS-HIPEC as a valid treatment resource. The eligibility criteria for patients' selection will be assessed by multidisciplinary teams, in high level, dedicated treatment centers, according to the performance index, PCI, histological grading, the perspective to obtaining a complete CRS, and the availability of sustaining chemotherapy protocols.

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Molecular approach to genetic and epigenetic pathogenesis of early-onset colorectal cancer

Gulcin Tezcan, Berrin Tunca, Secil Ak, Gulsah Cecener, Unal Egeli

Gulcin Tezcan, Berrin Tunca, Secil Ak, Gulsah Cecener, Unal Egeli, Department of Medical Biology, Faculty of Medicine, Uludag University, 16059 Bursa, Turkey

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Correspondence to: Berrin Tunca, PhD, Department of Medical Biology, Faculty of Medicine, Uludag University, Görükle Kampüsü, 16059 Bursa, Turkey. btunca@uludag.edu.tr
Telephone: +90-224-2954161
Fax: +90-224-4428863

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Abstract

Colorectal cancer (CRC) is the third most frequent cancer type and the incidence of this disease is increasing gradually per year in individuals younger than 50 years old. The current knowledge is that early-onset CRC (EOCRC) cases are heterogeneous population that

includes both hereditary and sporadic forms of the CRC. Although EOCRC cases have some distinguishing clinical and pathological features than elder age CRC, the molecular mechanism underlying the EOCRC is poorly clarified. Given the significance of CRC in the world of medicine, the present review will focus on the recent knowledge in the molecular basis of genetic and epigenetic mechanism of the hereditary forms of EOCRC, which includes Lynch syndrome, Familial CRC type X, Familial adenomatous polyposis, MutYH-associated polyposis, Juvenile polyposis syndrome, Peutz-Jeghers Syndrome and sporadic forms of EOCRC. Recent findings about molecular genetics and epigenetic basis of EOCRC gave rise to new alternative therapy protocols. Although exact diagnosis of these cases still remains complicated, the present review paves way for better predictions and contributes to more accurate diagnostic and therapeutic strategies into clinical approach.

Key words: Early-onset; Colorectal cancer; Epigenetic mechanism; Genetic mechanism; Clinical outcome

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Core tip: Early-onset colorectal cancer (EOCRC) cases are heterogeneous population that include both hereditary and sporadic forms of the colorectal cancer (CRC). EOCRC cases have some distinguishing clinical and pathological features than elder age CRC. Recent findings about molecular genetics and epigenetic basis of EOCRC gave rise to new alternative therapy protocols. We herein discuss the latest findings about genetic and epigenetic features of EOCRC.

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INTRODUCTION

Colorectal cancer (CRC) is the third most frequent cancer type and despite improvements in diagnosis and treatment, this disease is the second leading cause of cancer death in developed countries^[1]. The highest incidence of CRC is observed in Western Europe, North America and Australia in western populations. It is notable that although the rate of this disease is relatively lower in the communities of the sub-Saharan Africa, South America and Asia, the rate is gradually increasing depending on assimilating life-style and dietary habits of the western countries^[2]. In more developed countries, screening programs for 50 years and elder people leads to early detection of CRC and opportunity for more satisfactory treatments; thus, death rates reduced approximately 2% per year^[3-5]. However, CRC screening is not common for young adults (between 20-40 ages), the incidence of this disease is increasing gradually per year in individuals younger than 50 years^[6]. The tumors of early-onset patients are more aggressive than elder cases^[7,8].

Because of the advances in our understanding concerning the molecular mechanism of elder age CRC, we can describe the presenting phenotype depending on the molecular characteristics of the tumor in majority of the cases^[9]. This vital knowledge contributes to the available studies in the literature for individual-specific and targeted therapies for CRC patients related to their drug responses. However, the molecular mechanism underlying the early-onset CRC (EOCRC) is poorly clarified in the relevant literature. Recent studies have revealed that EOCRC might evolve in a different pathway and the molecular basis of these cases may be unique for individuals^[10]. Therefore, determining identifiable markers of this disease for early diagnoses is required to develop unique treatment protocols and increase the survival of the patients. However, to date, little knowledge has been gained about the molecular basis of young age. Given this gap to be highlighted, the aim of our review is to synthesize and evaluate the current literature regarding the genetic and epigenetic pathogenesis of EOCRC at molecular level.

MOLECULAR PATHOGENESIS OF EOCRC

In comparison to elder CRC, EOCRC cases have some distinguishing clinical and pathological features^[11,12]. These tumors are pathologically recognized with low-grade tumor differentiation, mucinous component and high signet ring cells frequency^[11,13]. Polyp development is contently observed during the follow-up period of EOCRC^[10]. The majority of early-onset tumors occur in the distal colon and the rectum^[14]. Previous studies underlined the significance of heritance as an indicator of EOCRC^[11,12]. Supporting these views, early-onset and hereditary forms of CRC demonstrate similar well-known pathological features^[11,13]. Nevertheless, the current knowledge is that EOCRC is a heterogeneous disease

with both familial and sporadic cases. The molecular basis of this heterogeneity has not yet been fully clarified in the literature, however, the severe histopathological properties and a possible genetic feature of the tumor may predispose to expedited tumor growth in young age patients as reported^[15,16].

HEREDITARY FORMS OF EOCRC

In the hereditary forms of CRC, the disease can be observed in one or more first and/or second degree relatives of the patient. Thus, familial CRC counts approximately 20% of all CRC patients^[17]. With almost 3% observation rate, the most frequently occurring familial CRC is Lynch syndrome (LS)^[18]. On the other hand, polyposis syndromes, such as familial adenomatous polyposis (FAP), MUTYH-associated polyposis (MAP), Juvenile polyposis syndrome (JPS) and Peutz-Jeghers syndrome (PJS), are less often observed familial colorectal syndromes^[17].

LS

LS is frequently right-sided, an autosomal dominant cancer predisposition. The majority of these tumors are synchronous and metachronous. Extracolonic sites of patients, such as brain, ovary, endometrium, renal pelvis, ureter, stomach, small intestine and skin, are also among a high cancer risk^[18]. LS is caused by various germline DNA mismatch repair (MMR) gene mutations^[19-21]. Approximately 90% of the identified LS mutations are observed in *MLH1* or *MSH2* genes and approximately 10% of the LS mutations were identified in *MSH6* and *PMS2* genes^[17,22]. The prevalence and characteristics of these mutations vary widely among populations. In 2010, we defined two frame-shift mutations (*MLH1* c.1843dupC and *MLH1* c.1743delG) and three missense mutations (*MLH1* c.293G < C, *MLH1* c.954_955delinsTA and *MSH2* c.2210G < A) uniquely in Turkish LS cases^[23]. In a study of Italian LS families, c.643_648 dupA, c.2156_2157 dupT, c.684_685 dupC and c.1701_1704 delT frameshift mutations and c.2206 G < T, that cause a truncating protein were first time determined in *MLH1* gene. Other truncating protein causing mutations, c.1089 G < T and c.2634-2 A < G, that results with a splice defect was originally reported in *MSH2* gene^[24]. In Malaysia population, two novel mutations, c.3341_3342insC and c.3885_3891delTAAAGC were characterized in *MSH6* and c.2395C > T mutation was defined in *PMS2* gene^[25]. Recently, an unidentified mutation of *MLH1*, c.2044_2045del was linked to LS in a Caribbean Hispanic family^[26].

Deficient MMR function of LS cases usually promotes microsatellite instability (MSI)^[27,28]. MSI is characterized by length alterations within simple repeated sequences that are called microsatellites^[28]. MSI is essential for deregulation of cell growth, differentiation and death^[29]. MSI also plays roles in modulating the response of patients to various chemotherapeutic agents^[27]. Losing the expression of MMR proteins *via* inactivation of MMR-

Table 1 MiRNA profile of lynch syndrome patients

MiRNA	Expression status	Function	Ref.
miR-155	Up	MMR deficiency	Valeri <i>et al</i> ^[46]
		MSI	Earle <i>et al</i> ^[48]
miR-26b	Down	MSS	Earle <i>et al</i> ^[48]
miR-31	Up	MSI	Earle <i>et al</i> ^[48]
miR-223	Up	MSI	Earle <i>et al</i> ^[48]
miR-486-5p	Down	MSI	Balaguer <i>et al</i> ^[47]
miR-622	Up	MSI	Balaguer <i>et al</i> ^[47]
miR-1238	Up	MSI	Balaguer <i>et al</i> ^[47]
miR-1362-5p	Down	MSI	Balaguer <i>et al</i> ^[47]
miR-132	Down	MMR deficiency	Kaur <i>et al</i> ^[49]
miR-345	Down	MMR deficiency	Kaur <i>et al</i> ^[49]

MSS: Microsatellite stable; MSI: Microsatellite instability; MMR: Mismatch repair.

deficient crypt foci genes causes an MSI phenotype^[30]. In these patients, the mutation rates of *ACVR2*, *TAF1B* and *ASTE1*, microsatellite-bearing target genes are higher than 80%^[29-33]. Recent studies indicated that in MSI cases, frameshift mutations of apoptotic genes, such as *APAF1*, *BAX* and *FLASH*, lead to intratumoral heterogeneity^[28]. The study of Markowitz *et al*^[34] demonstrated the relation with DNA repair defects with a specific pathway of CRC progression and three different mutation of *TGFBR2* gene in 1995^[34]. However, the latest study of de Miranda *et al*^[35] showed the transcription and translation of *TGFBR2* with a 1 nucleotide deletion at its microsatellite sequence still produced a functional *TGFBR2* protein. This protein is required for phosphorylation of *SMAD2*, which is phosphorylated in most of the MSI CRC tissues^[35].

The *MMR* gene modifications of LS occur by two hit usually point mutations or large rearrangements may give rise to the first hit. Accordingly, gene conversion or loss of the wild-type allele evokes the second hit^[36]. However, recent observations demonstrated the high rate of promoter methylation occurrence as the second hit^[37,38]. These findings emphasize the role of epigenetic events in formation of LS^[37,38]. Indeed, depending on the studies, germ line hemiallelic methylation of *MLH1* and epimutations of *MSH2* lead to LS with insufficient *MLH1* or *MSH2* protein expression in mutation negative families^[39-42]. Ligtenberg *et al*^[43] state that germ line 3' end deletions of *EPCAM* gene that is located upstream of *MSH2*, correlate with MSI and a loss of *MSH2* protein even though there was no identifiable mutation in *MSH2* gene^[44]. Kuiper *et al*^[44] found *EPCAM* deletions in approximately 2.3% of *MSH2*-deficient families. This study affirms the epigenetic transgenerational inheritance and the possibility of aberrant promoter methylation occurrence in neighbouring tumor suppressor genes by loosing of polyadenylation signals^[28]. In addition, current evidence in the empirical studies supports the role of miRNAs that is responsible for translational rearrangement of proteins, in regulation of MMR genes expressions^[45]. In comparison to sporadic MSI tumors, LS patients have a typical miRNA profile. Valeri *et al*^[46]

demonstrated the association of reduced expression of *MSH2*, *MLH1* and *MSH6* and induction of a mutator phenotype and MSI with over expression of miR-155 in LS. In another study, Balaguer *et al*^[47] determined the up-regulation of miR-622 and miR-1238 in these patients. MSI status modulates the miRNA expression levels^[48]. Earle *et al*^[48] defined the increased expression of miR-155, miR-31, miR-223 and miR-26b in MSI tumors. In addition, Earle *et al*^[48] linked over expression of miR-31 and miR-223 to LS. Not only miRNA regulates gene expression in an epigenetic way but also miRNA expressions may be regulated epigenetically. With containing a CpG island in the promoter region, most of the miRNAs are favorable for aberrant methylation which can give rise to dysregulation of miRNA^[49,50]. Kaur *et al*^[49] identified a correlation between miR-345 and miR-132 hypermethylation and MMR deficiency (Table 1).

Familial CRC type X

MMR germline mutations are observed in approximately 60% of the families, fulfilling clinical criteria for LS^[51]. Although familial colorectal cancer type X (FCCTX) accomplish the same clinical criteria with LS, the morphological features, such as right-sided tumor location, poor differentiation, expansive growth pattern, tumor-infiltrating lymphocytes, peritumorous lymphocytes, Crohn-like reactions and lack of dirty necrosis, are not common in FCCTX as LS^[52]. In addition, despite these, families demonstrate clinical features in which CRCs with MSI, FCCTX is not related to germline MMR gene mutations^[51,53]. The age onset of FCCTX is relatively older than LS cases and this disease differ from LS with the tumorigenic pathways^[54,55]. Basically, two individual molecular pathways involve in these families. One of these pathways is loosing of tumor suppressor gene loci genes, such as *TP53*, *APC*, *SMAD4* and *DCC*, somatic mutations of *APC* and *KRAS* and *MGMT* promoter methylation. At the second partway, there is no loosing of tumor suppressor gene loci genes and rarely presenting promoter methylation^[56]. Therikildsen *et al*^[57] linked to FCCTX tumors with gain of genetic material in two separate regions encompassing, 20q12-13.12 and 20q13.2-13.32. This study revealed that gain of material on chromosome 20q and loss on chromosome 18 differentiate FCCTX from LS. Findings of Dominguez-Valentin *et al*^[58] showed that gaining mutations of *GNAS* gene which is located in 20q13.32 and encodes for the Gα-subunit may cause FCCTX *via* activation of the Wnt and ERK1/2 MAPK signalling pathways. Moreover, other 20q located genes, *CDH26*, *SRC* and *ASIP* that play role in proliferation and migration may have a potential to cause FCCTX^[58]. Dominguez-Valentin *et al*^[58] defined the up-regulation of *PTGER1* in these tumors which can cause tumor growth through altered prostaglandin E2 function^[59,60]. Recently, an *SEMA4A* gene variant c.232G > A was determined in Austrian kindred with FCCTX. This study revealed that *SEMA4A* (V78M) lead to activation of MAPK/Erk and PI3K/Akt signaling. Moreover, *SEMA4A* mutations, c.1451G > C and c.977C > T and the single-nucleotide polymorphism c.2044C

Table 2 Molecular characterization of familial colorectal cancer type X patients

Molecular features		Ref.
Germline <i>MMR</i> gene mutations	-	Lindor <i>et al</i> ^[51] Klarskov <i>et al</i> ^[52] Sánchez-Tomé <i>et al</i> ^[53]
Tumor suppressor gene loci loss		
<i>APC</i> mutations	77%	Francisco <i>et al</i> ^[56]
<i>KRAS</i> mutations	46%	Francisco <i>et al</i> ^[56]
<i>MGMT</i> methylation	36%	Francisco <i>et al</i> ^[56]
Chromosome gains	20q, 19 and 17	Therkildsen <i>et al</i> ^[57]
Chromosome loss	8p, 15, 18	Therkildsen <i>et al</i> ^[57]
Signaling by G protein coupled receptor	up-regulated	Dominguez-Valentin <i>et al</i> ^[58]
(GNAS, F2R, F2RL2, EDN1, EDNRA, GRM8, GNA2, GNG11, GNG12, HCRT, PTGER1, P2RY2, RAMP2, MC1R, TUBB3, VIP) SEMA4A variants		Schulz <i>et al</i> ^[61]

> T were determined as associated with the FCCTX phenotype^[61]. Recent knowledge about the molecular characterization of FCCTX is summarized in Table 2.

FAP

FAP is an autosomal dominant cancer syndrome^[62]. FAP is diagnosed with 100 or more adenomatous polyps in colon or rectum in patients with younger than 40 age^[62]. Patients with FAP carry germline mutations of the adenomatous polyposis coli (*APC*) gene located on chromosome 5q21-q22^[63]. *APC* protein is a large scaffolding protein which involves in Wnt signaling pathway. In this protein complex, *APC* leads to down regulation of b-catenin activity and play a central role in a destruction complex of Axin, GSK-3 β and casein kinase 1. This complex directs a series of phosphorylation events on β -catenin that target it for ubiquitination and subsequent proteolysis^[64]. In the absence of *APC* protein, b-catenin binds to several transcription factors of the TCF/LEF and initiates the altered expression of genes associated with proliferation, differentiation, migration and apoptosis. Moreover, the depletion of *APC* can lead to abnormal chromosome segregation and aberrant mitosis^[65,66]. FAP occurs when there are mutations between codons 168-1580 and with severe disease between codons 1250-1464 of *APC* gene^[67,68]. The majority of *APC* mutations are either frameshift or nonsense mutations resulting in a truncated protein^[69]. The two most frequently described germline mutations are located at codon 1309 (c3927_3931delAAAGA) and codon 1061 (c.3183_87delACAAA)^[70]. Although two-thirds of FAP patient disease is inherited, the rest of the cases have no family history and carry unique mutations. Almost all *APC* mutations results with a colonic phenotype but variable for extra-colonic manifestations, such as desmoid tumor, hepatoblastoma, thyroid carcinoma, medulloblastoma, a litany of benign lesions and brain tumors, particularly medulloblastomas^[71-73]. Lamberti *et al*^[74] found that *GSTT1* polymorphism showed an uncertain association

with extra-intestinal manifestations in a study of 411 FAP patients. Recent studies demonstrated the enrichment of pyloric gland adenomas of the stomach, in addition to fundic gland polyps and foveolar-type adenomas in patients with FAP^[75,76]. Hashimoto *et al*^[75] analyzed the genetic alterations in these FAP-associated gastric lesions and they demonstrated that, as well as *APC* mutations, these cases had *GNAS* and *KRAS* mutations.

KRAS mutations have been observed in the early development of approximately 40% of colon cancers. Simultaneous *APC* depletion and *KRAS* mutation results with an augmentation in adenomas^[76] and induce the spread of stem cell marker carrying cells within the tumor epithelium^[77]. Phelps *et al*^[78] stated that in FAP adenomas, intestinal differentiation is required two consecutive steps. In the first step, after *APC* loss, CtBP1 contributes to adenoma initiation and in the following step, *KRAS* activation and β -catenin nuclear localization promote adenoma progression to carcinomas. On the other hand, Obrador-Hevia *et al*^[79] analysed somatic *APC* and *KRAS* mutations, beta-catenin immunostaining, and qRT-PCR of *APC*, *MYC*, *AXIN2* and *SFRP1* genes in sixty adenomas from six FAP patients with known pathogenic *APC* mutations. Based on this study, the Wnt pathway was constitutively activated in all *APC*-FAP tumors, with alterations occurring both upstream and downstream of *APC*. Thus, Obrador-Hevia *et al*^[79] suggest that for Wnt signalling activation in *APC*-associated FAP adenomas, oncogenic *KRAS* is not essential.

FAP may also pursue a different way to Wnt signalling pathway alterations through epigenetic mechanisms. Although epigenetic alterations of Wnt signalling are an effective factor for FAP formation, *APC* mutations exist in almost all FAP patients. Romero-Giménez *et al*^[80] evaluated the possible role of germline hypermethylation of the *APC* promoter in FAP families that were negative for *APC* mutations in 21 FAP families and they did not identify signs of abnormal promoter methylation, indicating that this form of epigenetic silencing is not a common cause of FAP. However, Kámory *et al*^[81] observed promoter hypermethylation that causes somatic inactivation of *APC* in 21 sporadic cases (30%). In the study of Zhang *et al*^[82] within FAP families, although methylation was not present in normal tissues, hypermethylation was determined in tumor tissues of one proband and her son. In addition, loss of heterozygosity was observed in another patient from the same FAP family. Segditsas *et al*^[83] declared similar findings with Zhang *et al*^[82]. They detected *APC* promoter methylation in 27%-45% of colorectal tumors and cell lines but did not detect in normal colorectum. However, they substantially observed that methylation was independent of the *APC* mutations and was not associated with the CpG island methylator phenotype. Although methylation caused the loosing of 1A isoform mRNA and a reduction in total *APC* transcript levels, *APC* gene expression was retained from promoter 1B^[83]. Moreover, a recent study of Pavicic demonstrated that promoter 1B deletions of *APC* are not very common^[84]. Thus, all these studies imply that

Table 3 Genetic and epigenetic alterations of familial adenomatous polyposis patients besides adenomatous polyposis coli

Alteration		Ref.
Gene		
GNAS	Mutation	Hashimoto <i>et al</i> ^[75]
MYC	Gene activation	Obrador-Hevia <i>et al</i> ^[79]
AXIN2	Gene activation	Obrador-Hevia <i>et al</i> ^[79]
SFRP1	Gene activation	Obrador-Hevia <i>et al</i> ^[79]
GSTT1	Polymorphism	Lamberti <i>et al</i> ^[74]
MGMT	Methylation	Wynter <i>et al</i> ^[86]
p14ARF	Methylation	Wynter <i>et al</i> ^[86]
p16INK4	Methylation	Wynter <i>et al</i> ^[86]
IGSF4	Methylation	Berkhout <i>et al</i> ^[85]
TIMP3	Methylation	Berkhout <i>et al</i> ^[85]
ESR1	Methylation	Berkhout <i>et al</i> ^[85]
CDH13	Methylation	Berkhout <i>et al</i> ^[85]
miRNA		
miR-143	Down regulation	Kamatani <i>et al</i> ^[87]
miR-145	Down regulation	Kamatani <i>et al</i> ^[87]
miR-126	Down regulation	Yamaguchi <i>et al</i> ^[88]
miR-20b	Down regulation	Yamaguchi <i>et al</i> ^[88]

even though *APC* promoter methylation occurs in early during colon neoplasia progression, it does not result in complete gene inactivation or act as a "second hit"^[84] and promoter-specific alterations of *APC* rarely leads to mutation-negative FAP^[84].

In addition to *APC*, hypermethylation of other genes are usually observed in both FAP-related and sporadic duodenal carcinomas^[85]. Wynter *et al*^[86] study showed that the methylation of *MGMT*, *p14ARF* and *p16INK4* genes promoter regions are frequently observed in both sporadic and familial adenomas. Berkhout *et al*^[85] defined the high methylation rate of the *IGSF4*, *TIMP3*, *ESR1*, *APC* and *CDH13*, in both of these cases, however, in the same study, *PAX6* gene was determined as hypermethylated only in FAP-related carcinomas. Recently, the role of altered miRNA expression in Wnt signalling regulation and FAP development has also been evaluated. Lately, the studies indicated the decreased expression of miR-143, miR-145, miR-126 and miR-20b as an early event of colorectal carcinogenesis in FAP tumors^[87,88]. Specifically, miR-126 and miR-20b play role in angiogenesis^[88]. Thus, downregulation of these miRNAs is an important genetic event for the initiation step in colorectal tumor development^[87]. Besides *APC* alterations, other genetic and epigenetic events determined in FAP patients were summarized in Table 3.

MAP

MAP is an autosomal recessive polyposis syndrome. Approximately 0.3%-1% of all CRCs is associated with MAP^[89,90]. Cases with MAP typically present multiple colon adenomas, thus at the first glance, these cases may be diagnosed as FAP. However, because they also can have *MMR* gene mutations, it can reverberate to phenotype as LS^[91]. Although existing of multiple colon adenomas, there is not any alteration in *APC* gene of these cases, but further analyses identified mutations in

Table 4 *MUTYH* mutations that vary with ethnicity

<i>MUTYH</i> mutation	Ethnicity	Ref.
c.231 C > T	Japan	Miyaki <i>et al</i> ^[99]
c.934-2A > C	Japan	Miyaki <i>et al</i> ^[99]
c.1376C > A	Finland	Alhopuro <i>et al</i> ^[100]
c.933 + 3A > C	North-Eastern Italy, Germany	Pin <i>et al</i> ^[101]
c.536A > G	Caucasians	Yamaguchi <i>et al</i> ^[92]
c.1187 G > A	Caucasians	Yamaguchi <i>et al</i> ^[92]

MUTYH gene which is a component of a base excision repair system and involves in protecting DNA from oxidative damage^[92]. Farrington *et al*^[93] reported that mutations of both *MUTYH* gene alleles increase the risk of endometrial tumors. These cases are rare and well known *MUTYH* mutations are linked to this disease are c.494A > G and c.1145G > A^[94-96]. However, *MUTYH* mutations can vary with ethnicity^[97]. c.536A > G and c.1187G > A in Caucasians, c.231 C > T and c.934-2A > C in Japan, c.1227_1228dup in Portugal, c.1376C > A in Finland were determined as the most frequent *MUTYH* mutations^[98-100]. In the North-Eastern Italy, c.933+3A > C (IVS10 + 3A > C), accounts for nearly 1/5 of all *MUTYH* mutations^[101]. In addition, because this mutation is also common in Germany, it is supposed to have a common origin in Western Europe^[101]. *MUTYH* mutations that vary with ethnicity are summarized in Table 4.

Germline *MUTYH* mutations may also lead to the mutation of cancer-related genes, such as the *APC* and/or the *KRAS* genes, via G to T transversion^[92]. In the study of Venesio *et al*^[102], mutated *MUTYH*-associated-polyposis adenomas exhibited only c.34G > T transversion in codon 12, or mutations in codon 13. They affirm that neither of these mutations was found in classical/attenuated familial polyposis adenomas.

JPS

JPS is a rare autosomal dominant disorder. JPS is diagnosed with numerous colon and rectum polyps or polyps with family history or juvenile polyps inside and outside of the intestine^[103]. 20%-50% of JPS demonstrates familial pattern and the average disease onset of cases are 16 to 18^[103]. JPS may coexist with Osler-Weber-Rendu syndrome [hereditary hemorrhagic telangiectasia (HHT)]. The most frequently encountered symptoms of HHT are Skin telangiectasia, epistaxis, intracranial haemorrhage, development of pulmonary arteriovenous fistulas, brain cavernous angioma and haemangioma^[104]. Almost 60% of JPS cases demonstrate mutations in *SMADH4* and *BMPRI1A* genes that are connected with TGF- β /BMP signal pathway^[105].

To date, a number of mutations leading JPS and/or HHT have been described in *SMAD4* gene. These mutations include point mutations that are resulting with a stop codon or a change in the coded amino acid into another one, codons 361, 533 and 534 mutations, small deletions and insertions^[103]. Specifically, Howe *et al*^[106] determined the mutation, c.1244-1247delAGAC, in the hot spot of the *SMAD4* gene which leads to a

Table 5 Pathogenic germline mutations of juvenile polyposis syndrome

Mutation	Effect	Ref.
<i>SMAD4</i> c.1244-1247delAGAC	Hotspot mutation serious course of JPS with numerous cases of polyps, tumors located in the stomach and intestines	Howe <i>et al</i> ^[106]
<i>BMPRI1A</i> c.230+452_333+441dup 1995	Frameshift mutation producing a truncated protein (p.D112NfsX2)	Yamaguchi <i>et al</i> ^[107]

JPS: Juvenile polyposis syndrome.

serious course of JPS with numerous cases of polyps and tumors located in the stomach and intestines. In addition, a considerable proportion of mutations in the *BMPRI1A* gene are nucleotide changes generating a stop codon (nonsense) or leading to amino acid changes (missense). These mutations are distributed evenly in the entire gene sequence, intronic mutations (intron 1, 3, 4 and 5) and deletions between codon 224 and 359^[103]. Yamaguchi *et al*^[107] identified a *BMPRI1A* mutation, which involves a duplication of coding exon 3 (c.230p452_333p441dup1995) that causes a frameshift mutation, producing a truncated protein (p.D112NfsX2) in a patient with JPS (Table 5).

In addition to mutations in *SMAD4* and *BMPRI1A* genes, Juvenile polyps also was observed in Cowden, Bannayan-Zonana, and Gorlin syndromes. Cowden and Bannayan-Zonana syndromes is occurred by *PTEN* mutations and Gorlin syndrome develops *via* germline *PTCH* mutations. *PTEN* and *PTCH* mutations have been excluded as the causative mutations in almost all JPS patients^[108-110].

To the best of our knowledge, there is a lack of knowledge about epigenetic regulation of JPS so far. However, recently, Ling *et al*^[111] defined *SMAD4* as a miR-224 target as a metastasis factor, yet the relation of miR-224 and *SMAD4* expression in formation of juvenile polyps has not been clarified.

PJS

PJS is a rare (approximately 1 in 200000 observation rate) autosomal dominant disease^[112,113]. PJS is characterized by occurrence of benign hamartomatous, Peutz-Jeghers-type polyps in the gastrointestinal tract in association with mucocutaneous pigmentation on the lips and oral mucosa^[114]. PJS is diagnosed with presence of a hamartoma associated with two of the following three signs: Family history of PJS, mucocutaneous lentiginosis or polyposis of the small-bowel^[115]. PJS patients face with abdominal symptoms during the first 10 years of life, almost 50% of patients experience the symptoms before the age of 20 years and they have an increased risk of developing gastrointestinal and extradigestive cancers^[115-117]. Cancer development localized in small intestine, stomach, pancreas and colon in most of the cases^[116]. In 93% of the affected individuals, there is a

risk of developing complicating cancers between aged 15-64 years^[116].

Percent of eighty to 90% of patients with PJS have family history^[118] and according to genetic analysis, 40%-60% of these cases have germline STK11 (also known as LKB1) mutations as the major cause of this disease^[112]. Because the downstream signalling pathway of STK11 has not been fully clarified, the knowledge about the mechanism of hamartomatous polyp formation and mucocutaneous pigmentation also insufficient at present. Studies demonstrated that induced *COX-2* gene expression has also been involved in the promotion of tumor formation from PJS polyps^[119,120]. On the other hand, PJS cases with wild-type STK11 demonstrates multiple causative loci such as chromosome1p, a pericentric inversion in chromosome 6, a second PJS locus at 19q13.4 and a heterozygous germline mutation in the MYH11 gene^[121-125]. Lately, Wang *et al*^[121] performed sequence analysing in three Chinese individuals with PJS and identified 2 variants, OR4C45 c.767-768insAG and ZAN c.5767insG, which occur in PJS cases independently of STK11.

More than 145 different STK11 germline mutations have been reported in the literature result in a truncated premature protein or in transcriptional splicing errors^[121,126-129] (Table 6). On the other hand, transcriptional silencing of this tumor suppressor gene by promoter hypermethylation has been shown as an alternative inactivation mechanism^[130-133]. In addition to germline mutations, and promoter methylation, Wang *et al*^[121] discovered four mutations in pre-microRNAs, MI0003131, MI0003530, MI0014206, and MI0005525, of which the corresponding mature miRNA, hsa-mir-492, hsa-mir-487b, hsa-mir-323b, hsa-mir-300 respectively.

SPORADIC FORMS OF EOCRC

The most well-defined hereditary form of CRC, LS, account for 2%-4% of total CRC and one-third of EOCRC cases^[134,135]. FAP cases are observed in less than 1% frequency in total CRC cases. Thus, 70% of all CRC and the majority of EOCRC cases are introduced in sporadic form^[136-138]. Sporadic EOCRCs are classified into two major groups. Chromosome unstable CRC (CIN) is characterized by gross chromosomal abnormalities and MSI^[135]. Although MSI tumors behave less aggressively compared to CIN, CIN or MSI tumors do not always appear separately^[139-141].

Sporadic EOCRC are morphologically characterized with poor cell differentiation, colloid component and lymphocytic stromal reaction^[8,12,142]. Therefore, these cases are likely to be confused with LS patients. However, while MMR defects are observed *via* MSI pathway in LS, in sporadic cases MSI is not frequent. Studies to date imply that colorectal tumors characterized by MSI may be distinct from microsatellite stabile (MSS) tumors in many molecular aspects, such as an association with the methylator phenotype, a higher frequency of *BRAF* mutations and a lower frequency of *KRAS*, *APC*, and *TP53* mutations. Thus, MSI and MSS colon tumors

Table 6 *STK11* mutations associated with colorectal cancer caused by peutz-jeghers syndrome

<i>STK11</i> mutation	Mutation type	Effect on protein	Ref.
c.511 G > A	Missense mutation	G171S	Dong <i>et al</i> ^[127]
c.595 G > A	Missense mutation	E199K	Dong <i>et al</i> ^[127]
c.622 G > A	Missense mutation	D208N	Dong <i>et al</i> ^[127]
c.644 G > A	Missense mutation	G215D	Dong <i>et al</i> ^[127]
c.941 C > A	Missense mutation	P314H	Resta <i>et al</i> ^[128]
c.1062 C > G	Missense mutation	F354L	Dong <i>et al</i> ^[127]
c.1100 C > T	Missense mutation	T367M	Dong <i>et al</i> ^[127]
c.842delC	Frameshift mutation	truncates	Dong <i>et al</i> ^[127] , Bartosova <i>et al</i> ^[129]
IVS2 + 1A > G	Intronic splice site mutation		Bartosova <i>et al</i> ^[129]
OR4C45 c.767-768insAG	Frameshift mutation	truncates	Wang <i>et al</i> ^[121]
ZAN c.5767insG	Frameshift mutation	truncates	Wang <i>et al</i> ^[121]

originate from different molecular backgrounds^[143]. In sporadic cases, MMR deficiency occurs mainly through epigenetic inactivation of the *MLH1* gene through biallelic promoter methylation instead of MSI^[136]. Both genetic and epigenetic inactivation of MMR genes result in a mutator phenotype, mutations in cancer related genes and CRC development^[144]. Kirzin *et al*^[137] identified *CTNWB1* as one of the most over-expressed genes in MSS-young patients compared to MSS-old patients and this leads to an over-activation of beta catenin in sporadic EOCRC. In addition, Fernandez-Rozadilla *et al*^[145] determined a heterozygous deletion in the 10q22-q23 region involving *BMPRI1A* gene of EOCRC cases with MMR proficiency. According to Luo *et al*^[146] CDC42, TEX11, QKI, CAV1 and FN1 proteins are representative elements of EOCRC specific networks. Moreover, we defined *REG1A*, *CK20* and *MAP3K8* gene expressions strongly upregulated (more than twofold) in early-onset MSS CRC compared with MSI CRC tumors^[147]. *CK20* expression is observed in the majority of colorectal tumors^[148,149], however, a limited number of studies have evaluated the relationship between *CK20* expression levels and MSI status^[147]. In one study, it was suggested that reduced or absent *CK20* expression in CRC is associated with both sporadic and hereditary MSI^[150]. In another study associated with EOCRC, *CK20* expression levels were also identified as relatively reduced in MSI tumors^[10]. It was determined that *CK20* expression levels are inversely correlated with numbers of aberrant microsatellite locus^[150]. We determined the upregulation of *CK20* expression levels in MSS tumors compared with MSI-low (MSI-L) and MSI-high (MSI-H) tumors^[150]. According to McGregor *et al*^[150] regulation of *CK20* gene expression involves molecular pathways that are altered by MSI-H. We defined 3.98-fold high *CK20* gene expression levels in MSS tumors with lymph nodes metastases than in MSI tumors with lymph nodes metastases^[147]. In addition, 17.5-fold upregulation was identified in *CK20* expression levels in low-grade MSS tumors of patients with recurrence and distant metastases^[147]. These results indicate that upregulation of *CK20* expression, specifically, is related to poor prognosis in patients with MSS tumors. Therefore, the results of our study indicate that *CK20* expression in MSS tumors allows for the determination

of the biological characteristics of EOCRC tissues^[147]. The encoded protein by *MAP3K8* gene is a member of the serine/threonine protein kinase family. In one of our study, *MAP3K8* expression in CRC was determined significantly elevated compared with normal mucosa^[149]. In addition, we determined *MAP3K8* expression levels more than two fold upregulated in early-onset MSS CRC compared with MSI CRC tumors^[147]. *MAP3K8* expression levels were significantly higher in the MSS tumors of patients with a short median survival. Thus, our observations revealed that upregulated *MAP3K8* expression was associated with a poor prognosis in patients with MSS tumors^[147]. Human *REG1A* belongs to the superfamily of calcium-dependent lectins. In several previous studies, *REG1A* was found to be upregulated in CRC^[151-153]. We also found that *REG1A* is upregulated in the tumors of early-onset sporadic CRC patients. Furthermore, 25.8-fold high *REG1A* gene expression levels were observed in MSS tumors with lymph nodes metastases. In addition, median survival and disease-free survival were significantly longer only for patients with MSI tumors with low *REG1A* expression compared with those with high expression of this gene. This result indicates that upregulated *REG1A* expression may be related to sporadic EOCRC tumor formation and characterization^[147]. Additionally, a recent study from Sengupta *et al*^[154] defined a relation with MSS CRC tumors and deletion in *RBFOX1* gene which encodes a highly conserved RNA-binding protein that regulates tissue-specific alternative splicing indicating important basic functions in development and differentiation in a British Bangladeshi MSS CRC population. This study showed that loss of *RBFOX1* activity may lead to aberrations in the splicing of genes associated with CRC^[154].

Different from MSS tumors, some sporadic EOCRC tumors belong to the MSI pathway^[28]. Sporadic EOCRC with MSI is likely to arise from sessile serrated polyps through the serrated neoplastic pathway^[155]. The *BRAF* gene, which plays an important role in the mitogen-activated protein kinase signalling pathway, is frequently mutated in these cases. *BRAF* V600E mutation is widely accepted as a prognostic factor of sporadic CRC with MSI and methylated *MLH1*^[156]. Although the frequency of *BRAF* V600E mutation is high in MSI tumors, this mut-

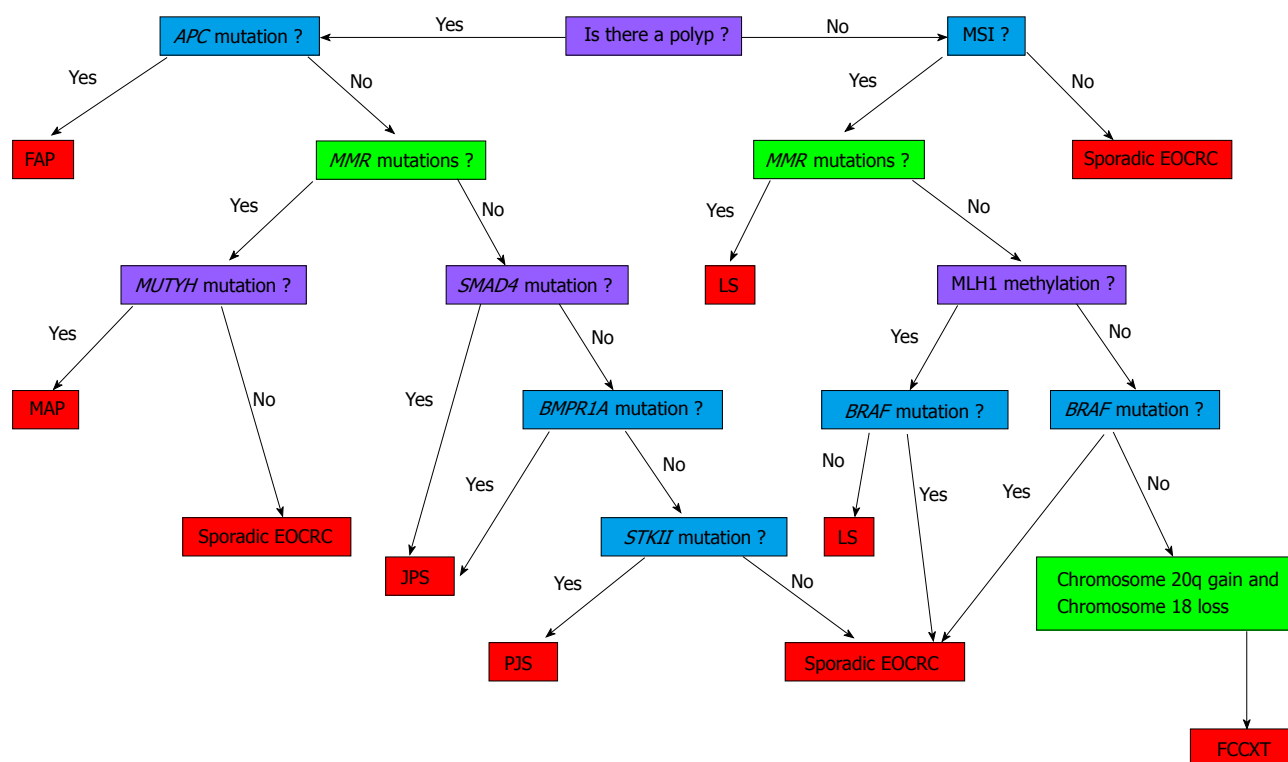


Figure 1 Genetic algorithm of early-onset colorectal cancer. MMR: Mismatch repair; EOCRC: Early-onset colorectal cancer; MSI: Microsatellite instability; APC: Adenomatous polyposis coli; FAP: Familial adenomatous polyposis; MAP: MUTYH-associated polyposis; LS: Lynch syndrome; JPS: Juvenile polyposis syndrome; PJS: Peutz-jeghers syndrome.

ation is not observed in LS cases, thus, this discrepancy between sporadic MSI cancer and LS might be used in a strategy for the detection of LS^[156].

The different attitude of sporadic and hereditary forms of EOCRC may also be caused by epigenetic modifications, such as miRNA expressions and their methylation patterns^[47,157]. Balaguer *et al.*^[47] demonstrated that miR-622, miR-362-5p and miR-486-5p could accurately classify the LS and sporadic MSI cases. The similarity of miRNA expression status of LS and sporadic MSI cases may be explained with occurrence of frameshift mutations in *TARBP2*, a miRNA processing gene, in both of these diseases^[158]. Moreover, in one of our study, using miRNA polymerase chain reaction arrays, the expression profiles of 38 different miRNAs associated with CRC were evaluated in 40 sporadic Turkish EOCRC patients^[157]. The expression of miR-106a was found to be upregulated, and miR-143 and miR-125b levels were found to be downregulated in sporadic EOCRC tissues compared with the normal tissues. In addition, 2.42-fold high expression level of miR-106a and 2.42-fold low expression level of miR-125b were observed in tumors with lymph node metastases compared with the normal colorectal mucosa samples^[157]. On the other hand, epigenetic regulations of sporadic EOCRC tumors also differ between each other depend on MSI status. Earle *et al.*^[48] described the different expression profile of miR-223, miR-155, and miR-92 between MSI and MSS CRCs. So far, Kaur *et al.*^[49] have investigated the association of miR-132 methylation and sporadic MSI CRC tumors located in

the proximal colon in a comparative study of Finnish and Australian population. In addition, different from MSS tumors, hypermethylation of miR-345 had a significant association with sporadic MSI in Finnish CRCs^[49].

CLINICAL OUTCOME OF GENETIC AND EPIGENETIC FEATURES OF EOCRC

A major challenge in CRC therapy is drug resistance. The current knowledge of CRC genetics has increased the sufficiency of applied conventional cytotoxic chemotherapy and targeted therapy. Genetic screening of EOCRC patients for hereditary cancer syndrome is determinative not only for the rate of cancer risk of relatives but also for appropriate treatment. A pyrimidine analogue, 5-fluorouracil (5-FU) which is widely used in CRC therapy, involves in induction of DNA replication stress response in cells through inhibiting thymidylate synthase. However, studies showed that *APC* mutations reduces the sensitivity to 5-FU^[159]. On the other hand, performance of MSI test is advisable for patients with strongly suspected on the basis of a known family history of colorectal and extracolonic cancers in the case of LS (Figure 1). Studies revealed that while adjuvant chemotherapy with a fluoropyrimidine does not have a beneficial effect on MSI cases and may even worsen the clinical picture, combination of oxaliplatin and infusional 5-FU/leucovorin regarded as more beneficial for these cases^[160,161]. According to Violette *et al.*^[162] increased expression of Reg genes caused *in vitro* resistance to

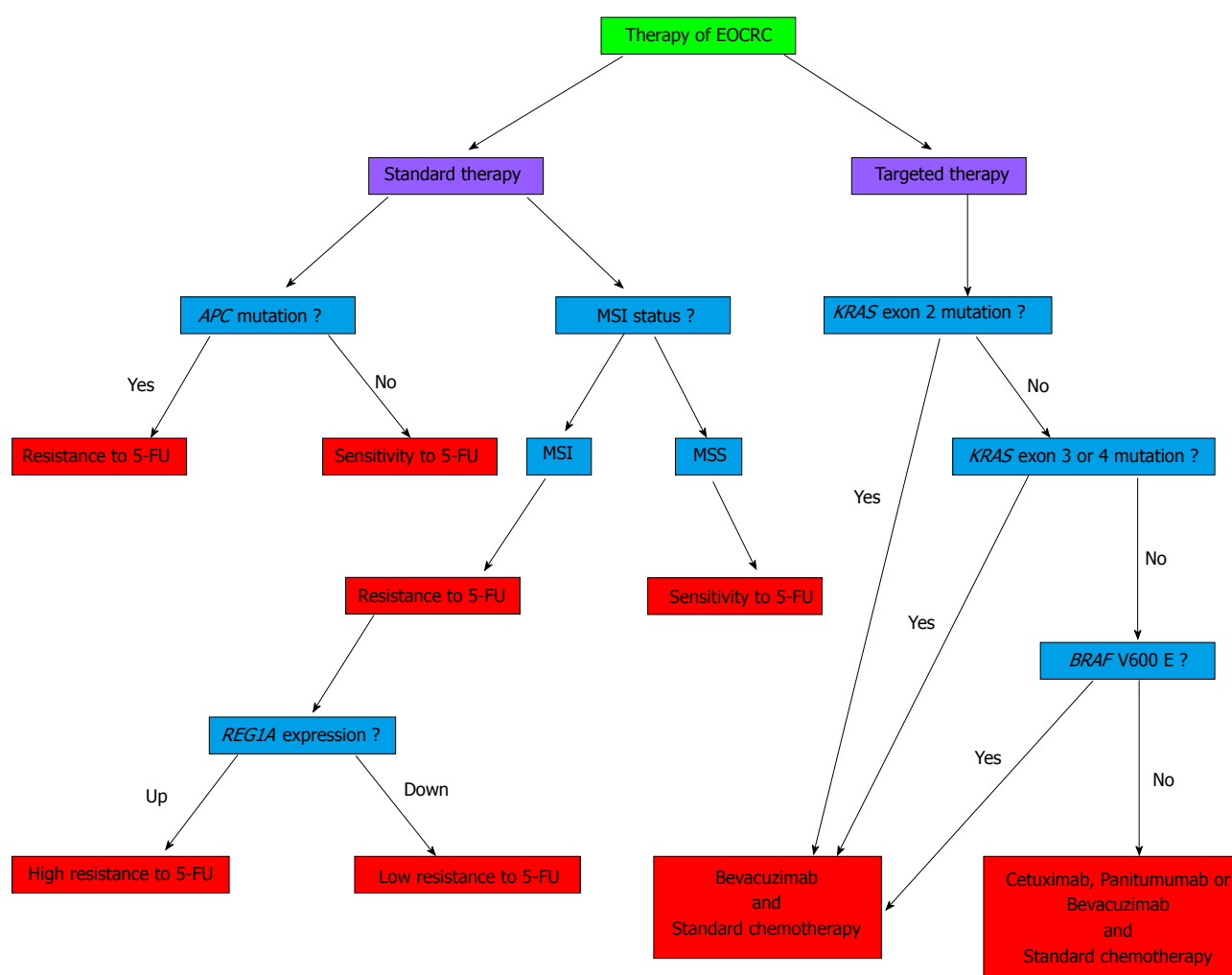


Figure 2 Therapy of early-onset colorectal cancer. EOCRC: Early-onset colorectal cancer; MSI: Microsatellite instability; MSS: Microsatellite stabile; 5-FU: 5-fluorouracil.

the 5-FU. Bishnupuri *et al*^[163] observed a mitogenic effect of the Reg IV protein, with subsequent changes in the expression of genes associated with apoptosis and metastasis. The Reg proteins are previously unappreciated regulators of antiapoptotic proteins in early tumorigenesis and may contribute to increased resistance to apoptotic death during therapy^[163]. As another mechanism of resistance to therapy, the result of our study of the poor prognosis of MSI tumors supports the hypothesis that high *REG1A* expression may contribute to increased resistance to apoptotic death during therapy in MSI tumors^[147]. Because of the important role of *REG1A* in tumorigenesis and development of metastasis in MSI tumors, the use of *REG1A*-specific inhibitors in CRC patients have MSI that may represent a novel significant approach to the treatment of cancer. In addition, according to recent studies, alterations in epigenetic regulation of these genes may also lead to resistance to chemotherapeutic agents. For example, Deng *et al*^[164] found out that reduced expression of miR-21 plays role in resistance to 5-FU therapy *via* targeting *MSH2*. However, miRNA studies have been performing in *in vitro* conditions and to prove the decisive importance of these markers further

advanced studies required.

Nowadays, the application of targeted therapy for CRC has been increasing. The goals of these therapies are interrupting the survival and proliferation of cancer cells^[165]. To date, United States Food and Drug Administration has approved several targeted drugs, such as cetuximab and panitumumab, the anti-EGFR antibodies that suppress the tumor angiogenesis and bevacizumab, an anti-VEGF antibody. Recently, different from bevacizumab, aflibercept and regorafenib have been used as new antiangiogenic agents^[166-168]. Although EGFR is overexpressed in most of the CRC cases, because of the down-stream modifications of EGFR signalling pathway, patients demonstrated different response to this therapy^[169]. Particularly, *KRAS* activating mutations in exon 2 avoid the sufficient therapy with EGFR inhibitors^[170,171]. A small number of patients with wild type *KRAS* exon 2 were demonstrated to have mutations exons 3 and 4 that are also caused *KRAS* activation^[172]. Activating mutations in the other genes that play role in downstream pathway of EGFR signalling, *NRAS*, *BRAF*, *PIK3CA* and *PTEN* are able to lead to resistance to anti-EGFR therapies^[173]. Thus, to predict the success of anti-EGFR monoclonal antibody

therapy, examination of downstream mutations of EGFR signalling pathway should be required before receiving an EGFR inhibitor^[170] (Figure 2). Second targeted signal pathway for CRC therapy is angiogenesis pathway. Bevacizumab is a monoclonal antibody that binds to VEGF-A preventing its interaction with VEGFR-2^[174]. Regorafenib demonstrated a multikinase inhibitor activity against VEGFR-2, VEGFR-3, TIE-2, PDGFR, FGFR, RET, c-Kit and RAF/MEK/ERK pathway^[175]. Aflibercept is a recombinant fusion protein and play a role in the inhibition of interactions between VEGF-A, VEGFB proteins and their specific receptors by acting as a trap receptor binding to VEGF-A and VEGFB^[176]. Thus, the blockage of the genes that encoded these proteins enhances the success of the therapy.

CONCLUSION

Genetic predispositions have been identified in EOCRC clearly distinct from the other types of CRC. The current knowledge about the molecular and genetic basis of EOCRC provides information regarding prognosis of this disease and response to therapies. A proportion of EOCRCs are hereditary forms. Hence, cases should be evaluated for existing of a germline mutation in one of the several MMR genes for suspicion of LS, in the APC gene for suspicion of FAP, or in one of the genes associated with a more uncommon syndrome. Identification of a hereditary syndrome in individuals also provides predictive mutational testing for non-symptomatic relatives. They are found to be positive for the mutation can take precaution for reduction of the risk of cancer-associated morbidity and mortality in this way. In addition, a better understanding of the genetic mechanism of EOCRC is highly likely to lead to develop more beneficial targeted therapies. To date, specifically, studies on MSI CRC, such as LS, herald new diagnostic and therapeutic strategies into clinical approach. It is notable that further research remains to be conducted to more finely characterize the underlying mechanism of sporadic EOCRC, which could allow improved prevention, diagnosis, and treatment of these cases.

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Novel therapeutic agents in the treatment of metastatic colorectal cancer

Sachin Gopalkrishna Pai, Jyotsna Fuloria

Sachin Gopalkrishna Pai, Northwestern Medicine Developmental Therapeutics Institute, Robert H. Lurie Comprehensive Cancer Center of Northwestern University, Chicago, IL 60611, United States

Jyotsna Fuloria, Department of Hematology and Oncology, Ochsner Medical Center, New Orleans, LA 70121, United States

Jyotsna Fuloria, Ochsner Cancer Institute, New Orleans, LA 70121, United States

Author contributions: Pai SG prepared the initial draft; Pai SG and Fuloria J coordinated the revisions; Fuloria J approved the final draft.

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Correspondence to: Jyotsna Fuloria, MD, Director, Department of Hematology and Oncology, Ochsner Medical Center, 1514 Jefferson Highway, New Orleans, LA 70121, United States. jfuloria@ochsner.org
Telephone: +1-504-8423708
Fax: +1-504-8424533

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Abstract

Over the past couple of decades considerable prog-

ress has been made in the management of metastatic colorectal cancers (mCRC) leading to a significant improvement in five-year survival. Although part of this success has been rightly attributed to aggressive surgical management and advances in other adjunct treatments, our understanding of the pathogenesis of cancer and emergence of newer molecular targets for colon cancer has created a powerful impact. In this review article we will discuss various targeted therapies in the management of mCRC. Newer agents on the horizon soon to be incorporated in clinical practice will be briefly reviewed as well.

Key words: Metastatic colorectal cancer; Molecular targeted drugs; Anti-angiogenesis inhibitors; Epidermal growth factor receptor inhibitors; Novel therapeutic agents

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Core tip: This article reviews the novel agents in the management of metastatic colorectal cancer. The core principles and the evidence behind the use of these agents are discussed. Clinically relevant features are highlighted to help the health care provider involved in the care of metastatic colorectal cancer patients.

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INTRODUCTION

In 2015, a total of 132700 new cases of colorectal cancer are expected to be diagnosed in the United States accounting for about 8% of all new cancer diagnoses. In this same year 49700 patients will die of metastatic colorectal cancers (mCRC), which will contribute to 8.4%

of all cancer related mortality^[1]. With the widespread use of screening colonoscopy and newer modalities like Stool DNA based screening, we can expect early diagnosis and curative treatments in patients diagnosed with early disease and hence better survival. However up to > 25% of patients will present with metastatic disease, where systemic treatment options will be desired. Widespread use of genetic screening and sharing platforms like the cancer Genome Atlas has led to a better understanding of carcinogenesis and as a consequence newer molecular targets for colon cancer have been discovered^[2]. In this review article we will discuss some of the well-known targetable pathways as well as shed light on some of the novel pathways where we can expect newer therapies to emerge.

ANTI-ANGIOGENESIS AGENTS

Anti-angiogenesis was proposed as an anticancer therapy over four decades ago^[3]. We know that angiogenesis is required for invasive tumor growth and metastasis and is an integral part of cancer progression^[4]. Angiogenesis is mediated through vascular endothelial growth factor (VEGF), the altered regulation of which is associated with several diseases including malignancy. VEGF is a heparin-binding growth factor specific for vascular endothelial cells that is able to induce angiogenesis *in vivo*^[5]. Three notable anti-VEGF agents have been approved by United States Food and Drug Administration (USFDA) for treating mCRC and will be reviewed here.

Bevacizumab

Bevacizumab is a recombinant humanized IgG-1 antibody against soluble VEGF-A which has a high binding specificity with VEGF-A. Once bound, Bevacizumab prevents its interaction with receptors on vascular endothelial cells and thereby truncates the abnormal downstream signaling. After success in early phase trials, this agent was tested in phase 3 clinical trials^[6]. In the pivotal trial which had 813 previously untreated patients with mCRC randomized to the two arms, the median duration of survival was 20.3 mo in the Irinotecan, 5-Fluorouracil and Leucovorin (IFL) plus Bevacizumab group, as compared with 15.6 mo in the IFL plus placebo group, corresponding to a hazard ratio for death of 0.66 ($P < 0.001$)^[7]. An Eastern Cooperative Oncology Group Study (E3200) showed median duration of survival for the group treated with FOLFOX4 and Bevacizumab was 12.9 mo compared with 10.8 mo for the group treated with FOLFOX4 alone (corresponding hazard ratio for death 0.75, $P < 0.001$), and 10.2 mo for those treated with Bevacizumab alone. Bevacizumab is approved by the USFDA in combination with either an Irinotecan or Oxaliplatin based regimen for the treatment of mCRC^[8,9].

Bevacizumab is generally well tolerated when administered in combination with chemotherapy for mCRC. Hypertension, proteinuria, epistaxis and thrombosis are

some of the common adverse events associated with its use^[6]. No clear guidelines exist on the management of hypertension but in most patients it is usually possible to control hypertension with standard antihypertensive medications. On occasion, it may be necessary to temporarily or permanently discontinue Bevacizumab if hypertension is severe or persistent^[10].

Routine use of Bevacizumab as maintenance therapy is controversial. A recent study found no clear benefits of continuing Bevacizumab after 4-6 mo of standard first-line chemotherapy plus Bevacizumab and given the cost and lack of clear benefit, it was not recommended^[11]. Whether a certain subgroup with high-risk disease such as high metastatic burden would benefit from this approach needs further investigation^[12].

Ziv-Aflibercept

Ziv-Aflibercept is a fusion protein consisting of human VEGF receptor extracellular domains fused to the Fc portion of human immunoglobulin G1, and works by inhibiting VEGF receptor. Aflibercept was used in a large phase 3 trial in combination with 5-Fluorouracil, Irinotecan and Leucovorin (FOLFIRI) and was found to confer a statistically significant survival benefit over FOLFIRI combined with placebo in patients with mCRC previously treated with an Oxaliplatin based regimen^[13]. Adding Aflibercept to FOLFIRI showed an improved overall survival relative to placebo plus FOLFIRI (HR = 0.817, 95%CI: 0.713-0.937, $P = 0.0032$) with median survival times of 13.50 mo vs 12.06 mo, respectively. Efficacy was maintained across demographic and baseline characteristics and stratification factors at randomization, irrespective of prior treatment with Bevacizumab, with a similar safety profile^[14].

Ramucirumab

Ramucirumab is a recombinant human monoclonal anti vascular endothelial growth factor-receptor 2 antibody which was recently approved by USFDA for use in combination with FOLFIRI for the treatment of patients with mCRC whose disease has progressed on first line Bevacizumab, Oxaliplatin- and Fluoropyrimidine-containing regimen. Approval was based on a study that enrolled 1072 patients (536 in each group) and patients were randomized either to receive Ramucirumab or placebo^[15]. PFS was significantly improved in patients who received Ramucirumab in combination with FOLFIRI compared to placebo [Median PFS was 5.7 and 4.5 mo; HR = 0.79 (95%CI: 0.70-0.90, $P < 0.001$]. Median overall survival was 13.3 mo (95%CI: 12.4-14.5) for patients in the Ramucirumab group vs 11.7 mo (10.8-12.7) for the placebo group (HR = 0.844, 95%CI: 0.730-0.976, log-rank $P = 0.0219$). Diarrhea, hypertension and fatigue were the common adverse events with the use of Ramucirumab, consistent with the previously known safety profile established in previously approved indications.

EPIDERMAL GROWTH FACTOR RECEPTOR AND OTHER KINASES

The epidermal growth factor receptor (EGFR) autocrine pathway has been known to affect a number of processes important to carcinogenesis including cell proliferation, apoptosis and angiogenesis. This has been the rationale for developing EGFR inhibitors, both monoclonal antibodies to prevent ligand binding as well as small molecule inhibitors of the tyrosine kinase enzymatic activity to inhibit auto-phosphorylation and downstream intracellular signaling^[16]. Although monoclonal antibodies like cetuximab were initially developed to treat head and neck cancer, traditionally known to highly express EGFR on immunohistochemistry, their use was extended to treating colorectal cancer.

Cetuximab: Cetuximab is a chimeric (mouse/human) monoclonal antibody used in the management of mCRC, which was initially approved by USFDA as a third line single agent in patients who have failed Oxaliplatin- or Irinotecan- based chemotherapy and who are intolerant to Irinotecan. In the pivotal trial which compared FOLFIRI plus Cetuximab vs FOLFIRI plus Bevacizumab as first-line treatment for patients with mCRC, 592 patients with KRAS exon 2 wild-type tumors were randomly assigned and received treatment. Median progression-free survival was 10.0 mo (95%CI: 8.8-10.8) in the Cetuximab group and 10.3 mo (95%CI: 9.8-11.3) in the Bevacizumab group (HR = 1.06, 95%CI: 0.88-1.26, $P = 0.55$); however, median overall survival was 28.7 mo (95%CI: 24.0-36.6) in the Cetuximab group compared with 25.0 mo (22.7-27.6) in the Bevacizumab group (HR = 0.77, 95%CI: 0.62-0.96, $P = 0.017$). Anti-EGFR monoclonal antibodies are well tolerated, the most important adverse event being cutaneous reaction including rash, pruritus, and nail changes. These adverse reactions can usually be medically managed and patients tend to continue on the drugs. Occasionally the drug may need to be discontinued due to intolerable side effects.

Panitumumab

Panitumumab is a fully humanized monoclonal antibody specific to EGFR. The efficacy of Panitumumab was established in the PRIME study which showed that in the wild-type KRAS stratum, Panitumumab-FOLFOX4 significantly improved PFS compared with FOLFOX4 (median PFS, 9.6 mo vs 8.0 mo, respectively; HR = 0.80; 95%CI: 0.66-0.97, $P = 0.02$). Also noted was a nonsignificant increase in OS for Panitumumab-FOLFOX4 vs FOLFOX4 (median OS, 23.9 mo vs 19.7 mo, respectively; HR = 0.83, 95%CI: 0.67-1.02, $P = 0.072$)^[17]. In an open-label, phase 3 head-to-head study of Panitumumab vs Cetuximab which enrolled patients with chemotherapy-refractory mCRC Panitumumab was non-inferior to Cetuximab. Median overall survival was 10.4 mo (95%CI: 9.4-11.6) with Panitumumab and 10.0 mo (9.3-11.0) with Cetuximab (HR = 0.97,

95%CI: 0.84-1.11)^[18]. Panitumumab has been shown to induce pathological near complete response or complete response when given along with neoadjuvant concurrent radiation therapy in patients with KRAS wild-type locally advanced rectal cancer^[19]. Panitumumab is generally well tolerated and has a similar side effect profile as Cetuximab.

Ras testing and use of egfr antibodies

EGFR expression as measured by immunohistochemistry on many occasions does not predict clinical benefit with the use of EGFR inhibitors^[20,21]. It has also been shown that mutations in the KRAS exon 2 (codons 12 and 13), which was down stream to EGFR, dictated the response to EGFR antibodies. Additional mutations like KRAS exon 3 (at codons 59 and 61) and exon 4 (at codons 117 and 146), NRAS exon 2 (at codons 12 and 13), exon 3 (at codons 59 and 61), and exon 4 (at codons 117 and 146), have been demonstrated to be negative predictive biomarkers for EGFR antibody treatment. These additional mutations now account for approximately 17% of patients with wild-type KRAS exon 2 status who harbor a mutation in other RAS exons^[22]. Testing for extended EGFR mutation is highly recommended and if truly wild type then use of EGFR antibodies is justified in those cases.

TYROSINE KINASE INHIBITORS

Regorafenib (BAY 73-4506) is a novel oral diphenylurea based multikinase inhibitor, shown to be a potent inhibitor of a wide variety of Tyrosine kinases which include several angiogenic, stromal receptor and oncogenic tyrosine kinases as well as intracellular signaling kinases in preclinical studies^[23]. A phase III trial in refractory mCRC, (CORRECT) randomized 760 patients between Regorafenib ($n = 505$) and placebo ($n = 255$). It showed a small but statistically significant improvement in OS (median 6.4 mo vs 5 mo, one-sided P value 0.005) and progression-free survival (median 1.9 mo vs 1.7 mo, one-sided P value < 0.000001) for Regorafenib^[24]. The most common side effects of Regorafenib are fatigue, hand-foot skin reaction (palmar-plantar erythrodysesthesia), diarrhea, mucositis and weight loss for which the patients need to be monitored closely^[25]. A novel germline mutation of PDGFR-beta might be associated with clinical response of colorectal cancer to Regorafenib^[26].

EMERGING AGENTS

Targeting cancer stem cells

Human cancers have been shown to harbor cancer stem cells which are thought to play an important role in cancer recurrence and metastasis. With the recent discoveries of small molecules that target highly conserved cell homeostasis pathways which have been implicated in the pathogenesis of colorectal cancer, gives us an

Table 1 Ongoing clinical trials in Immunotherapy in colorectal cancer

Drug name	Class	Phase	ClinicalTrials.gov Identifier	Sponsor	Remarks
AMP-224	PD-1 inhibitor	1	NCT02298946	NCI	Combination with stereotactic body radiation therapy
MPDL3280A	Engineered anti-PDL1 antibody	1	NCT01375842	Genentech	Administered as single agent
Varlilumab and nivolumab	Monoclonal antibodies that binds to CD27 and PD-1	1/2	NCT02335918	Celldex therapeutics/bristol-myers squibb	Phase II to determine objective response rate
MPDL3280A and bevacizumab	Engineered anti-PDL1 antibody	1b	NCT01633970	Genentech	Assess the safety, pharmacology and preliminary efficacy of the combination
Avelumab	Antibody targeting PDL-1	1	NCT01772004	EMD serono	Open-label, dose-escalation trial
MEDI4736	Anti PDL-1	2	NCT02227667	Memorial sloan Kettering cancer center	Study to evaluate the efficacy of MEDI4736

Available from: URL: <http://www.clinicaltrials.gov>, accessed on 4/25/2015.

exciting avenue in treating mCRC. BBI608, an orally-administered first-in-class cancer stem cell inhibitor, has been tried in a Phase 1 study after excellent preclinical evidence. This has shown some promising anticancer activity in patients with CRC^[27]. An open label, multicenter, Phase 2 study of BBI608 in combination with cetuximab, Panitumumab or Capecitabine in patients with advanced colorectal cancer is ongoing (ClinicalTrials.gov Identifier: NCT01776307). Another phase 1 dose escalation study with LGK974 is currently ongoing and recruiting patients with special emphasis on those with B-RAF mutant colorectal cancer with documented Wnt pathway alteration (ClinicalTrials.gov Identifier: NCT01351103).

BRAF

BRAF mutations have been shown to be the cause of sporadic CRCs through altered mismatch repair pathway and occur mutually exclusive of KRAS mutations^[28]. At this time BRAF mutation is known to confer a poor prognosis in mCRC, but is not a validated target for anti-cancer therapy^[29,30]. Although this mutation is found in a relatively small proportion of CRC (5%-8%), targeting BRAF has been unsuccessful as feedback stimulation of EGFR pathway has been suggested as the reason for the treatment failure^[31]. Current studies are focused on dual blockade of BRAF and EGFR or of the subsequent downstream pathway. Initial experience of combining BRAF inhibitor Vemurafenib with EGFR inhibitor Panitumumab has been safe, although the response has been modest^[32]. Another Phase II Study of Irinotecan and Cetuximab with or without Vemurafenib in BRAF mCRC is still recruiting patients (ClinicalTrials.gov Identifier: NCT02164916). Another potential strategy is the use of ERK inhibitor that is thought to suppress MAPK activity, which is usually upregulated in patients on RAF inhibitors and may overcome resistance. ERK inhibitors are currently in early phase clinical trials^[33].

Immunotherapy

The advent of immune check point blockade has been an exciting field in cancer immunotherapy. Already of considerable success in other types of cancers like melanoma and squamous cell lung cancer where Anti

PD-1 drugs are approved by USFDA, various groups are studying the efficacy in colorectal cancer. Mismatch-repair status has been useful in predicting clinical benefit of immune checkpoint blockade with Pembrolizumab, with higher response in Microsatellite Instability High (MSI-High) tumors^[34]. The table summarizes the current ongoing trials mainly targeting PD-1 - PDL-1 immune checkpoint pathway (Table 1).

Targeting kras with reolysin

Biological strategies like Reovirus Serotype 3 - Dearing Strain (Reolysin), a naturally occurring ubiquitous, non-enveloped human Reovirus, have been explored in mCRC for targeting KRAS. Reovirus has been shown to replicate selectively in RAS-transformed cells causing cell lysis. Activating mutations in RAS or mutations in oncogenes signaling through the RAS pathway may occur in as many as 80% of human tumors and can be targeted by this approach. A multicenter phase 1 study Reolysin in combination with FOLFIRI and Bevacizumab in FOLFIRI naive patients with KRAS mCRC is ongoing (ClinicalTrials.gov Identifier: NCT01274624).

TAS-102

TAS-102 is a novel oral nucleoside and works as an antimetabolite. TAS-102 is a combination of trifluridine, a nucleoside analog, and tipiracil hydrochloride, a thymidine phosphorylase inhibitor. In a double-blind, randomized, placebo-controlled phase 2 trial, 112 patients were allocated to TAS-102 and 57 allocated to placebo. Median overall survival was 9.0 mo (95%CI: 7.3-11.3) in the TAS-102 group and 6.6 mo (4.9-8.0) in the placebo group (hazard ratio for death 0.56, 80%CI: 0.44-0.71, 95%CI: 0.39-0.81, $P = 0.0011$) on a median follow up of 11.3 mo (interquartile range 10.7-14.0 mo). Hematological toxicities were the important side effects to consider in patients on TAS- 108 arm, 57 (50%) neutropenia of grade 3 or 4, 32 (28%) leucopenia and 19 (17%) experiencing anemia. Serious adverse events were reported in 21 (19%) patients in the TAS-102 group. Recent data from RECURSE study has shown that median overall survival improved from 5.3 mo with placebo to 7.1 mo with TAS-102. Hazard ratio for death

in the TAS-102 group vs the placebo group was 0.68 (95%CI: 0.58-0.81, $P < 0.001$), and this data led to its FDA approval^[35].

CONCLUSION

In conclusion, mCRC treatment is a rapidly evolving field with many novel agents under investigation. Although many targeted drugs have been approved and are already in clinical use, there is a clear need for further research and development of more effective treatments. Over the coming years, as understanding of the biology of the disease improves, newer treatment modalities will be investigated. The optimum use and sequencing of these agents, especially in combination with chemotherapy and other targeted agents will need to be better defined.

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Long-term outcomes after stenting as a “bridge to surgery” for the management of acute obstruction secondary to colorectal cancer

Javier Suárez, Javier Jimenez-Pérez

Javier Suárez, Department of General Surgery, Coloproctology Unit, Complejo Hospitalario de Navarra, 31008 Pamplona, Spain

Javier Jimenez-Pérez, Department of Gastroenterology, Endoscopy Unit, Hospital de La Ribera, 46600 Alzira, Spain

Author contributions: Suárez J and Jimenez-Pérez J equally contributed to this work.

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Correspondence to: Javier Suárez, MD, Department of General Surgery, Coloproctology Unit, Complejo Hospitalario de Navarra, c/Irunlarrea - 3, 31008 Pamplona, Spain. fj.suarez.alecha@cfnavarra.es
Telephone: +33-848-422179
Fax: +34-848-422303

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the time of initial diagnosis in cases of colorectal cancer. Emergency surgery has been classically considered the treatment of choice in these patients. However, in the majority of studies, emergency colorectal surgery is burdened with higher morbidity and mortality rates than elective surgery, and many patients require temporal colostomy which deteriorates their quality of life and becomes permanent in 10%-40% of cases. The aim of stenting by-pass to surgery is to transform emergency surgery into elective surgery in order to improve surgical results, obtain an accurate tumoral staging and detection of synchronous lesions, stabilization of comorbidities and performance of laparoscopic surgery. Immediate results were more favourable in patients who were stented concerning primary anastomosis, permanent stoma, wound infection and overall morbidity, having the higher surgical risk patients the greater benefit. However, some findings laid out the possible implication of stenting in long-term results of oncologic treatment. Perforation after stenting is related to tumoral recurrence. In studies with perforation rates above 8%, higher recurrences rates in young patients and lower disease free survival have been shown. On the other hand, after stenting the number of removed lymph nodes in the surgical specimen is larger, patients can receive adjuvant chemotherapy earlier and in a greater percentage and the number of patients who can be surgically treated with laparoscopic surgery is larger. Finally, there are no consistent studies able to demonstrate that one strategy is superior to the other in terms of oncologic benefits. At present, it would seem wise to assume a higher initial complication rate in young patients without relevant comorbidities and to accept the risk of local recurrence in old patients (> 70 years) or with high surgical risk (ASA III/IV).

Key words: Self-expanding metallic stent; Colorectal cancer; Obstructive colorectal cancer; Colorectal cancer chemotherapy; Colorectal cancer surgery

Abstract

Obstructive symptoms are present in 8% of cases at

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Core tip: Self-expanding metal stents placement as a bridge to surgery in patients with obstructive left-colon cancer is controversial. Stent insertion is beneficial regarding perioperative morbidity, being patients with advanced age or with important comorbidity the ones who could obtain more benefit of transforming emergency surgery into elective surgery. But, on the other hand, an increase of local recurrence rate has been shown after stent placement when compared with emergency surgery, compromising oncologic outcome of these patients. Without definitive data, it seems cautious to consider emergency surgery and assume a higher initial complication rate in young patients without relevant co-morbidities avoiding the risk of local recurrence and stenting, accepting the risk of local recurrence but with a lesser perioperative complications rate, in old patients with high surgical risk.

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INTRODUCTION

Colorectal cancer is one of the most frequently diagnosed cancer in developed countries^[1], with over 400000 new cases and more than 200000 cancer related deaths per year in Europe^[2]. Some patients present colorectal obstruction at the time of diagnosis. Although in previous studies this situation was reported in up to 30% of patients^[3], recent papers conclude that obstructive symptoms are present in 8% of cases at the time of initial diagnosis in cases of metastatic tumors^[4] and also independently of the tumoral stage^[5]. Emergency surgery has been classically considered the treatment of choice in these patients, although patients operated on emergency basis have poorer prognosis than those undergoing elective surgery^[6]. Ascanelli *et al*^[7] found a 5-year survival rate of 59% in patients electively operated in contrast with 39% in patients surgically treated on emergency basis. For some authors, this worse prognosis correlates with a lower quality surgery due to the emergency situation^[8,9]. However, other studies suggest that poorer long-term prognosis in patients undergoing emergency surgery is due to a more advanced tumoral stage^[10].

Some studies have been recently published supporting the possibility of performing colonic segmental resection with primary anastomosis in emergency surgery with a complication rate comparable to that of elective surgery. Zorcolo *et al*^[11] analysed surgical outcomes in 323 patients and found that primary

anastomosis can be performed in emergency surgery with low morbidity and mortality rates in selected patients. However, in the majority of studies, emergency colorectal surgery is burdened with higher morbidity and mortality rates than elective surgery. In a series of 989 patients, Tekkis *et al*^[12] proved, after multivariate analysis, that emergency surgery is significantly associated with a higher postoperative mortality (20% vs 12.8%) as well as ASA classification and patient age. In another recent study comparing 171 surgically treated patients with obstructive left colon cancer by means of resection and primary anastomosis after intraoperative lavage and 1053 patients operated on elective basis, emergency surgery patients were older and with a more advanced tumoral stage. Besides, both postoperative mortality (4.1% vs 0.9%: $P = 0.001$) and morbidity (11.7% vs 7.6%: $P = 0.07$) rates were higher in obstructed patients^[13].

In this clinical scenario, not all patients are candidates for surgery with primary anastomosis and so, many patients require temporal colostomy which deteriorates their quality of life and becomes permanent in 10%-40% of cases^[3,14].

BENEFITS OF SELF-EXPANDABLE METAL STENTS

Self-expandable metal stents can restore large bowel transit achieving colonic decompression. Initially used in patients with non resectable malignant tumors, stents were then indicated in patients with resectable colorectal tumors and obstructive symptoms as a bridge to surgery procedure. The aim of stenting is to transform, in left colon cancer, emergency surgery into elective surgery in order to allow, with lower morbidity, mortality and stoma requirements, accurate tumoral staging and detection of synchronous lesions with CT-colonoscopy or conventional colonoscopy^[15,16], stabilization of comorbidities and improvement of the nutritional status before surgery and performance of laparoscopic surgery^[17]. Tejero *et al*^[18] reported the outcomes of the first two patients treated with this strategy in 1994.

Although the definition of clinical success can be different in published papers, the most commonly used is to consider clinical success as the resolution of obstructive symptoms within the first 72 h after stent placement. In a systematic review including 1785 patients and 1845 stents, Watt *et al*^[19] reported a clinical success rate of 92% (46%-100%). Concerning technical success, defined as the passage of the guide wire and the stent across the stricture with further appropriate stent release and expansion, the same authors reported a 96.2% success rate. A multicenter European prospective study, including 182 stented patients under the bridge to surgery indication, reported similar results for both technical (98%) and clinical success (94%) rates^[20].

The advantages of stenting were confirmed in retrospective studies. Watt *et al*^[19] found that the

rate of primary anastomosis performance in patients treated with elective surgery was two-fold higher than in patients operated on emergency basis. Patients electively operated presented lower stoma requirements, lower complication rate and shorter hospital stay. However, results were not so consistent in randomized control trials. Pirllet *et al*^[21] randomized 60 patients with obstructive left colon cancer into two groups, emergency surgery vs stenting plus elective surgery. No differences were found concerning stoma performance (56% vs 43.3%; $P = 0.30$), mortality, morbidity or hospital stay. However, stenting technical success rate was as low as 46.7% with a perforation rate of 6.7%.

In a Dutch study, 98 patients with obstructive left colon tumors were randomized for emergency surgery or emergency stenting. No differences were found regarding 30-d mortality, overall mortality, morbidity and permanent stoma at the end of follow-up. However, patients included in the emergency surgery arm, presented a higher rate of initial stoma confection (absolute risk difference: 0.23, 95%CI: 0.04-0.40, $P = 0.016$) as well as a reduced rate of stoma related complications (between-group difference: -12.0, 95%CI: -23.7-0.2, $P = 0.046$). Stenting technical success rate was 70.2% and perforation rate 12.8%^[22].

The low rates of technical success at the time of stenting in both studies and the high perforation rate of the Dutch publication are surprising, worrisome, and, to a certain extent, question the results of both studies considering that in most published papers reported technical success rates are higher than 85% and perforation rate does not exceed 5%. There is no comment in the French paper about the expertise of participant endoscopists concerning stenting, while the Dutch study mentions that colonic stenting was done by endoscopists who had placed at least 10 colonic stents. According to the recently published clinical guideline of the European Society of Gastrointestinal Endoscopy regarding stenting for obstructive colonic and extracolonic cancer, one of the recommendations is that colonic stent placement should be performed or directly supervised by an experienced operator who has performed at least 20 colonic stent placement procedures^[23]. These data might have influenced the study results.

Nevertheless, perioperative results of SEMS insertion are actually better known. In a recent meta-analysis published by Huang *et al*^[24] including 7 randomized control trials comparing emergency surgery and stenting plus further elective surgery (382 patients), results were more favourable in patients who were stented concerning primary anastomosis (OR = 0.28; 95%CI: 0.12-0.62; $P = 0.002$), permanent stoma (OR = 2.01; 95%CI: 1.21-3.31; $P = 0.007$), wound infection (OR = 0.31; 95%CI: 0.14-0.68; $P = 0.004$) and overall morbidity (OR = 0.30; 95%CI: 0.11-0.86; $P = 0.03$). No differences were found regarding mortality, anastomosis dehiscence and intra-abdominal infection.

Uncovered SEMS has lesser tendency to migrate

than covered SEMS but showed higher tumor in growth rates. Globally, both types are equally effective and safe. Surgery might be performed 5 to 10 d after stent placement^[23].

This benefit may not be the same in all groups of patients and, in old patients these benefits can be greater. Gorissen *et al*^[25] demonstrated that in-hospital mortality of patients older than 75 was higher in patients undergoing emergency surgery than in those who received a stent as a bridge to surgery procedure (21% vs 8%; $P = 0.228$). In a study published in 2007 and based on a decision model (Markov Chain Monte Carlo), authors conclude that stenting is cheaper and more effective than emergency surgery due to a lower mortality and lower permanent stoma requirements. A low perforation rate with stenting and a high surgical risk were determinant factors to obtain these beneficial results with stenting, having the higher risk patient the greater benefit^[26].

STENTING AND LONG-TERM ONCOLOGIC OUTCOMES

Although initial studies were focused on short-term results of bridge to surgery stenting, some results laid out the possible implication of stenting in long-term results of oncologic treatment. Maruthachalam *et al*^[27] could demonstrate that peripheral blood levels of a tumoral marker, CK20 mRNA, increased after stent placement while did not modify after performing a diagnostic colonoscopy in patients with colorectal cancer. The consequence of this finding on tumoral behaviour is unknown. In a recent prospective multicenter study including 519 patients with stage III colonic cancer and receiving adjuvant therapy with FOLFOX, the presence of circulating tumoral cells after surgery did not correlate with a poorer disease-free survival or overall survival^[28].

Another study reported an increased perineural tumoral invasion in patients with obstructive left colon cancer and treated with a stent under the bridge to surgery indication in comparison with patients surgically treated on emergency basis. In spite of this finding, no significant differences were found regarding overall survival or disease-free survival between the two groups of patients. Even more, perineural invasion did not correlate with tumoral recurrence or 5-year survival^[29]. Anyhow, the finding of an increased perineural invasion and lymph node involvement after stenting has been confirmed by other authors^[30].

Kim *et al*^[31] reported a shorter overall survival (38.4% vs 65.6%; $P = 0.025$) and 5-year disease free survival (48.3% vs 75.5%; $P = 0.024$) in patients with obstructive left colon cancer treated with a stent plus elective surgery than in patients with non-obstructive tumors surgically treated on elective basis. Very likely, this poor prognosis associated with stenting is not due to the stent but to the fact that stented patients presented with a large bowel obstruction.

Table 1 Data of recurrence and survival in studies comparing self-expandable metallic stents by-pass to elective surgery and emergency operation for obstructive colorectal cancer

Ref.	Perforation rate	Recurrence SEMS vs EO	Survival SEMS vs EO
Ghazal <i>et al</i> ^[43] Saida <i>et al</i> ^[45]	0 -	RR: 17.2% vs 13.3%; $P = 0.228$ RR of Dukes B: 23% vs 14%; $P = 0.51$)	3 yr-OS: 48% vs 50% 5 yr-OS: 40% vs 44%. Log-rank test: $P = 0.84$ DFS of Dukes B: Log-rank test: $P = 0.71$
Alcántara <i>et al</i> ^[46]	0	RR: 53.3% vs 15.3%; $P = 0.055$	DFS: 25.4 m vs 27 m; $P = 0.096$ OS: Log-rank test: $P = 0.843$
Tung <i>et al</i> ^[34]	0		5 yr-OS: 48% vs 27%; $P = 0.076$ 5 yr-DFS: 52% vs 48%; $P = 0.63$
Pessione <i>et al</i> ^[47] Gianotti <i>et al</i> ^[40]	0 1.2%		2 yr-OS: 66.6% vs 28.5% HR: 0.412 $P = 0.007$ OS: Log-rank test: $P = 0.004$
van den Berg <i>et al</i> ^[42]	1.7%	5 yr-RR of stage I - II: 33% vs 26%; $P = 0.81$ 5 yr-RR of stage III: 35% vs 51%; $P = 0.24$ 3 yr-RR of stage IV: 32% vs 58%; $P = 0.30$	5 yr-OS of stage I - II: Log-rank test: $P = 0.85$ 5 yr-OS of stage III: Log-rank test: $P = 0.48$ 5 yr-OS of stage IV: Log-rank test: $P = 0.08$
Kim <i>et al</i> ^[29]	3.3%	RR: 35% vs 35%; $P = 1.000$ LR: 0% vs 1.6%	5 yr-OSR: 67.2% vs 61.6%; $P = 0.386$ 5 yr-DFS: 61.2% vs 60%; $P = 0.932$ 5 yr-CRSR: 77% vs 65%; $P = 0.233$
Sabbagh <i>et al</i> ^[33]	4.2%	Patients with no perforation or metastases 34% vs 28 %	Patients with no perforation or metastases 5 yr-OSR: 30% vs 67%; $P = 0.001$ 5 yr-DFS: 27% vs 43%; $P = 0.16$ 5 yr-CSMR: 29% vs 22%; $P = 0.62$
Kavanagh <i>et al</i> ^[44]	4.3%	RR 17.3% vs 23%	OS: Log-rank test: $P = 0.13$ CSM: Log-rank test: $P = 0.21$ CSMR: 13% vs 15.3%
Dastur <i>et al</i> ^[48] Gorissen <i>et al</i> ^[25]	5.2% 8%	RR: 31.6 vs 28.2; $P = 0.824$ LRR: 23% vs 15%; $P = 0.443$ LRR in young patients: 32% vs 8%; Log-rank test: $P = 0.038$	3 yr-OS: 48% vs 46%; $P = 0.54$ CSMR: 24.1% vs 37.2%; $P = 0.180$
Sloothaak <i>et al</i> ^[32]	11.5%		4 yr-DFS: 30% vs 49%; Log-rank test: $P = 0.149$ 4 yr-DSS: 66% vs 87%; Log-rank test: $P = 0.061$ 4 yr-OS: 58% vs 67%; Log-rank test: $P = 0.468$ Stent-related perforation vs no perforation 4 yr-DFS: 0% vs 45%; Log-rank test: $P = 0.007$ 4 yr-DSS: 60% vs 69%; Log-rank test: $P = 0.099$ 4 yr-OS: 50% vs 62%; Log-rank test: $P = 0.478$ 5yOSR: 49% vs 40%; OR: 0.98; 95%CI 0.9-1.07
Erichsen <i>et al</i> ^[49]	Non-reported	5 yr-RR: 38% vs 29%; OR: 1.12; 95%CI: 0.99-1.28	
Choi <i>et al</i> ^[50]	Non-reported		5yOSR: 97.8% vs 94.3%; $P = 0.469$

RR: Recurrence rate; LRR: Local recurrence rate; OS: Overall survival; OSR: Overall survival rate; DFS: Disease-free survival; DFSR: Disease-free survival rate; CRSR: Cancer related survival rate; CSM: Cancer-specific mortality; CSMR: Cancer-specific mortality rate; DSS: Disease-specific survival; EO: Emergency operation; SEMS: Self-expandable metallic stents.

Going beyond these findings with unclear significance, more relevant data are available now.

Perforation after stenting and tumoral recurrence

Results of stent-in 2 trial showed that, although no significant statistical differences were found regarding disease free survival, cancer related survival and overall survival when comparing patients treated with a stent and further elective surgery and patients who underwent emergency surgery, tumoral recurrence was significantly higher in patients who had been stented and presented a colonic perforation than in those also stented but without any secondary complication (4 year disease free survival: 0% vs 45%; $P = 0.007$). However, this fact had no influence on overall survival (4 year overall survival: 50% vs 62%; $P = 0.478$)^[32]. Gorissen *et al*^[25] also reported a slightly higher recurrence rate in the

group of stented patients (31.6% vs 28.2%; $P = 0.824$). This difference was due to an increased local recurrence in these patients (23% vs 15%; $P = 0.443$). Patients younger than 75 years had a significantly higher local recurrence rate (32% vs 8%; $P = 0.038$) and, after multivariate analysis, stenting almost reached statistical significance as a risk factor for local recurrence (OR = 12.45, 95%CI: 0.99-156.08; $P = 0.051$). However, it is paramount to remark that the perforation rate in these two studies was 11.5% and 8% respectively (Table 1).

Oncologic benefits of stenting and further elective surgery

In addition to colonic perforation, other factors can affect oncologic evolution of these patients. Quality of surgery could be better in previously stented patients. Sabbagh *et al*^[33] reported a significant higher lymph node retrieval

Table 2 Data of lymph node count, administration of adjuvant chemotherapy and laparoscopic surgery in studies comparing self-expandable metallic stents by-pass to elective surgery and emergency operation for obstructive colorectal cancer

Ref.	Lymph node count SEMS vs EO	Adjuvant chemotherapy SEMS vs EO	Laparoscopic surgery SEMS vs EO
Ghazal <i>et al</i> ^[43]		80% vs 76.7%	
Saida <i>et al</i> ^[45]		66% vs 53%; $P = 0.54$	
Alcántara <i>et al</i> ^[46]	17.7 vs 24.2; $P = 0.099$		
Tung <i>et al</i> ^[34]	23 vs 11; $P = 0.005$	75% vs 54%; $P = 0.2$	
Gianotti <i>et al</i> ^[40]	23 vs 18; $P = 0.08$	46.7% vs 34%; $P = 0.28$	38.7% vs 0%; $P = 0.000$
van den Berg <i>et al</i> ^[42]	Lymph node harvest > 12 62.7% vs 60.7%; $P = NS$	39 vs 39; $P = NS$	
Kim <i>et al</i> ^[29]	28.9 vs 24.4; $P = 0.25$	84% vs 65.7%; $P = 0.085$	
Sabbagh <i>et al</i> ^[33]	22 vs 15; $P = 0.002$	56.2% vs 43.6%; $P = 0.28$	
Kavanagh <i>et al</i> ^[44]	17 vs 17; $P = 0.29$	36% vs 46%; $P = 0.29$	27% vs 12%; $P = 0.1$
Gorissen <i>et al</i> ^[25]		41.6 vs 25.6%; $P = 0.13$	59.6% vs 23%; $P = 0.001$
Sloothaak <i>et al</i> ^[32]	15 vs 13; $P = 0.180$	13 vs 15; $P = 1.000$	

SEMS: Self-expandable metallic stents; EO: Emergency operation.

in the surgical specimen of patients electively operated after initial bridge to surgery stenting, reaching statistical significance in some published papers. In a French study, the number of removed lymph nodes was 22 in the stenting group and 15 in the emergency surgery group ($P = 0.002$). Results were similar in an Asian publication (23 vs 11; $P = 0.005$)^[34]. Significant differences were not reached in other reports (Table 2). In this sense, several studies have correlated the number of removed lymph nodes with survival^[35,36]. Furthermore, Tung *et al*^[34] reported a higher percentage of curative resection surgery in patients previously stented (91.6% vs 54.1%; $P = 0.01$).

Moreover, stent placement is associated with a decreased postoperative complication rate, which is relevant regarding survival^[24]. In a recent analysis including 12075 patients, it has been shown that post-operative complications are associated with shorter survival (HR = 1.24; 95%CI: 1.15-1.34; $P = 0.001$). Analysing complications, infectious complications had a significant influence on long-term survival (HR = 1.31; 95%CI: 1.21-1.42; $P = 0.001$)^[37].

Another potential benefit could be the percentage of patients receiving adjuvant chemotherapy. A non-statistically significant higher percentage of patients received adjuvant chemotherapy after SEMS placement in seven of ten studies (Table 2).

Finally, the number of patients who can be surgically treated with laparoscopic surgery is larger in patients operated on elective basis after bridge to surgery stenting than in the group of patients undergoing emergency surgery. Laparoscopic surgery could have a beneficial effect on long-term survival. In a randomized study published by Lacy *et al*^[38] including 219 patients with colonic cancer, laparoscopic surgery was significantly related to lower recurrence rate (HR = 0.47; 95%CI: 0.23-0.94, $P = 0.03$), cancer-related mortality (HR = 0.44; 95%CI: 0.21-0.92; $P = 0.03$) and overall mortality (HR = 0.59; 95%CI: 0.35-0.98; $P = 0.04$) when compared with open surgery. A similar finding has been reported from COLOR II trial; in patients with

stage-III rectal cancer disease-free survival rate was 64.9% in the laparoscopic surgery group and 52% in the open surgery group (difference 12.9 percentage points, 95%CI: 2.2-23.6)^[39]. In Gorissen *et al*^[25] publication, 59.6% of stented patients and 23.2% of patients who underwent emergency surgery were operated by means of laparoscopic surgery ($P < 0.001$). Gianotti *et al*^[40] also found significant differences concerning laparoscopic surgery performance when comparing stented patients and emergency surgery patients (63.3% vs 0%; $P = 0.001$) (Table 2).

Stenting vs emergency surgery: Which strategy is more beneficial regarding oncologic outcomes?

At present, there are no consistent studies able to demonstrate that one strategy is superior to the other in terms of oncologic benefits.

In a multicenter French study, 5-year overall survival was lower in the group of stented patients than in the emergency surgery group after excluding patients with colonic perforation or metastases at the time of hospital admission (30% vs 67%; $P = 0.001$)^[33]. However, the type of patient (more stage IV patients in one center) and the type of treatment (stenting only in one center) was different in each participating hospital, fact which was not taken into account in multivariate analysis. Moreover, it really attracts attention that with a similar 5-year cancer related mortality (29% vs 22%; $P = 0.62$), overall survival differences are considered attributable to one therapeutic strategy.

In stent-in 2 trial, there was a non significant benefit in the emergency surgery group concerning 4-year disease free survival (Stenting: 30% vs Emergency Surgery: 49%; $P = 0.149$) and 4-year overall survival (Stenting: 58% vs Emergency Surgery: 67%; $P = 0.468$) in relation to colonic perforation after stenting^[32] and, a higher rate of local recurrence in young patients was reported by Gorissen^[25].

However, these results have not been reproduced in other studies with lower stent-related perforation rates. Kim *et al*^[29] reported a similar overall recurrence rate

in both groups of patients (Stenting: 35%; Emergency Surgery: 35%; $P = 1$), with non-significant better results concerning 5-year disease free survival (66.7% vs 54.8%; $P = 0.948$) and 5-years overall survival (100% vs 77.9%; $P = 0.103$) in the stenting group. In this study no case of local recurrence was registered in the stenting group. Tung *et al.*^[34] also reported an almost significant benefit in the stenting group regarding 5-year overall survival (48% vs 27%; $P = 0.076$) and Gianotti *et al.*^[40] demonstrated that stenting was the only parameter related to long-term survival (HR = 0.412; 95%CI: 0.217-0.785; $P = 0.007$). Stent related perforation rate in these three studies was 3.3%, 0% and 1.2% respectively. In a recent meta-analysis including 8 clinical trials, four of them reporting long-term results, no significant differences were found regarding 1-year survival (HR = 1.07; 95%CI: 0.87-1.31; $P = 0.51$), 2-year survival (HR = 1.14; 95%CI: 0.98-1.34; $P = 0.10$) and 3-year survival (HR = 1.08; 95%CI: 0.90-1.31; $P = 0.39$) although it was always better in the stenting group^[41]. Other studies which evaluate long-term results comparing stenting plus elective surgery vs emergency surgery do not find statistical differences in favour of any of the two strategies. Table 1 includes data regarding stent-related perforation, recurrence and survival. Oncologic evolution seems to be better in stented patients while the perforation rate is lower than 8% (Table 1).

In summary, we can't assure that stenting has a deleterious or beneficial effect on oncologic prognosis unless in those cases in which the patient presents a stent-related perforation.

Quality of life

The relevance of choosing one treatment strategy or the other concerning its influence on patient's quality of life has been seldom studied. In the Dutch study, quality of life was assessed with EORTC QLQ-C30 and QLQ-C38 questionnaires and no differences were found comparing stenting with emergency surgery, in spite of the more frequent stoma-related complications in the stenting group^[22].

Other studies have described different parameters directly related with quality of life. Permanent stoma performance is significantly higher in patients undergoing emergency surgery according to Tung *et al.*^[34] (25% vs 0%; $P = 0.03$) and Gianotti (26% vs 6.3%; $P = 0.01$)^[40] publications. In another paper it was also described that stented patients presented milder abdominal pain (4 vs 5; $P = 0.02$) and lower postoperative requirements of acetaminophen (8 tablets vs 16 tablets; $P = 0.04$) or morphine (40 mg vs 60 mg; $P = 0.001$)^[17]. On the other hand, other studies did not find differences regarding permanent stoma performance^[22,42].

Another interesting aspect to be assessed is the quality of bowel movements, as it is clearly related with the surgical technique. Ghazal *et al.*^[43] showed that patients operated on emergency basis performing a subtotal colectomy had a significantly larger number

of bowel movements than patients treated with a stent and elective surgery (6 vs 2; $P = 0.013$). In this sense, total colectomy was less common in surgically treated patients after bridge to surgery stenting in both Kavanagh *et al.*^[44] (4.3% vs 23%; $P = 0.027$) and Saida *et al.*^[45] (2% vs 30%; P value is not reported) studies.

CONCLUSION

Placement of a bridge to surgery self-expandable metal stent is beneficial for the surgical treatment of patients with an obstructive colorectal cancer. This benefit is not identical for every patient, being those patients with an advanced age or with important comorbidity the ones who would obtain more benefit of transforming emergency surgery into elective surgery.

Stenting has no demonstrated influence on survival although patients who present a stent related perforation have a higher risk of tumor recurrence and shorter disease free survival. In studies with perforation rates above 8%, higher recurrences rates in young patients^[25] and lower disease free survival^[32] have been shown. Each medical team must be well aware of their perforation rate in order to implement improvement measures if needed.

According to the literature, in these clinical setting, we have to choose between a treatment with more perioperative complications and another therapeutic strategy which might increase the risk of tumor recurrence. It seems cautious, as it has been suggested by others^[23,32], to consider emergency surgery and assume a higher initial complication rate in young patients without relevant co-morbidities avoiding the risk of local recurrence and stenting, accepting the risk of local recurrence but with a lesser perioperative complications rate, in old patients (> 70 years) with high surgical risk (ASA III/IV).

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Role of self expandable stents in management of colorectal cancers

Erdinc Cetinkaya, Ahmet Bulent Dogrul, Mehmet Bulent Tirnaksiz

Erdinc Cetinkaya, Department of General Surgery, Ankara Numune Education and Training Hospital, 06100 Sıhhiye, Ankara, Turkey

Ahmet Bulent Dogrul, Mehmet Bulent Tirnaksiz, Department of General Surgery, Hacettepe University Medical School, 06100 Sıhhiye, Ankara, Turkey

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Correspondence to: Ahmet Bulent Dogrul, MD, Assistant Professor, Department of General Surgery, Hacettepe University Medical School, Hacettepe Mh., 06100 Sıhhiye, Ankara, Turkey. ahmetdogrul@yahoo.com
Telephone: +90-312-3051675
Fax: +90-312-3104071

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Abstract

Acute malignant colorectal obstruction is a complication of colorectal cancer that can occur in 7%-29% of

patients. Self-expanding metallic stent placement for malignant colorectal obstruction has gained popularity as a safe and effective procedure for relieving obstruction. This technique can be used in the palliation of malignant colorectal obstruction, as a bridge to elective surgery for resectable colorectal cancers, palliation of extracolonic malignant obstruction, and for nonmalignant etiologies such as anastomotic strictures, Crohn's disease, radiation therapy, and diverticular diseases. Self-expanding metallic stent has its own advantages and disadvantages over the surgery in these indications. During the insertion of the self-expanding metallic stent, and in the follow-up, short term and long term morbidities should be kept in mind. The most important complications of the stents are perforation, stent obstruction, stent migration, and bleeding. Additionally, given the high risk of perforation, if a patient is treated or being considered for treatment with antiangiogenic agents such as bevacizumab, it is not recommended to use self-expanding metallic stent as a palliative treatment for obstruction. Therefore, there is a need for careful clinical evaluation for each patient who is a candidate for this procedure. The purpose of this review was to evaluate self-expanding metallic stent in the management of the obstruction of the colon due to the colorectal and extracolonic obstruction.

Key words: Colorectal cancer; Obstruction; Metallic stents; Self-expandable; Extracolonic obstruction

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Core tip: Self-expanding metallic stent placement for malignant colorectal obstruction has gained popularity as a safe and effective procedure for relieving obstruction. This technique can be used in the palliation of malignant colorectal obstruction, as a bridge to elective surgery for resectable colorectal cancers, palliation of extracolonic malignant obstruction, and for nonmalignant etiologies such as anastomotic strictures, Crohn's disease, radiation therapy, and diverticular diseases. In this review we

aimed to evaluate the placement technique, indications and complications of self-expanding metallic stent in colorectal obstructions.

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INTRODUCTION

Colorectal cancers are one of the most common cancers worldwide, and it is the second most common diagnosed cancer in women and third in men^[1]. Acute malignant colorectal obstruction is a complication of colorectal cancer that can occur in 7%-29% of patients^[2]. It is a life-threatening condition that needs prompt evaluation. Large bowel obstruction causes colonic dilatation, bacterial translocation, and electrolyte and fluid imbalance, and has an increased risk of colonic necrosis and perforation^[3]. The main treatment of malignant colonic obstruction is resection of the tumor; however, in the past two decades, the use of self-expanding metallic stents (SEMS) has drawn interest since it was first reported in 1991 by Dohmoto^[4] for palliation of malignant colonic obstruction.

The major indications of SEMS for colonic stenting are palliation of malignant obstruction and preoperative decompression^[5]. Additionally, extracolonic obstructions due to other malignancies and some benign diseases have been shown to be treated by SEMS^[3]. Although SEMS placement for treating malignant obstruction seems safe and effective and has some advantages over surgery, short term and long term morbidities should be kept in mind. In this review, we aimed to evaluate SEMS in the treatment of colorectal and extracolonic cancers with advantages and disadvantages of this technique over surgery.

STENT PLACEMENT TECHNIQUE

SEMS placement can be performed by using endoscopic guidance with or without the use of fluoroscopy. It can be inserted through the scope (TTS) or over the guidewire^[5]. Most of the SEMS are inserted endoscopically with TTS with the use of fluoroscopy^[6]. SEMS placement with endoscope has advantages over the radiologic placement when the obstruction is proximal to the rectosigmoid region or in the presence of a tortuous colon. Kim *et al.*^[7] evaluated the technical feasibility and clinical effectiveness of fluoroscopically guided placement of SEMS in 42 patients for acute malignant colorectal obstruction; clinical success was achieved in 98% of the patients. They stated that fluoroscopically guided placement was feasible without endoscopic assistance,

even in lesions proximal to the splenic flexure and transverse colon. In a multicenter retrospective study, Geraghty *et al.*^[8] aimed to determine the outcomes after SEMS; TTS endoscopy technique was found to be more successful than radiological placement alone (90.3% vs 74.8%, $P < 0.001$) for large bowel obstruction. In another retrospective study, Kim *et al.*^[9] compared the SEMS placement technique in 111 patients; while the technical success rate was significantly higher in the endoscopic method than in the radiologic method (100% vs 92.1%, respectively, $P = 0.038$), the clinical success rate did not differ significantly between the two groups (91.8% vs 97.1%, respectively, $P = 0.424$). They concluded that endoscopic and radiologic placement technique have their own advantages and disadvantages, but when an obstructive lesion is located in the tortuous, curved angulation of the sigmoid or descending colons, it is more difficult to pass the stenotic lesion using the radiologic method alone.

Bowel preparation before stent placement is not necessary, and oral bowel cleansing is contraindicated in symptomatic bowel obstruction, but enema can be used for facilitating the stent placement by preparing the bowel distal to the stenosis^[5].

Antibiotic prophylaxis before SEMS placement is not recommended routinely because of the low risk of fever and bacteremia after the insertion^[5]. However, antibiotic prophylaxis should be considered especially in patients with complete obstruction who have dilated colon and a risk of microperforation during insertion^[10].

Operator experience is an important matter in the placement of the stents. In a retrospective study, SEMS placement was performed in 334 patients, and technical and clinical success was higher for operators who had performed more than 10 procedures^[8]. In another study, Small *et al.*^[11] reported that the complication rate was higher when stents were placed by endoscopists who were not experienced in pancreaticobiliary endoscopy.

Technical success is usually defined as stent placement appropriately across the entire length of the stenosis, and clinical success is defined as resolution of colonic obstruction within the first days after the stent placement^[6]. Technical and clinical success rates vary between the studies. In a systematic review focusing on 88 studies published in 2007 by Watt *et al.*^[12] the median rate of technical success was 96.2%, ranging from 66.6% to 100%, and clinical success was achieved in 92% of the cases, ranging from 46% to 100%. It was stated that the etiology of the primary obstruction and indication for the stent placement appeared to have little effect on the rates of technical and clinical success. In a recent meta-analysis that included seven randomized clinical trials, pooled data showed a mean success rate of 76.9% (range: 46.7%-100%)^[13]. In another meta-analysis, Cennamo *et al.*^[14] compared randomized trials in terms of endoscopic stenting and surgical decompression for colorectal cancer obstruction; the stents were successfully inserted in 73.5% of patients, with

clinical relief of obstruction in 72% of patients.

Covered and uncovered SEMS can be used for colonic stenting. In a meta-analysis, in which Zhang *et al.*^[15] compared covered and uncovered stents, uncovered stents were found to be associated with a lower late migration rate, a higher tumor ingrowth rate, and a prolonged stent patency. No significant difference was found in technical success, clinical success, tumor overgrowth, early migration, perforation and overall complications between type of stents. In another meta-analysis including a total of 1376 patients, Yang *et al.*^[16] compared covered and uncovered SEMS in terms of technical success, clinical success and stent patency, and no significant difference was found between the two groups. Uncovered stents were found to be more prone to tumor ingrowth, but covered stents had the higher risk of stent migration over uncovered stents. Each type of stent have their own advantages and disadvantages. The main advantage of covered stents is a reduction in the risk of tissue ingrowth, whereas they are more prone to migrate.

INDICATIONS OF SEMS

Palliation of malignant obstruction

Acute malignant colorectal obstruction is a complication of colorectal cancer that can occur in 7%-29% of patients^[2]. Bowel obstruction can be caused by intrinsic disease or extrinsic compression. Large bowel obstruction causes colonic dilatation, bacterial translocation, electrolyte and fluid imbalance, and has an increased risk of colonic necrosis and perforation, so this gastrointestinal emergency needs urgent evaluation. The main treatment modalities for malignant colorectal obstruction are surgical resection or diverting colostomy. Resection is a suitable procedure in patients with less advanced cancer. Permanent stoma creation is a procedure for relieving symptoms of obstruction in patients with nonresectable tumors^[12]. Emergent surgery should be performed for the patients with colonic perforation and ischemia/necrosis. If there are no signs of systemic toxicity, SEMS can be performed in patients with a partial obstruction or with complete obstruction. SEMS is an alternative procedure for relieving the obstruction of the colon. In the literature, several studies have been published showing the feasibility and safety of SEMS in the management of acute malignant obstruction. In 2007, Watt *et al.*^[12] compared the safety and efficacy of SEMS with surgery through a systematic review. SEMS was found to be effective and safe in overcoming left-sided malignant colorectal obstructions, with high levels of technical and clinical success, shorter hospital stay, and lower rate of serious adverse events than surgery. Zhao *et al.*^[17] compared surgery with SEMS in the relief of obstruction in a meta-analysis, and the SEMS group showed a lower clinical success rate (99.8% vs 93.1%, $P = 0.0009$) but shorter length of hospital stay (18.84 d vs 9.55, $P < 0.00001$) and time to initiation of chemotherapy (33.36 d vs 15.53 d, $P < 0.00001$), and

lower rate of stoma formation (54.0% vs 12.7%, $P < 0.00001$). Hospital mortality was significantly lower in the SEMS group, and no difference was found in overall complications between the two groups. Surgery was found to be associated with short term complications and SEMS with late term complications^[17]. Liang *et al.*^[18] compared SEMS with surgery in the same indication mentioned above in a meta-analysis; the success rate of SEMS was found to be 93.9%, and no significant difference was found in mortality between the groups. The hospitalization time was shorter in the SEMS group ($P < 0.01$); however, long term complications were higher than surgery ($P = 0.03$). However, it was mentioned that all of the studies reported only the complications of colostomy or Hartmann's procedure, and none of them considered the complications of the stoma. They stated that morbidity and mortality would be much higher in the multi-stage surgery than with SEMS. In a recent study Young *et al.*^[19] compared the stent insertion and surgical decompression in patients with incurable large bowel obstruction in terms of improving quality of life. Stent related perforations or deaths were not reported. They found that surgery group had significantly reduced quality of life compared with the stent group. The patients in the stent group was found to have significantly lower permanent stoma rates, reductions in post procedure stay, earlier return of bowel function and shorter hospital stay. Thirty-day mortality for the stent group was 8% and for the surgery was 15% ($P = 0.67$). No significant difference was found in survival rates between treatment groups ($P = 0.61$)^[19].

Placement of SEMS as a bridge to elective surgery

SEMS have been suggested to relieve colon obstruction and act as a "bridge to surgery" for resectable colon cancers. There are conflicting results on this subject. In the literature, systematic reviews with meta-analysis have been published in order to evaluate preoperative SEMS placement as a bridge to elective surgery with emergency resection for acute malignant left-sided colonic obstruction. In the most recent meta-analysis that included seven randomized clinical trials, Huang compared emergency surgery and SEMS group. The pooled data showed a mean success rate of colonic stent placement of 76.9% (range: 46.7%-100%). Compared with the emergency surgery group, the SEMS group achieved significantly lower rates of permanent stoma (9% vs 27.4%, $P < 0.01$), primary anastomosis (67.2% vs 55.1%, $P < 0.01$), lower overall complications (33.1% vs 53.9%, $P = 0.03$) and lower wound infections (6.7% vs 18.1%, $P < 0.01$). No significant difference was found between the two groups in anastomotic leakage, mortality, or intra-abdominal infection. In this setting, SEMS placement for relieving obstructive symptoms allows time for the optimization of the medical condition, bowel preparation, and staging the disease^[13]. Although there are some advantages of SEMS placement preoperatively compared to the emergency surgery, long term oncological outcome, especially in patients with

resectable colon cancer, should be kept in mind.

There has been a major concern about the oncological outcome of the patients with resectable colon cancer who received SEMS placement as a bridge to surgery. In the literature, it was shown that placement of SEMS preoperatively in patients with resectable colon cancer impairs the oncological outcome, because of the dissemination of cancer cells during the procedure^[20], and because stent placement will be complicated by perforation and associated with ulceration as well as perineural and lymph node invasion of the tissues^[21]. Alcántara *et al.*^[22] compared short-term results and long-term outcomes of patients who underwent stent placement preoperatively with intraoperative colonic lavage with primary anastomosis. More relapses occurred in the SEMS group, but this finding was not significant, and no differences were found in survival. In another study, Sloothak *et al.*^[23] compared 5-year overall recurrence rates in the SEMS placement as a bridge to surgery group with the emergency surgery group; the SEMS group was found to have higher recurrence rate (42% vs 25%, $P = 0.027$). In a larger prospective study that evaluated the long-term oncological outcome between the same groups, in patients aged ≤ 75 years, stent as a bridge to surgery was associated with a higher local recurrence rate compared with emergency surgery (32% vs 8%, $P = 0.038$) without a difference in the overall survival rates^[24]. These findings suggest that use of SEMS in the treatment of curable patients with left-sided malignant colonic obstruction will impair the oncologic outcome. In the recent guidelines, as stent seems to impact the oncological safety with no reduction in postoperative mortality, SEMS as a bridge to elective surgery in curable patients with left-sided malignant colonic obstruction is not recommended. However, this procedure may be a good option for selected patients with a high risk of postoperative mortality, and patients over 70 years old and/or with American Society of Anesthesiologists score $\geq III$ ^[5].

Palliation of extracolonic malignant obstruction

Colonic obstructions also can occur due to tumor invasion, peritoneal seeding, or extraluminal compression resulting from advanced extracolonic malignancy^[25]. Outcomes of SEMS placement in the treatment of extracolonic malignancies are unclear. There have been published studies that compared the clinical outcomes of SEMS between patients with colon cancer and with extracolonic malignancies. Kim *et al.*^[25] performed SEMS placement for colorectal cancer in 149 patients and for extracolonic malignancy in 60 patients. Advanced gastric cancer, pancreatic cancer, and ovarian cancer were the most common causes of obstruction in that study. The clinical success rates, complications, and stent patency were similar between the two groups. In another study, Kim *et al.*^[26] evaluated the clinical outcomes and complications of SEMS compared with emergency surgery for relieving obstruction; technical and clinical success rates were higher in the emergency surgery

group. SEMS related complications occurred in 64.5% of the patients, including reobstruction (36.8%), stent migration (10.5%), perforation (13.2%), and bleeding (3.9%). In a retrospective study of palliative stent placement for extracolonic malignancies, clinical success was significantly higher in patients with colorectal cancer than in those with extracolonic malignancies (94.1% vs 20%, $P < 0.0001$). Two procedure related deaths occurred in the extracolonic malignancy group. Colon stenting for this purpose was found to be less successful in comparison with patients with colorectal cancer^[27].

SEMS for nonmalignant etiologies

Colonic stents have been used in a variety of non-malignant conditions as colonic strictures, including anastomotic strictures, Crohn's disease, radiation therapy, and diverticular disease^[3]. In a retrospective study, Keränen *et al.*^[28] evaluated a total of 21 patients with 23 SEMS procedures for benign colorectal obstruction; eight of the patients had an obstruction in the surgical anastomosis, two patients had anastomotic strictures due to Crohn's disease, 10 patients had the obstruction due to diverticular disease, and one patient had a stricture after radiation therapy. Technical success was achieved in all patients, and clinical success was achieved in 76% of the patients; complications occurred for 9 patients in 10 out of 23 procedures. They concluded that SEMS placement for benign colon strictures may be a good option for the patients who are not fit for surgery. Pommergaard *et al.*^[29] performed a retrospective study that included 45 patients with benign and malignant colonic obstruction: Technical and clinical success was 97.4% of the patients with malignant etiology, complications occurred in 21%, and mortality rate was 2.6%. For benign etiology, technical success was 85.7%, and clinical success was 71.4%, and complications occurred in 71.4% in this group with a mortality rate of 28.6%.

COMPLICATIONS OF SEMS

Stent related complications can occur in patients with malignant colon obstruction in the palliative or bridge to surgery setting. The most important complications of the stents are perforation, stent obstruction, stent migration, and bleeding. The most seen complication is the stent obstruction because of the tumor ingrowth or overgrowth^[30]. The main stent related complications are discussed above.

Perforation

The incidence of colonic perforation after SEMS placement varies between the studies. Perforation is the most feared complication of SEMS. In a review that included a total of 2287 patients, Datye *et al.*^[31] found overall perforation rate was 4.9%. No significant difference was found in the perforation rates for palliation and bridge to surgery (4.8% vs 5.4%, $P = 0.66$). The mortality rate after perforation was 16.2%. Most of the perforations

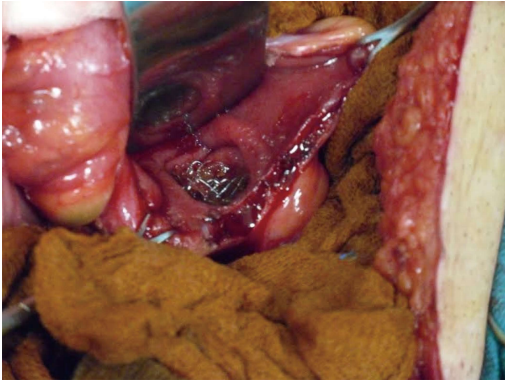


Figure 1 Perforation of the rectum due to the self-expanding metallic stent detected intraoperatively.

(over 80%) occurred within 30 d of stent placement, and almost half of these were noted during or within one day of the procedure; the majority of them were related to dilation, the guidewire, or the stent. In another systematic review, Watt *et al*^[12] reported the median rate of perforation caused by either the guidewire or stent was 4.5% (range: 0%-83%). The perforation rate was not found to be affected by the indication for stent placement. Colon perforation can be immediate or delayed, and is more likely to occur in the distal colon where sharp angulation and redundancy make stent deployment challenging^[32]. Baron *et al*^[10] identified the reasons that can cause perforation after stent placement in four different types: (1) guidewire or catheter malpositioning; (2) dilation of the stricture before or after stent placement; (3) stent-induced perforation; and (4) caused by proximal colonic distention away from the site of stent placement because of inadequate colonic decompression or excessive air insufflation. Delayed perforation can be tumor related, drug related (bevacizumab), and stent related^[33]. Figure 1 shows the perforation of the rectum due to the SEMS intraoperatively.

In the literature, published results have shown that patients who have undergone palliative stenting can be treated with chemotherapy without antiangiogenic agents^[34]. Safety of SEMS in the colon or rectum of patients who are receiving the anti-angiogenic agent bevacizumab as a component of chemotherapy has been studied in the literature. Radiotherapy and bevacizumab may increase the risk of perforation. In a retrospective study that includes 201 patients undergoing stenting for incurable malignant obstruction, bevacizumab therapy was found to increase the risk of perforation by 19.6-fold over patients who did not receive bevacizumab^[35]. In another review, Small *et al*^[11] determined long-term efficacy, incidence of complications, and risk factors of SEMS placement for colonic obstruction; the incidence of perforation was higher in patients with bevacizumab treatment compared with untreated patients (15.4% vs 6.8%). The complication rate was found to be associated with SEMS placement in men, completely obstructed bowel, with balloon-dilated strictures, and with post-stent bevacizumab treatment. Given the high risk of



Figure 2 Occluded self-expanding metallic stent due to the tumor ingrowth.

perforation, if a patient is treated or considered to be treated with antiangiogenic agents like bevacizumab, it is not recommended to use SEMS as a palliative treatment for obstruction^[34]. However, there is no strong evidence for the newer antiangiogenic agents like aflibercept and regorafenib, and because of the similar mechanisms, perforation risk should be kept in mind^[5]. Based upon these data, it is suggested that colonic stent placement be avoided if possible in patients who are or who will be receiving bevacizumab.

Stent obstruction

The most common complication is stent obstruction because of the tumor ingrowth or overgrowth^[31]. Figure 2 shows the occluded SEMS due to the tumor ingrowth. In a meta-analysis that included 13 studies, 11 of them reported stent related complications, rate of perforation was 10.1%, stent migration was 9.2%, and stent obstruction was 18.3%^[17]. It is believed that covered stents provide resistance to tumor ingrowth, thus helping to reduce reconstruction events, while uncovered stents are believed to minimize stent migration^[12,36,37]. For the uncovered stents, the main disadvantage is the tumor ingrowth, but the membrane of the covered stents can provide a barrier to prevent this. In a systematic review that compared covered SEMS with uncovered SEMS, uncovered SEMS showed a higher tumour ingrowth rate (RR = 5.99; 95%CI: 2.23-16.10, $P = 0.0004$)^[15]. The factors that are associated with the stent obstruction are: Demographic factors, underlying malignancy, length of stent, site of stricture, degree of stent expansion, and chemotherapy after stent insertion^[30]. However, Im *et al*^[38] performed a study in order to evaluate clinical outcomes, long term complications, and patency of SEMS in patients with malignant colorectal obstruction, and stent patency was not found to be associated with demographic characteristics of patients, site of obstruction, or palliative chemotherapy. In a retrospective study, Suh *et al*^[30] analyzed the predictive factors for stent occlusion, and insufficient stent expansion (< 70%) 48 h after stent insertion was significantly associated with stent occlusion during the follow-up.

If SEMS is inserted for the relief of obstruction in advanced and incurable colorectal cancer, the stent patency should be maintained until the death of the patients^[30]. but if it is used in the preoperative setting, after 1-2 wk, the stent will be removed en bloc at the time of surgical resection^[6]. In a systematic review, 14 studies reported duration of patency; the median of reported study mean durations was 106 d (range: 68-288 d) in the palliative stent population^[12]. Suh *et al*^[30] reported stent patency mean and median 184 and 141 d respectively.

There have been no accurate data in the management of occluded SEMS in malignant colorectal obstruction. In most of the patients with stent obstruction, this can be treated by stent-in-stent placement. In a retrospective case series, Yoon *et al*^[39] determined the effectiveness of stent-in-stent SEMS insertion for the treatment of SEMS obstruction in cases with malignant colorectal obstruction. In this study the clinical success was reported in 75% of the cases; 9 of them had persistent symptoms, 8 of them underwent palliative surgery, but at the end of the follow-up, 16 of 36 patients (44.4%) remained free of obstruction symptoms until death. The success rate was found to be slightly lower than that of primary SEMS placement^[40]. In another study, Yoon *et al*^[39] compared the clinical outcomes of the patients who underwent second intervention because of the obstruction of the first successful SEMS placement for colorectal obstruction with second SEMS insertion or palliative surgery. No significant difference was found in the median overall survival (8.2 mo vs 15.5 mo) and progression-free survival (4.0 mo vs 2.7 mo) between the stent and surgery groups. However, the median lumen patency in the stent group was 3.4 mo and 7.9 mo in the surgery ($P = 0.003$). Male gender and having an obstruction in the right colon were identified as prognostic factors of lumen patency in second SEMS; additional chemotherapy after a second intervention was found to be a prognostic factor with a longer lumen patency in the palliative surgery group.

Stent migration

In a systematic review including 54 studies, Watt *et al*^[12] evaluated stent for all indications reported that median rate of migration was 11%, ranging from 0% to 50%. Stent migration can occur at any time following the insertion, but is usually detected within one week of insertion. Migrations tend to occur with stents which are too narrow in diameter and/or too short in relation to the stricture they are placed in^[41]. In another systematic review, Khot *et al*^[42] reported stent migration in 54 (10%) of 551 technically successful cases; 26% of the stent migration occurred within 3 d, and the remaining occurred after 3 d. Factors that were associated with the migration were laser pretreatment, chemotherapy, and benign tumor. Covered and small diameter (< 24 mm) SEMS were also found to be associated with stent migration^[15,35,36]. Chemotherapy with the mechanism of tumor shrinkage increases the stent migration^[43-45].

Others

After the SEMS placement, abdominal pain and bleeding can occur in the follow-up. Bleeding is usually minor after the procedure, and generally no intervention will be required. Abdominal or rectal pain is common and varies between 7.4% and up to 62.5% in patients with SEMS placement within 5 cm of the anal verge^[46,47]. Mild abdominal pain generally requires no specific treatment; if needed, use of analgesics will be enough for relieving the pain.

CONCLUSION

SEMS placement can be an alternative method in the treatment of patients with colorectal cancer who have acute malignant colorectal obstruction. SEMS offers favourable results compared to surgery in the setting of colorectal obstruction in advanced disease. Use of SEMS in the treatment of curable patients with left-sided malignant colonic obstruction will impair the oncologic outcome; therefore, SEMS as a bridge to elective surgery in curable patients with left-sided malignant colonic obstruction is not recommended. Although SEMS placement seems to be safe and effective and has some advantages over surgery, short term and long term morbidities should be kept in mind, and it will be preferred for patients who are at increased risk for complications of emergency surgery.

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Role of microRNA-7 in digestive system malignancy

Wan-Qun Chen, Ling Hu, Geng-Xin Chen, Hai-Xia Deng

Wan-Qun Chen, Hai-Xia Deng, Guangdong Provincial Hospital of Chinese Medicine, Guangzhou 510120, Guangdong Province, China

Wan-Qun Chen, Ling Hu, Institute of Gastroenterology, Guangzhou University of Chinese Medicine, Guangzhou 510004, Guangdong Province, China

Geng-Xin Chen, Guangdong Provincial Hospital of Chinese Medicine, Guangdong Provincial Key Laboratory of Clinical Research on Traditional Chinese Medicine Syndrome, Guangzhou 510120, Guangdong Province, China

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Correspondence to: Hai-Xia Deng, MD, PhD, Guangdong Provincial Hospital of Chinese Medicine, No. 111 Dade Road, Guangzhou 510120, Guangdong Province, China. hxd6870@126.com
Telephone: +86-20-81887233
Fax: +86-20-81887233

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Abstract

There are several malignancies of the digestive system (including gastric, pancreatic and colorectal cancers, and hepatocellular carcinoma), which are the most common types of cancer and a major cause of death worldwide. MicroRNA (miR)-7 is abundant in the pancreas, playing an important role in pancreatic development and endocrine function. Expression of miR-7 is downregulated in digestive system malignancies compared with normal tissue. Although there are contrasting results for miR-7 expression, almost all research reveals that miR-7 is a tumor suppressor, by targeting various genes in specific pathways. Moreover, miR-7 can target different genes simultaneously in different malignancies of the digestive system. By acting on many cytokines, miR-7 is also involved in many gastrointestinal inflammatory diseases as a significant carcinogenic factor. Consequently, miR-7 might be a biomarker or therapeutic target gene in digestive system malignancies.

Key words: MicroRNA-7; Digestive system malignancy; Tumor biomarker; Target gene; Inflammation

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Core tip: MicroRNA (miR)-7 targets different genes in various complicated pathways and plays diagnostic, prognostic, anti-metastatic, and therapeutic roles in digestive system malignancies. MiR-7 might be a biomarker or therapeutic target gene in digestive system malignancies, even in the precancerous lesions (inflammatory disease).

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INTRODUCTION

MicroRNAs are small noncoding RNAs consisting of 18-25 nucleotides that post-transcriptionally regulate expression of target genes, and are involved in cell proliferation, epithelial-mesenchymal transition (EMT), apoptosis, migration, invasion and metastasis^[1-3]. miRNAs have emerged as potential critical regulators of carcinogenesis and tumor progression^[4,5].

The digestive system is composed of many ducts and glands, and because of its complicated physiology and anatomy, numerous diseases may occur, especially malignancies including the third, fourth and eighth most common cancers worldwide: Colorectal cancer (CRC), gastric cancer (GC) and esophageal cancer, respectively^[6,7], as wells as the leading cause of cancer-related death: Pancreatic cancer (PC)^[8]. According to the 2014 cancer statistics, the combined cancer mortality rates have been continuously declining for the past two decades. However, the incidence of some digestive system malignancies, including cancers of the esophagus, liver, anus and pancreas, is increasing. Moreover, with rising death rates for cancers of the liver, anus and pancreas, and other non-digestive cancers, cancer is still the second leading cause of death following heart disease^[8]. Therefore, it is necessary for us to explore novel molecular mechanisms, and screen for the most effective therapeutic methods to avoid the majority of patients succumbing to these digestive malignancies.

MicroRNA (miR)-7 is an evolutionarily conserved miRNA that is involved in the development of the eye and pancreas in *Drosophila*. Li *et al*^[9] reported that miR-7 is repressed by the transcription factor Yan, which is degraded while mediating epidermal growth factor receptor (EGFR) signaling. Also, miR-7 is expressed abundantly in human pancreas and endocrine cells and has a specific role in endocrine cell differentiation and function^[10]. It has been demonstrated that miR-7 is a tumor suppressor in breast, lung and ovarian cancers, and glioblastoma, mainly focusing on its relationship with EGFR^[11-15]. Accumulating evidence shows that miR-7 can simultaneously target a variety of mRNAs involved in diverse signaling pathways in different tumors. However, no specific review has described the role of miR-7 in digestive tract malignancies. In this review, we focus on current research on miR-7 in order to elucidate its role in digestive system malignancies or their precancerous lesions, with reference to its expression, signaling pathways, and role as a circulatory biomarker.

EXPRESSION OF MIR-7

By comparing the differential expression of miRNAs in

pancreatic islets (endocrine) and acinar (exocrine) tissue in rats, using microarray and quantitative polymerase chain reaction (qPCR), Bravo-Egana *et al*^[16] revealed that miR-7 was ranked highest among the 17 miRNAs preferentially expressed in islets, suggesting that it acts as an endocrine miRNA. Another two studies reported that miR-7 was expressed at a high level during human pancreatic islet development^[10,17]. For malignancy, it has been demonstrated that miR-7 is downregulated in cancer tissue of digestive malignancies such as GC^[18-20], CRC^[21,22] and hepatocellular carcinoma (HCC)^[23] by comparison with normal tissues, suggesting that it acts as a suppressor. A similar conclusion was drawn in a study of hydroxycamptothecin-resistant GC cells^[24]. In some inflammatory diseases, such as gastritis and Crohn's disease^[25], the level of miR-7 is also lower than that in normal tissue, which suggests that it is an inflammation-related miRNA participating in the process of digestive cancer.

In contrast, using the same method, Suto *et al*^[26] discovered that miR-7 level was higher in CRC tissue than in adjacent normal tissue, induced by EGFR mutations. However, it was found that the aforementioned results would be opposite when the EGFR protein expression was positive in CRC. Finally, they concluded that low miR-7 expression resulted in poorer prognosis than high expression. Ahmed *et al*^[27] identified the expression of miR-7 in stool samples from 40 cases of colon cancer (TNM stages 1-4), and found that miR-7 was one of the 12 increased miRNAs, which they then recognized as a diagnostic gene. In HCC, Fang *et al*^[28] speculated that owing to inactivation of the transcriptional regulators and/or failure to promote miR-7 expression, there is no alteration of its expression between tumor and adjacent normal tissues. However, miR-7 and miR-21 are overexpressed in esophageal squamous cell carcinoma (ESCC) and related to its differentiation^[29].

From Table 1, we can speculate the reasons for the divergent views about the expression of miR-7 under different conditions, including^[30]: (1) heterogeneity of different malignancies/diseases; (2) different study sample sizes; and (3) the standards were not the same (*e.g.*, whether or not to include patients with prior cytotoxic therapy). Based on the published studies, we conclude that miR-7 could be an oncogene or tumor suppressor in the digestive system depending on the specific gene targeted (Table 2).

GC

The pathogenesis of GC has been extensively studied, and there is a consensus that intestinal gastric carcinogenesis is a multistep process starting with chronic gastritis triggered by *Helicobacter pylori*, progressing through atrophy, intestinal metaplasia and dysplasia to carcinoma (Correa model)^[31]. Thus, inflammation is a significant event in gastric carcinogenesis, whereas miR-7 is an inflammation-mediated miRNA inversely

Table 1 Expression of microRNA-7 in the digestive system

Cancer	Sample	Sap No.	Method	Exp	Role	Ref.
Colon	Stool	60	qPCR	↑	Diagnostic	[27]
CRC	Tissue	80	qPCR	↓	Diagnostic/therapeutic	[21]
CRC	Tissue	8	RT-PCR	↓	Therapeutic	[22]
CRC	Tissue	105	qRT-PCR	↑	Prognostic	[26]
ESCC	Tissue	34	Microarray/qRT-PCR	↑	Differentiation	[29]
GC	Tissue	40	ISH/IHC	↓	Inhibits metastasis/EMT	[18]
GC	Tissue	23	Microarray	↓	Inhibits invasion/metastasis	[19]
			qRT-PCR			
GC	Tissue	28	RT-PCR	↓	Represses inflammation	[20]
HCC	Tissue	10	Microarray	-	Therapeutic/diagnostic/prognostic	[28]
			qRT-PCR			
HCC	Tissue	12	qRT-PCR	↓	Tumor suppressor	[23]
HCC	Tissue	429	Chip assay	↓	Prognostic	[43]
CD	Tissue	-	RT-PCR	↓	Therapeutic	[25]

↓: MiR-7 is downregulated; ↑: MiR-7 is upregulated; -: There is no alteration for expression of miR-7, or no mention. Sap No.: Sample number; Exp: Expression; ISH/IHC: *In situ* hybridization/immunohistochemistry; CRC: Colorectal cancer; GC: Gastric cancer; HCC: Hepatocellular carcinoma; ESCC: Esophageal squamous cell carcinoma; EMT: Epithelial-mesenchymal transition; qPCR: Quantitative polymerase chain reaction.

Table 2 Function of microRNA-7 by targeting diverse genes

Cells	Function of miR-7	Target	Ref.
CCA	Reduces migration, invasion and metastasis	LAT1	[41]
CRC	Inhibits proliferation, invasion and metastasis, and induces G1 arrest	PAX6	[21]
CRC	Inhibits proliferation and induces apoptosis	XRCC2	[22]
CRC	Suppresses proliferation, induces G1 arrest, and induces apoptosis	YY1	[37]
GC	Suppresses invasion and metastasis	IGF1R	[18]
GC	Inhibits proliferation, invasion and metastasis	EGFR	[19]
HCC	Decreases invasion and migration	PIK3CD/mTOR/p70S6K	[28]
HCC	Suppresses colony formation and induces cell cycle arrest	CUL5	[23]

CCA: Cholangiocarcinoma; CRC: Colorectal cancer; GC: Gastric cancer; HCC: Hepatocellular carcinoma; miR-7: MicroRNA; XRCC2: X-ray repair complementing defective repair in Chinese hamster cells 2; IGF1R: Insulin-like growth factor 1 receptor; EGFR: Epidermal growth factor receptor; mTOR: Mammalian target of rapamycin; CUL5: Cullin 5.

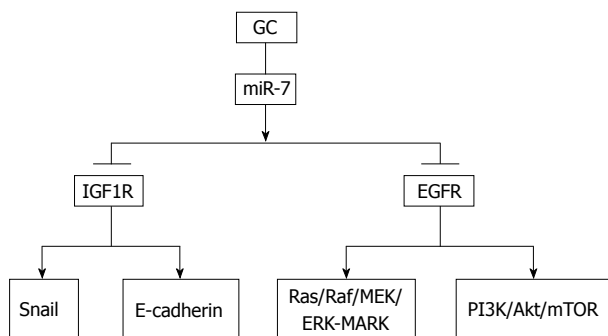


Figure 1 Pathway of microRNA-7 in gastric cancer. It has been revealed that miR-7 targets mainly IGF1R and EGFR. IGF1R: Insulin-like growth factor 1 receptor; EGFR: Epidermal growth factor receptor; GC: Gastric cancer; miR-7: MicroRNA-7; PI3K: Phosphoinositide 3-kinase; mTOR: Mammalian target of rapamycin.

correlated with many proinflammatory cytokines and inflammatory factors such as interleukin-1 β and tumor necrosis factor- α . Three genes, *LPHN2*, *BASP1* and *MAFG*, targeted by miR-7 are induced in the cyclooxygenase (COX)-2/prostaglandin (PG) E2 pathways, which shows that miR-7 plays a significant part in gastric tumorigenesis with an inflammatory response^[20].

MiR-7 suppresses GC cell invasion and metastasis both *in vitro* and *in vivo* by targeting the miR-7/insulin-like growth factor 1 receptor/Snail axis, which shows its EMT function and suggests that it can act as a therapeutic biomarker to prevent GC metastasis^[18]. Xie *et al.*^[19] demonstrated that restoration of miR-7 significantly inhibited tumor cell viability, invasion and migration by suppressing EGFR expression. These results suggest that targeting miR-7 is a potential therapeutic option for GC (Figure 1).

PC

PC is one of the major leading causes of cancer mortality; the 5-year survival rate for pancreatic adenocarcinoma is < 5%, and most patients die within the first 2 years^[8]. Therefore, there is an urgent need to explore novel therapeutic methods. In accordance with the expression of miR-7 in the pancreas, miR-7-3, which is one of the three endogenous genes potentially transcribed in the human genome, is upregulated by targeting mitogen-activated protein kinase (MAPK), suggesting that miR-7 is negatively modulated by an EGFR-MAPK feedback loop^[32]. In an *in vitro* study, in

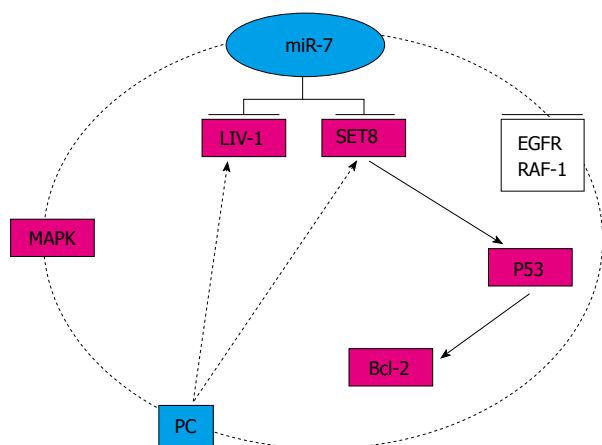


Figure 2 Pathway of microRNA-7 in pancreatic cancer. In PC, there is an EGFR-MAPK-miR-7 negative feedback loop, and LIV-1 and SET8 are two other targets. EGFR: Epidermal growth factor receptor; MAPK: Mitogen-activated protein kinase; PC: Pancreatic cancer; miR-7: MicroRNA-7; SET8: SET domain containing 8.

which miR-7 targeted SET domain containing 8 leading to increased p53 expression and decreased Bcl-2 level, curcumin suppressed cell growth, migration and invasion, and induced apoptosis in PC cells, indicating that targeting miR-7 is a useful therapeutic option for PC^[33]. Although knockdown of LIV-1 (a zinc transporter) can upregulate expression of miR-7 in PC cells, the exact role of miR-7 in the maintenance of cancer-stem-cell-related phenotypes in PC remains unclear^[34]. Future research will focus on identifying the exact pathway of miR-7 in PC, and only in this way, can research proceed from bench to bedside (Figure 2).

CRC

CRC is related to the mutation of genes such as P53, APC, SMAD4, PIK3CA, KRAS, ARID1A, SOX9 and FAM123B. Some minimal tailoring of therapy (selecting a chemotherapeutic agent based on toxicity, or not using anti-EGFR in those with KRAS-mutated tumors) can be offered to patients, however, the dream of truly individualized therapy remains elusive^[35,36]. Based on the present studies, miR-7 can target specific genes to modulate the correlated pathways, and its decreased expression continuously participates in the process of CRC. MiR-7 is a tumor suppressor, which is mediated through the YY1-P53-Wnt signaling pathway, and plays pivotal roles in many cellular processes, such as development, differentiation, proliferation and apoptosis^[37]. By targeting EGFR and *v-raf-1* murine leukemia viral oncogene homolog 1 (RAF-1), a low level of miR-7 suggests poor prognosis for CRC, and miR-7 precursor, alone or in combination with a monoclonal antibody, could be a novel therapy against CRC^[26]. XRCC2 (X-ray repair complementing defective repair in Chinese hamster cells 2) participates in homologous recombination, and its relationship with miR-7 has been studied. *In vitro*, overexpression of miR-7 suppressed

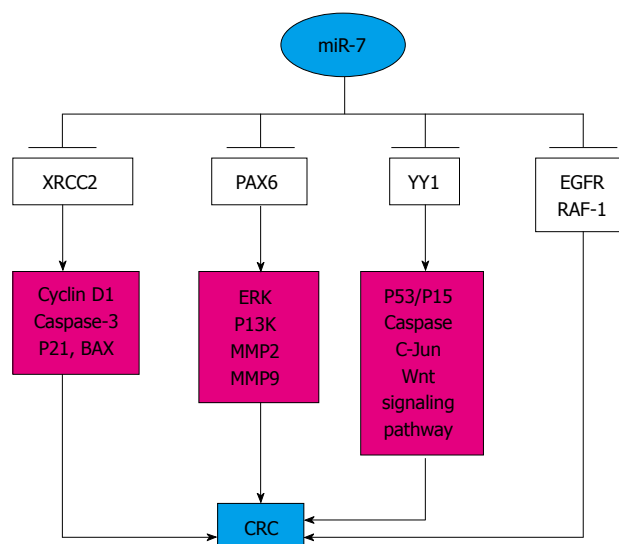


Figure 3 Pathway of microRNA-7 in colorectal cancer. MiR-7 can target various genes involving different pathways and act as a suppressor. CRC: Colorectal cancer; EGFR: Epidermal growth factor receptor; miR-7: MicroRNA-7. XRCC2: X-ray repair complementing defective repair in Chinese hamster cells 2; ERK: Extracellular signal-regulated kinase.

proliferation and induced apoptosis of CRC cells by directly targeting XRCC2 through decreasing cyclin D1 and increasing p21, caspase-3 and BAX expression^[22]. In addition, the expression of paired box (PAX) 6 is inversely correlated with that of miR-7, and simultaneous activation of the extracellular signal-regulated kinase and phosphoinositide 3-kinase (PI3K) signaling pathways and regulation of the levels of matrix metalloproteinase (MMP) 2 and MMP9 could modulate the expression of PAX6 and miR-7 in opposing ways, which suggests that miR-7 is a promising therapeutic target for CRC^[21]. Thus, further mechanisms mediated by miR-7 should be explored, which could be a promising approach for individually tailored therapy of CRC (Figure 3).

HCC

HCC, the third most common cause of cancer mortality worldwide, which develops from activation of cellular oncogenic pathways and abrogation of tumor suppressor pathways including the p53/p21^{WAF1} pathway, the p16^{INK4a}/CDK4/RB1/E2F pathway, the Wnt/ β -catenin signaling pathway, transforming growth factor- α , c-myc, transcription factor NF- κ B, insulin/IGF-I, and receptor tyrosine kinases and their downstream activators^[38,39]. Several studies have shown that miR-7 participates in several pathways by targeting different genes in HCC. MiR-7 regulates the PI3K/Akt/mammalian target of rapamycin *in vitro* and *in vivo*, which functions downstream of EGFR, suggesting that miR-7 is a potential target for treating or diagnosing/prognosing HCC^[28]. Likewise, ectopic expression of cullin 5, a novel target gene of miR-7, inhibits HCC cell proliferation, arrests cell cycle progression, and suppresses colony formation, although the exact pathway remains unclear^[23]. Moreover,

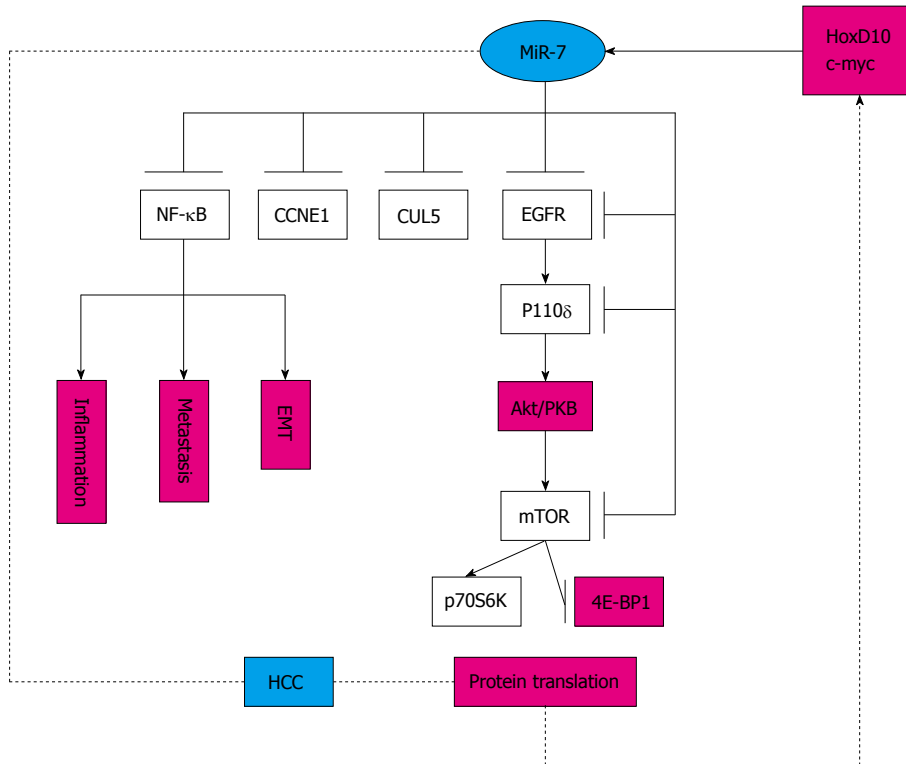


Figure 4 Pathway of miR-7 in hepatocellular carcinoma. MiR-7 can target various genes in the specific signaling pathway. HCC: Hepatocellular carcinoma; miR-7: MicroRNA-7; NF-κB: Nuclear factor κB; CCNE1: Cyclin E1; CUL5: Cullin 5; EGFR: Epidermal growth factor receptor; mTOR: Mammalian target of rapamycin; EMT: Epithelial–mesenchymal transition.

a member of the highly conserved cyclin family, CCNE1 (cyclin E1), is inversely correlated with miR-7 expression in HCC cell lines and clinical samples, indicating that it is a downstream mediator for miR-7, and miR-7 might be a candidate for the treatment of HCC^[40]. Most studies in this field have been *in vitro* experiments, except one^[28]. The detailed mechanisms remain to be elucidated, thus, the exact role of miR-7 in HCC needs further research (Figure 4).

OTHER DIGESTIVE MALIGNANCIES

MiR-7 also plays a role in other digestive tract malignancies such as cholangiocarcinoma and ESCC^[29,41]. However, the expression and exact role of miR-7 in these two malignancies need to be verified.

INFLAMMATORY DISEASE

Inflammation makes a significant contribution to carcinogenesis and progression of malignancies^[42]. In some conditions, inflammation such as chronic atrophic gastritis is defined as a precancerous lesion of GC. MiR-7 also participates in some inflammatory diseases, in addition to malignancies. The role of miR-7 in the progression from chronic inflammation to GC has been studied more thoroughly compared with other inflammatory diseases. Using established mouse models, Kong *et al.*^[20] have demonstrated that downregulation of miR-7 induced by PGE2 associated with inflammation, and activation

of EGFR are critical steps in gastric carcinogenesis. Although the COX-2/mPGES-1/PGE2/EP2 pathway has been identified in gastric tumorigenesis, whether there is a similar mechanism mediated by miR-7 has not been established in other malignancies. It has been revealed that the expression of miR-7 is decreased in actively inflamed colonic tissues from patients with Crohn's disease, which is regulated by hCD98^[25]. Similarly, chronic hepatitis has an important influence on HCC development, and hepatocyte nuclear factor 4α and NF-κB form a feedback circuit, for which miR-7 and miR-124 could be the targets^[43]. These findings suggest that miR-7 is involved in many inflammatory diseases by activating many inflammatory/proinflammatory cytokines, and it will be intriguing to demonstrate the role of miR-7 in the regulation of alimentary inflammatory responses and carcinogenesis.

CIRCULATORY BIOMARKER

The diagnostic and prognostic biomarkers for some digestive malignancies including GC and CRC are still limited. Common circulatory markers such as carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 have inadequate sensitivity, therefore, exploring specific biomarkers is a significant breakthrough.

MiRNAs are stable in serum, plasma and body fluids (*e.g.*, stools and gastric juice), and their expression differs between tumor and non-tumor tissue. Some miRNAs, like miR-21, have been subjected to meta-

analysis and concluded to be diagnostic biomarkers for GC^[44]. Wang *et al.*^[45] designed their study with three phases. In the discovery phase, they detected 723 miRNAs in 80 serum samples using microarrays; in the training phase they experimented on another 112 plasma samples using qPCR; and finally, they confirmed the results with 49 samples using a logistic model, and screened miR-7 as one of a panel that yielded high diagnostic accuracy to diagnose CRC. Compared with CEA, miR-7 has a higher receiver operating characteristic curve, sensitivity and specificity (0.897, 82% and 89%, respectively). Similarly, by analyzing the serum from 12 acute pancreatitis patients and three healthy controls, Liu *et al.*^[46] identified miR-7 as one of the three diagnostic and prognostic biomarkers. Although several systematic reviews^[44,47-49] have investigated biomarkers for GC, none has shown that miR-7 could be a biomarker of GC.

CONCLUSION

Several studies have identified possible mechanisms mediated by miR-7 in specific malignancies of the digestive system, including some inflammatory diseases. No study has investigated miR-7 comprehensively, which may explain why different studies have discovered different targets for miR-7, or it may be because miRNA can form one-to-one, one-to-multiple or multiple-to-one relationships with its target genes^[50]. Disruption of homeostasis in the digestive system is due to many pathways acting together in a complicated manner, which contributes to the progression from inflammatory diseases to malignancy. Furthermore, the genetic abnormalities in tumors are highly heterogeneous, and no two tumors are exactly alike, which raises a serious challenge. Consequently, more research should be conducted to verify whether miR-7 could be a biomarker or therapeutic target gene for digestive system malignancies.

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Retrospective Study

Impact of *RAS* and *BRAF* mutations on carcinoembryonic antigen production and pattern of colorectal metastases

May Cho, Chie Akiba, Cecilia Lau, David Smith, Milhan Telatar, Michelle Afkhami, Stephen Sentovich, Kurt Melstrom, Marwan Fakih

May Cho, Chie Akiba, Cecilia Lau, Marwan Fakih, Department of Medical Oncology, City of Hope National Medical Center, Duarte, CA 91010, United States

David Smith, Department of Statistics, City of Hope National Medical Center, Duarte, CA 91010, United States

Milhan Telatar, Michelle Afkhami, Department of Pathology, City of Hope National Medical Center, Duarte, CA 91010, United States

Stephen Sentovich, Kurt Melstrom, Department of Surgical Oncology, City of Hope National Medical Center, Duarte, CA 91010, United States

Author contributions: Fakih M designed and supervised the trial; Fakih M and Cho M wrote the paper; Telatar M, Afkhami M, Sentovich S and Melstrom K contributed to the analysis; Cho M, Akiba C and Lau C collected and analysed data; Smith D did the statistical analysis.

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Correspondence to: Marwan Fakih, MD, Professor, Department of Medical Oncology, City of Hope National Medical Center, Building 51, Room 101, 1500 E Duarte St., Duarte, CA 91010, United States. mfakih@coh.org
Telephone: +1-626-2564673-63087
Fax: +1-626-3018233

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Abstract

AIM: To investigate the impact of *RAS* and *BRAF* mutations on the pattern of metastatic disease and carcinoembryonic antigen (CEA) production.

METHODS: In this retrospective study, we investigated the impact of *RAS* and *BRAF* mutational status on pattern of metastatic disease and CEA production. Only patients presenting with a newly diagnosed metastatic colorectal cancer (CRC) were included. Patients' characteristics, primary tumor location, site of metastatic disease and CEA at presentation were compared between those with and without *RAS* and *BRAF* mutations.

RESULTS: Among 174 patients, mutations in *KRAS*, *NRAS* and *BRAF* were detected in 47%, 3% and 6% respectively. *RAS* mutations (*KRAS* and *NRAS*) were more likely to be found in African American patients (87% vs 13%; *P* value = 0.0158). *RAS* mutations were associated with a higher likelihood of a normal CEA (< 5 ng/mL) at presentation. *BRAF* mutations were more likely to occur in females. We were not able to confirm

any association between mutational status and site of metastatic disease at initial diagnosis.

CONCLUSION: No association was found between *RAS* and *BRAF* mutations and sites of metastatic disease at the time of initial diagnosis in our cohort. Patients with *RAS* mutations were more likely to present with CEA levels < 5 ng/mL. These findings may have clinical implications on surveillance strategies for *RAS* mutant patients with earlier stages of CRC.

Key words: *RAS*; *BRAF*; Carcinoembryonic antigen; Pattern of metastatic disease; Surveillance

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Core tip: We investigated the impact of *RAS* and *BRAF* mutations on pattern of colorectal cancer (CRC) metastases and carcinoembryonic antigen (CEA) production. Patients with *RAS* mutations were more likely to present with CEA levels < 5 ng/mL. No association was found between *RAS* and *BRAF* mutations and sites of metastatic disease at the time of initial diagnosis in our cohort. Our study is the first study to link low CEA production with a *RAS* mutant status at the time of initial presentation of metastatic CRC. These findings may have clinical implications on surveillance strategies for *RAS* mutant patients with earlier stages of CRC.

Cho M, Akiba C, Lau C, Smith D, Telatar M, Afkhami M, Sentovich S, Melstrom K, Fakih M. Impact of *RAS* and *BRAF* mutations on carcinoembryonic antigen production and pattern of colorectal metastases. *World J Gastrointest Oncol* 2016; 8(1): 128-135 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v8/i1/128.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v8.i1.128>

INTRODUCTION

Colorectal cancer (CRC) continues to be the second leading cause of cancer-related death in the United States. It is projected that 136830 individuals will be diagnosed with CRC in 2014 in the United States, 50310 of whom will succumb to this disease^[1]. While significant progress has been made in the treatment of metastatic CRC (mCRC) over the last two decades, cure amongst these patients remains rare and is only achievable in approximately 20% of patients who are amenable to metastases resection^[2,3].

It is estimated that 20% of patients with CRC present with metastatic disease while another 30% develop metastatic disease after an initial presentation with local or regional disease^[2,4]. Patients with limited oligometastatic disease are the ones who benefit the most from aggressive surgical strategies^[5]. Therefore, early identification of metastatic disease remains key in improving the outcome of patients with metastatic disease. Indeed, intensive surveillance strategies in

patients with earlier stages of CRC have been associated with an increased rate of metastectomies in several prospective and retrospective clinical trials^[6]. However, these surveillance strategies are not standardized amongst different medical societies and do not take into account the molecular heterogeneity of CRC^[7]. It has been recently shown that certain oncogenic alterations have significant impact on disease biology, response to treatment, and overall outcome. For example, *BRAF* mutations, present in 5%-10% of CRCs, are associated with worse prognosis, a worse overall survival after disease recurrence, and a tendency to metastasize to the peritoneum and distant lymph nodes^[8,9]. The impact of *KRAS* and *NRAS* mutations, which occur in approximately 50% of CRCs, on the pattern of metastatic disease at initial presentation has been more controversial^[10-13].

To better understand the impact of the commonly tested *RAS* (*KRAS* and *NRAS*) and *BRAF* mutations on metastatic disease pattern and on surveillance strategies, we conducted a single institute retrospective study that investigates the impact of *RAS* and *BRAF* mutations on the pattern of metastatic disease and carcinoembryonic antigen (CEA) production.

MATERIALS AND METHODS

Study population

We retrospectively reviewed all cases with metastatic colon cancer patients who presented to City of Hope Comprehensive Cancer Center from 2007 to 2014. Inclusion on study required all the following criteria: (1) confirmed CRC by pathology; (2) availability of imaging studies confirming metastatic disease at the time of presentation; (3) availability of *KRAS* or *BRAF* testing by PCR or by ONCO44 or ONCO48 next generation sequencing; and (4) available CEA level at the time of presentation of metastatic CRC.

Patients' characteristics including age, gender, race, location of the primary tumor, CEA, and sites of metastatic disease at the time of presentation were reviewed and collected from corresponding electronic medical records. Primary tumor location was categorized as right or transverse colon, left colon, and rectum. Metastatic sites were categorized into 3 groups: (1) lung; (2) liver; and (3) mesenteric or distal lymph nodes or peritoneum. The study was approved by the local institutional review board.

RAS and *BRAF* analysis

To allow for a more powerful sample size, we included *RAS* and *BRAF* analysis performed by either a CLIA certified next generation sequencing or a CLIA certified PCR assay.

Onco 44: Genomic DNA is extracted from micro-dissected cells from formalin-fixed, paraffin-embedded tissue with minimum 30% tumor cellularity. A targeted DNA library is generated using the Ion AmpliSeq™ Cancer

Table 1 *RAS* and *BRAF* status and patient demographics

		All		<i>BRAF</i> MT		<i>BRAF</i> WT		<i>P</i>	<i>RAS</i> MT		<i>RAS</i> WT		<i>P</i>
		<i>n</i>	%	<i>n</i>	%	<i>n</i>	%		<i>n</i>	%	<i>n</i>	%	
Age	< 60	87	50	5	6	82	94	1.00	44	51	43	49	1.00
	≥ 60	87	50	6	7	81	93		43	49	44	51	
Gender	Male	103	59	3	3	100	97	0.052	49	48	54	52	0.54
	Female	71	41	8	11	63	89		38	54	33	46	
Race	White	122	70	7	6	115	94	0.53	57	47	65	53	0.015
	Asian, PI	41	24	4	10	37	90		23	56	18	44	
	Black	8	5	0	--	8	100		7	87	1	13	
	Unknown	3	2	0	--	3	100		0	--	3	100	

n: Number of patients; PI: Pacific Islander; MT: Mutant; WT: Wild type.

Hotspot Panel Kit, and sequenced by semiconductor-based next-generation sequencing technology on an Ion Torrent PGM. The Onco 44 panel is designed to target 713 mutations in 44 key cancer genes that include *KRAS*, *NRAS*, and *BRAF*. Tested *KRAS* mutations include codon 12, 13, 61 and 146. Tested *NRAS* mutations include codon 12, 13, 61.

Onco 48: The Onco48 Panel is designed to target 2800 mutations in 48 key cancer genes. The difference between Onco 44 and 48 is the additional sequencing of 4 target genes: *EZH2*, *GNA11*, *GNAQ*, and *IDH2*. In addition, the Onco48 panel identifies the rare *KRAS* codon 117 and *NRAS* codon 146 mutations.

***KRAS*-PCR:** DNA was extracted from micro-dissected cells from formalin-fixed, paraffin-embedded tissue with minimum 30% tumor cellularity. A PCR based fragment analysis with 5% sensitivity using "Shift Termination" technology was used to detect mutations in the *KRAS* gene. This assay is CLIA approved and detects 6 mutations on codon 12 and 1 mutation on codon 13 of exon 2.

***BRAF*:** Genomic DNA is extracted from micro-dissected cells from formalin-fixed, paraffin-embedded tissue with minimum 30% tumor cellularity. A real-time PCR assay with 1% sensitivity was performed to detect the c.1799 T > A (V600E) mutation in the *BRAF* gene.

CEA assay

CEA was tested via Siemens Advia Centaur chemiluminescent immunoassay and normal range is 0.5 ng/mL to 4.5 ng/mL.

Statistical analysis

We tested for differences in proportions between rate of mutations vs clinical and demographic factors with Fisher's Exact Tests. We also tested for differences in proportions between rate of mutations vs site of metastatic disease, location of primary disease, and CEA (cut point of 5 ng/mL) with Fisher's Exact Tests. For testing the association between metastases site and CEA as a continuous variable, we transformed CEA using

the natural logarithm and used it as the independent variable in a logistic regression. The dependent variable in the logistic regression was presence or absence of a given metastases location.

KRAS and *NRAS* mutations were categorized under *RAS* mutations, irrespective of the testing methodology. Comparative analysis was performed on 4 distinct subgroups: *RAS* mutant, *RAS* wild type, *BRAF* mutant, and *BRAF* wild type populations.

RESULTS

The study population consisted of 174 patients who presented with metastatic colon cancer patients and documented *RAS* and *BRAF* mutational analysis. Genomic evaluation for *KRAS*, *NRAS*, and *BRAF* was performed by next generation sequencing using ONCO44 or ONCO48 in 122 patients. 52 patients were evaluated for *KRAS* (no *NRAS* evaluation) and *BRAF* mutation by PCR. Eighty-seven (50%) of patients had an identifiable *RAS* mutations (47% *KRAS* and 3% *NRAS*). Only 11 patients (6%) had *BRAF* mutation (Table 1).

RAS and *BRAF* mutations and patients' demographics

The median age of the study population was 60 years (range 23 to 87 years). There was no difference in *RAS* or *BRAF* mutation status by age, or gender. However, females had a trend towards a higher incidence of *BRAF* mutation. No distinct variations were noted in *KRAS* or *BRAF* mutations by race, with the exception of an increased rate of *RAS* mutations among African Americans. However, African American representation on this study was low (5%), limiting the interpretation of this finding (Table 1).

RAS and *BRAF* status, primary tumor location and pattern of metastases

There was no difference in primary tumor sites by *KRAS* or *BRAF* status, with the exception of a lower likelihood of *BRAF* mutations among rectal cancers. In addition, no difference in tumor spread pattern at the time of metastatic disease presentation was noted among the 4 molecular subgroups (Table 2).

Table 2 *RAS* and *BRAF* status and primary tumor location and pattern of metastasis

		All		<i>BRAF</i> MT		<i>BRAF</i> WT		<i>P</i>	<i>RAS</i> MT		<i>RAS</i> WT		<i>P</i>
		<i>n</i>	%	<i>n</i>	%	<i>n</i>	%		<i>n</i>	%	<i>n</i>	%	
Primary lesion	Rectal	43	25	0	--	43	100	0.022	23	53	20	47	0.23
	Left colon	79	45	5	6	74	94		34	43	45	57	
	Right colon	52	30	6	12	46	88		30	58	22	42	
Site of metastasis	Lung	72	42	3	4	69	96	0.36	41	57	31	42	0.17
	Liver	98	56	5	5	93	95	0.54	46	47	52	53	0.44
	Peritoneal	49	28	5	10	44	90	0.3	23	47	26	53	0.74

n: Number of patients; MT: Mutant; WT: Wild type.

Table 3 *RAS* and *BRAF* status and carcinoembryonic antigen levels

		All		<i>BRAF</i> MT		<i>BRAF</i> WT		<i>P</i>	<i>RAS</i> MT		<i>RAS</i> WT		<i>P</i>
		<i>n</i>	%	<i>n</i>	%	<i>n</i>	%		<i>n</i>	%	<i>n</i>	%	
< 5 ng/mL		60	34	4	7	56	93	1.00	37	62	23	38	0.037
≥ 5 ng/mL		114	66	7	6	107	94		64	56	50	44	

n: Number of patients; MT: Mutant; WT: Wild type.

***RAS* and *BRAF* status and CEA production**

Thirty-four percent of the total cohort were non-CEA producers (CEA < 5 ng/mL). Patients with liver metastases were more likely to produce CEA (OR = 0.639; *P* < 0.0001) while patients with peritoneal/mesenteric metastases were less likely to produce CEA (OR = 1.315; *P* = 0.0010). Patients with *RAS* mutation were more likely to be low-CEA producers at the time of metastatic disease presentation (Table 3). There was no significant association between *BRAF* mutation status and CEA production.

DISCUSSION

In this study we sought to explore correlations between *RAS* and *BRAF* mutational status, patient demographics, metastatic disease pattern, and CEA production. No distinct demographic characteristics were associated with *RAS* or *BRAF* status, with the exception of *BRAF* mutations which were less likely to occur with a rectal primary. Although not statistically significant, females were more likely to harbor a *BRAF* mutation. These findings are consistent with prior reports^[9,12,14,15]. We were not able to confirm an association between *BRAF* mutations and age or a right colon primary, contrary to previous reports^[9,12,14,15]. This discordance is likely related to our more limited sample size, especially that the percentages of *RAS*-mutant and *RAS*-wild type patients with right colonic primaries were in line with the above referenced studies. We also investigated the impact of race on *RAS* and *BRAF* mutational status. The only positive association was for *RAS* mutation and African American race. Several studies have previously evaluated the impact of race on *RAS* and *BRAF* mutational status^[16,17]. The N0147 adjuvant clinical trial in patients with stage III colon cancer reported an

increased likelihood of *BRAF* mutation amongst White and an increased *KRAS* mutation frequency in African Americans^[18]. In addition, N0147 reported a lower frequency of *KRAS* mutations in Asians, a finding not supported by our study.

Contrary to the current literature, we did not find an association between *BRAF* mutation and peritoneal metastases at the time of presentation, likely due to our small *BRAF* mutant sample size. Several studies have reported an increased likelihood of peritoneal dissemination in *BRAF* mutant mCRC patients^[8,9,19]. Yaeger *et al*^[9] reported that patients with *BRAF* mutations were more likely to present with peritoneal metastases at initial diagnosis and less likely to have liver-limited metastases. Moreover, the 2-year cumulative incidence of peritoneal metastases was higher with *BRAF* mutated tumors^[9]. Tran *et al*^[8] reported a higher rate of peritoneal and distant lymph node metastases and a lower rate of lung metastases in *BRAF* mutated tumors. Similarly, Russo *et al*^[19] reported a higher likelihood of *BRAF* mutations in patients with distant lymph node metastases at the site of first recurrence. Finally, Kawazoe *et al*^[12] retrospectively studied the clinical-pathological features of *BRAF* mutations in Japanese patients with metastatic CRC and found that peritoneal metastases are more frequently observed in *BRAF* mutated patients. Since the presence of peritoneal metastases has been identified as a poor prognostic factor, a higher incidence of peritoneal metastases in *BRAF* tumors may partly explain the poor prognosis associated with this subgroup^[12,20,21]. These studies are summarized in Table 4.

Our study did not confirm an association between *RAS* mutations and lung metastases at initial mCRC presentation. There is discordance among studies on the impact of *RAS* mutational status on lung metastases at the time of initial mCRC presentation. However,

Table 4 *BRAF* status and pattern of colon cancer metastases

Ref.	n (% <i>BRAF</i>)	End point	% <i>BRAF</i> MT vs % <i>BRAF</i> WT	P
Tran <i>et al</i> ^[8]	524 (11%)	Rate of peritoneal metastases	46% vs 24%	0.001
		Rate of distant lymph node metastases	53% vs 38%	0.008
		Rate of lung metastases	35% vs 49%	0.049
Yaeger <i>et al</i> ^[9]	515 (18%)	Peritoneal involvement at presentation	26% vs 14%	< 0.01
Kawazoe <i>et al</i> ^[12]	264 (5%)	Peritoneal metastasis	50% vs 18%	0.009

n: Total number of patients; %*BRAF*: %patients with *BRAF* mutation; MT: Mutant; WT: Wild type.

Table 5 *RAS* status and pattern of colon cancer metastases

Ref.	n (%MT)	Results	P
Cejas <i>et al</i> ^[29]	110 (34% <i>KRAS</i> MT)	Frequency of <i>KRAS</i> mutation in primary tumor of patients with lung vs liver metastases	0.054
Tie <i>et al</i> ^[10]	Cohort A 161 (48.4% <i>KRAS</i> MT)	59% vs 32%	0.003
		Mutation frequencies in lung in <i>KRAS</i> MT vs WT	
		62% vs 38%	0.003
Kim <i>et al</i> ^[23]	Cohort C 859 (33.8% <i>KRAS</i> MT) 143 (43.4% <i>KRAS</i> MT)	Mutation frequencies in brain in <i>KRAS</i> MT vs WT	0.007
		56.5% vs 43.5%	0.003
		Relapse in lung in <i>KRAS</i> MT	
		HR 2.1, 95%CI: 1.2-3.5	0.007
		Lung as initial metastatic site in <i>KRAS</i> MT vs WT	0.003
Vauthey <i>et al</i> ^[25]	193 (18% All <i>RAS</i> MT)	45.3% vs 22.1%	< 0.001
		Liver as initial metastatic site in <i>KRAS</i> MT vs WT	
		37.3% vs 70.6%	< 0.001
Yaeger <i>et al</i> ^[11]	918 (48% All <i>RAS</i> MT)	Distant lymph node as initial metastatic site in <i>KRAS</i> MT vs WT	0.025
		6.7% vs 19.1%	< 0.001
		3-yr lung RFS rate in patients undergoing curative resection of liver metastases in <i>RAS</i> MT vs WT	
Kemeny <i>et al</i> ^[24]	169 (30% <i>KRAS</i> MT)	34.6% vs 59.3%	< 0.01
		Lung as site of first metastasis in <i>RAS</i> MT vs WT	< 0.01
		22% vs 0%	< 0.001
		Cumulative incidence of lung as subsequent metastasis at 2 yr after diagnosis in <i>RAS</i> MT vs WT	
		32.5% vs 19%	< 0.01
Pereira <i>et al</i> ^[13]	494 (41% <i>KRAS</i> MT)	3-yr cumulative recurrence rate to lung after hepatic resection and HAI in <i>KRAS</i> MT vs WT	0.05
		58% vs 32%	< 0.01
		3-yr cumulative recurrence rate to brain after hepatic resection and HAI in <i>KRAS</i> MT vs WT	
Pereira <i>et al</i> ^[13]	494 (41% <i>KRAS</i> MT)	14.5% vs 2%	< 0.01
		3-yr cumulative recurrence rate to bone after hepatic resection and HAI in <i>KRAS</i> MT vs WT	0.002
Pereira <i>et al</i> ^[13]	494 (41% <i>KRAS</i> MT)	13.4% vs 2%	0.002
		Time to lung metastasis (median months) in <i>KRAS</i> MT vs WT	
		15.2 vs 22.4 (HR 1.4)	

n: Total number of patients; %MT: %patients with mutation; MT: Mutant; WT: Wild type; RFS: Recurrence free survival; HAI: Hepatic arterial infusion.

clinical studies have consistently shown an association between *KRAS* mutation and lifetime likelihood of lung metastases in patients with mCRC, but not at initial presentation (Table 5). In our previous study, conducted on a different patient data set, Sharma *et al*^[22] reported no predictive role for *KRAS* mutations on the site(s) of metastatic disease at the time of presentation. Pereira *et al*^[13] retrospectively evaluated patients with mCRC who were tested for *KRAS* mutation at MD Anderson Cancer Center. They did not report an increase rate of lung

metastases in *KRAS* mutated patients at the time of diagnosis of mCRC. However, *KRAS* mutation was found to have a shorter time to lung metastases and a two-fold greater odd of developing lifetime lung metastases in a cohort of a liver-limited CRC. However, several other studies reported that *KRAS* mutant patients were more likely to present with lung metastases than *KRAS* wild type patients. Kim *et al*^[23] reported on the initial metastatic disease patterns in South Korean patients with mCRC. Lung metastases were more frequent

as the initial metastatic site in *KRAS* mutant patients while liver and distant lymph node metastases were less likely^[23]. Yaeger *et al.*^[11] reported on the impact of *KRAS* mutations on the pattern of metastatic spread in CRC. In this retrospective study, *KRAS* mutant patients had a higher incidence of lung metastases at initial presentation compared to *KRAS* wild type patients. In addition, *KRAS* mutated patients had higher cumulative incidence of lung, bone and brain metastases at two years from initial mCRC presentation. Fewer patients had liver-limited disease at the initial presentation in *KRAS* mutated patients than *KRAS* wild type patients^[11]. *KRAS* mutations have also been associated with a higher risk of lung relapse while *NRAS* mutations were associated with increased local recurrence after curative resection of primary CRC or after curative intent hepatectomy^[10,24,25]. Review of patients with stage II and III primary CRC who participated in VICTOR clinical trial showed an association between *KRAS* mutations and an increased relapse rate in the lung. Relapse in the liver was similar between *KRAS* mutant and wild type patients^[10]. Kemeny *et al.*^[24] reported on the pattern of metastatic disease recurrence in patients who underwent hepatic resection and adjuvant HAI plus systemic chemotherapy. The three-year cumulative incidence of lung metastases was higher in the *KRAS* mutant patients. The cumulative incidence of bone and brain metastases was also increased in the *KRAS* mutant patients. Similarly, Vauthey *et al.*^[25] reported that patients with *KRAS* mutant tumors who underwent curative intent liver resection at MD Anderson cancer center had a lower three-year lung RFS in comparison to patients with *KRAS* wild type tumors. Based on the above studies (summarized in Table 5), *KRAS* mutant mCRC patients have an increased lifetime risk of developing lung metastases. However, the impact of *KRAS* mutational status on the incidence of lung metastases at the initial time of diagnosis of metastatic disease remains controversial. Whether the lack of association between lung metastases at presentation and *KRAS* mutations is related to a limited sample size on those studies vs being the result of tumor biology remains unclear.

We have studied the impact of *RAS* and *BRAF* mutational status on CEA levels at the time of initial diagnosis of metastatic disease. We did not find any difference in CEA levels between *BRAF* mutant and *BRAF* wild type mCRC at initial presentation. In contrast, *RAS* mutant mCRC patients were more likely to be non-CEA producers (62% *RAS*-MT vs 38% *RAS*-WT) (Table 3). Our findings are in contrast to a study by Selcukbiricik *et al.*^[26] which reported a higher percentage of patients with CEA > 5 ng/mL among the *KRAS* mutant cohort. Selcukbiricik study was limited by stage heterogeneity (stages I-IV) and did not include an analysis of the impact of *RAS* mutation within the stage IV disease cohort. Our study also showed an association between CEA levels and site of metastatic disease. CEA was more likely to be elevated in patients with liver metastases

and lower in patients with peritoneal or mesenteric recurrence, which is consistent with prior reports^[27].

Our study has several limitations. This is a single institution study with a relatively small size. Modest associations between *RAS* and *BRAF* status and other clinical variables may have therefore been missed due to the lack of adequate power. In addition, the diagnosis of metastatic disease on this study could have been made during surveillance for disease recurrence or during the work-up of symptomatic disease. Therefore, the conclusions derived from this study may not be clearly generalizable to the surveillance population or to the population presenting with symptomatic stage IV disease. Other limitations include the inclusion of patients with *KRAS* PCR mutation assay (no ONCO48 analysis). This implies that some patients may have been assigned to the *RAS* wild type subgroup without ruling out the possibility of *NRAS* or non-exon 2 *KRAS* mutations. The likelihood of this event impacting our overall results is low as only 52 patients (30%) of our study population was analyzed by *KRAS*-PCR only. Given that less than 10% of the general population carries a non-exon 2 *KRAS* mutation or *NRAS* mutations, we expect that less than 10 patients may have been inappropriately labeled.

In summary, our study is the first study to link low CEA production with a *RAS* mutant status at the time of initial presentation of metastatic CRC. If validated in larger studies, especially in surveillance settings, our findings would have major clinical significance. It has been recently confirmed that *RAS* mutations increase the risk of systemic disease recurrence after a curative resection in patients with stage III colon cancer^[28]. Reliable screening strategies are especially important in this high risk population in order to diagnosis early recurrence and increase the likelihood of curative-intent metastectomies. If CEA is confirmed as a less reliable screening strategy, intense radiographic screening will be especially important as a complement to CEA screening in this population.

COMMENTS

Background

It is estimated that 20% of patients with colorectal cancer (CRC) present with metastatic disease while another 30% develop metastatic disease after an initial presentation with local or regional disease. Patients with limited oligometastatic disease are the ones who benefit the most from aggressive surgical strategies. Therefore, early identification of metastatic disease remains key in improving outcome. It has been recently shown that certain oncogenic alterations have significant impact on disease biology, response to treatment, and overall outcome. To better understand the impact of the commonly presented *RAS* (*KRAS* and *NRAS*) and *BRAF* mutations on metastatic disease pattern and on surveillance strategies, the authors conducted a single institute retrospective study that investigates the impact of *RAS* and *BRAF* mutations on the pattern of metastatic disease and carcinoembryonic antigen (CEA) production.

Research frontiers

BRAF mutations, present in 5%-10% of CRCs, are associated with worse prognosis, a worse overall survival after disease recurrence, and a tendency to metastasize to the peritoneum and distant lymph nodes. The impact of *KRAS* and *NRAS* mutations, which occur in approximately 50% of CRCs, on the

pattern of metastatic disease at initial presentation has been more controversial. No studies have reported on the impact of either *RAS* or *BRAF* mutations on CEA production.

Innovations and breakthroughs

The authors did not find any difference in CEA levels between *BRAF* mutant and *BRAF* wild type mCRC at initial presentation. In contrast, *RAS* mutant mCRC patients were more likely to be non-CEA producers (62% *RAS*-MT vs 38% *RAS*-WT).

Applications

The study is the first study to link low CEA production with a *RAS* mutant status at the time of initial presentation of metastatic CRC. If validated in larger studies, especially in surveillance settings, the authors' findings may indicate that CEA surveillance is less reliable in curatively resected *RAS* mutant CRC patients. Alternative surveillance strategies may be required in this patients population.

Terminology

The ONCO 44/48 is the next-generation sequencing technology at the City of Hope which is designed to target 713 mutations in 44 and 48 key cancer genes.

Peer-review

It is a well-written paper.

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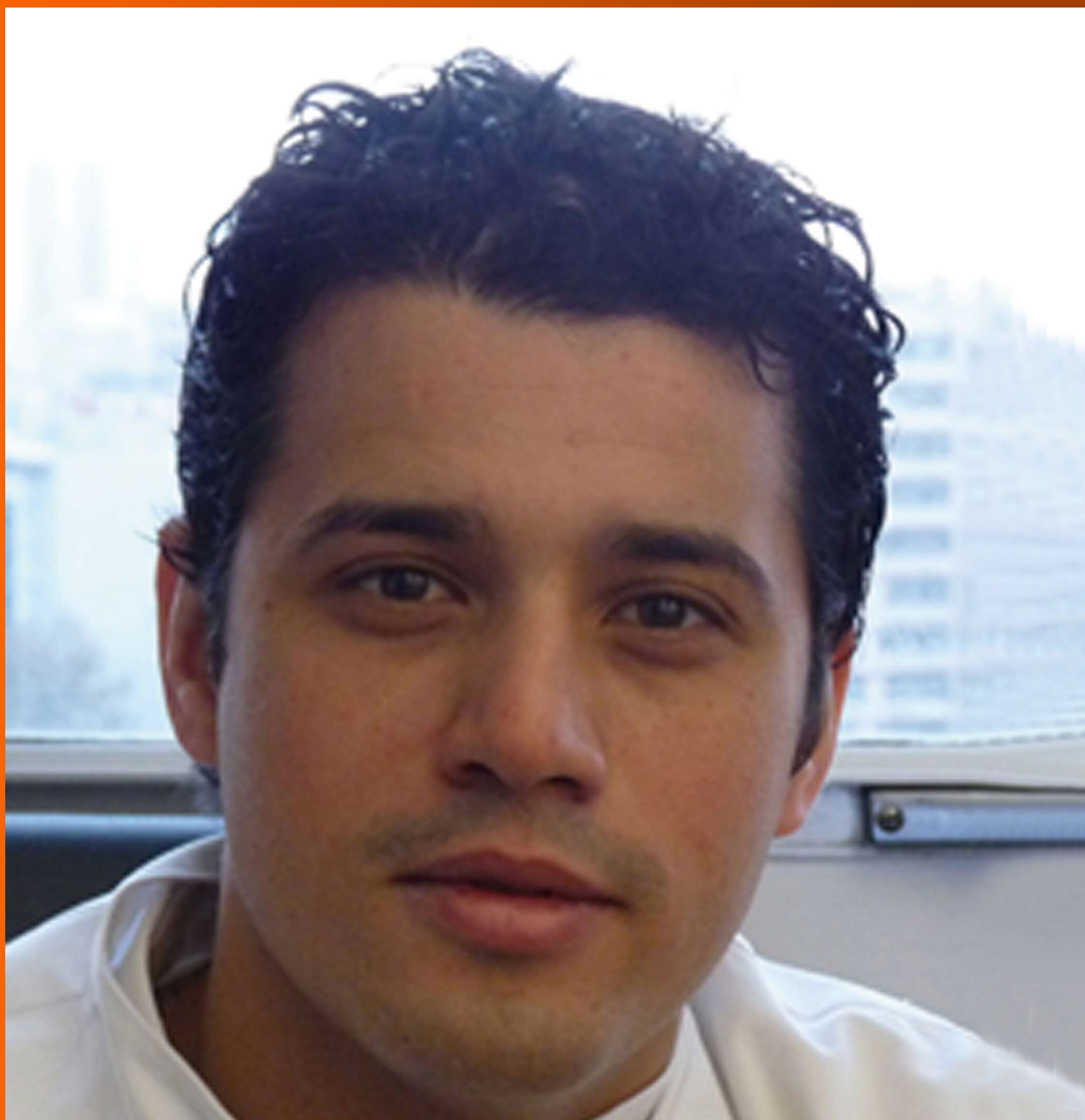
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World J Gastrointest Oncol 2016 February 15; 8(2): 136-234





TOPIC HIGHLIGHT

- 136 Clinical significance of lymphadenectomy in patients with gastric cancer
Tóth D, Plósz J, Török M
- 147 Role of *Helicobacter pylori* in gastric cancer: Updates
Khatoon J, Rai RP, Prasad KN
- 159 Endoscopic approach to the diagnosis of pancreatic cystic tumor
Kawaguchi Y, Mine T
- 165 Advancement in treatment and diagnosis of pancreatic cancer with radiopharmaceuticals
Xu YP, Yang M

REVIEW

- 173 Molecularly targeted therapy for advanced hepatocellular carcinoma - a drug development crisis?
Thillai K, Ross P, Sarker D
- 186 Neoadjuvant radiotherapeutic strategies in pancreatic cancer
Roeder F
- 198 Ubiquitin proteasome system research in gastrointestinal cancer
Zhong JL, Huang CZ

ORIGINAL ARTICLE

Case Control Study

- 207 Risk factors for the development of colorectal carcinoma: A case control study from South India
Iswarya SK, Premarajan KC, Kar SS, Kumar SS, Kate V

Retrospective Cohort Study

- 215 Correlation of *Helicobacter pylori* and interleukin-8 mRNA expression in high risk gastric cancer population prediction
Chongruksut W, Limpakan (Yamada) S, Chakrabandhu B, Ruengorn C, Nanta S

EVIDENCE-BASED MEDICINE

- 222** Evaluation of antibody-dependent cell-mediated cytotoxicity activity and cetuximab response in *KRAS* wild-type metastatic colorectal cancer patients

Lo Nigro C, Ricci V, Vivenza D, Monteverde M, Strola G, Lucio F, Tonissi F, Miraglio E, Granetto C, Fortunato M, Merlano MC

CASE REPORT

- 231** Rectal neuroendocrine tumor with uncommon metastatic spread: A case report and review of literature

Tsoukalas N, Galanopoulos M, Tolia M, Kiakou M, Nakos G, Papakostidi A, Koumakis G

Contents

World Journal of Gastrointestinal Oncology
Volume 8 Number 2 February 15, 2016

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World Journal of Gastrointestinal Oncology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-85381891
Fax: +86-10-85381893
E-mail: editorialoffice@wjnet.com
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2016 Gastric Cancer: Global view

Clinical significance of lymphadenectomy in patients with gastric cancer

Dezső Tóth, János Plósz, Miklós Török

Dezső Tóth, Department of General Surgery, Kenézy Teaching Hospital, 4043 Debrecen, Hungary

János Plósz, Department of Internal Medicine, Kenézy Teaching Hospital, 4043 Debrecen, Hungary

Miklós Török, Department of Pathology, Kenézy Teaching Hospital, 4043 Debrecen, Hungary

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Correspondence to: Dezső Tóth, MD, PhD, Associate Professor, Department of General Surgery, Kenézy Teaching Hospital, 2-26 Bartók Street, 4043 Debrecen, Hungary. detoth@gmail.com
Telephone: +36-30-9388867
Fax: +36-52-511797

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Abstract

Approximately thirty percent of patients with gastric

cancer undergo an avoidable lymph node dissection with a higher rate of postoperative complication. Comparing the D1 and D2 dissections, it was found that there is a significant difference in morbidity, favoured D1 dissection without any difference in overall survival. Subgroup analysis of patients with T3 tumor shows a survival difference favoring D2 lymphadenectomy, and there is a better gastric cancer-related death and non-statistically significant improvement of survival for node-positive disease in patients with D2 dissection. However, the extended lymphadenectomy could improve stage-specific survival owing to the stage migration phenomenon. The deployment of centralization and application of national guidelines could improve the surgical outcomes. The Japanese and European guidelines enclose the D2 lymphadenectomy as the gold standard in R0 resection. In the individualized, stage-adapted gastric cancer surgery the Maruyama computer program (MCP) can estimate lymph node involvement preoperatively with high accuracy and in addition the Maruyama Index less than 5 has a better impact on survival, than D-level guided surgery. For these reasons, the preoperative application of MCP is recommended routinely, with an aim to perform "low Maruyama Index surgery". The sentinel lymph node biopsy (SNB) may decrease the number of redundant lymphadenectomy intraoperatively with a high detection rate (93.7%) and an accuracy of 92%. More accurate stage-adapted surgery could be performed using the MCP and SNB in parallel fashion in gastric cancer.

Key words: Gastric cancer; Surgery; Lymphadenectomy; Sentinel node biopsy; Maruyama computer program

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Core tip: Comparing the D1 and D2 dissections, it was found that there is a significant difference in postoperative morbidity and mortality, favoured D1

dissection without any difference in overall survival. The implementation of centralization and application of national guidelines could improve the surgical outcomes. More accurate stage-adapted surgery could be performed using the Maruyama computer program and sentinel lymph node biopsy in parallel fashion in gastric cancer.

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INTRODUCTION

In most cases, modern, optimal treatment of patients with different neoplasms can be achieved with a stage adapted, combined modality therapy according to international protocols. In case of solid tumors, the lymph node (LN) involvement and its exact number is the most important prognostic factor. Adjuvant chemotherapy, as well as the oncological outcome is terminated by the tumor-node-metastasis stage. Preoperative imaging techniques provide a much more accurate determination of the T and M stage than that of the N stage. The correct status of LN metastases can be obtained only by histology following an optimally extended node dissection. The removal of further LNs on the other hand, increases operative time, the rate of complications, and if negative may be considered unnecessary.

Almost three hundred thousand patients with gastric adenocarcinoma do not have LN metastasis in the one million new cases each year^[1,2]. The depth of tumor invasion^[3,4], the metastatic LN status and R0 resection are the most important independent prognostic factors for overall and disease free survival (OS, DFS)^[5-7]. Moreover, a lot of study proved that LN metastasis is an independent risk factor for local recurrence as well as the time interval between radical gastrectomy and hepatic metastasis in patients after R0 resection^[8-10].

The aim of this review is to report the latest issues from 2014 according to lymphadenectomy in gastric cancer and compare these results with earlier studies.

LN INVOLVEMENT

Successful estimation of LN involvement may help to define which patients would or would not benefit from an extended LN dissection in association with gastrectomy^[11]. However, preoperative diagnostic tools have a low sensitivity and specificity for defining these patient subpopulations. Sensitivity, specificity and accuracy of spiral computer tomography for detection of pathologic LN involvement are 73.1%, 50.0% and 84.2%, respectively^[11,12]. Endoscopic ultrasonography has an accuracy of 68.6%, with a sensitivity and specificity of 66.7%

and 73.7%, respectively^[11,13]. The real problem of these imaging procedures is that exclusion of endoscopic ultrasonography, only the size of the LNs is taken into account.

In association with T stage, LN involvement can be found in 15% of patients with carcinoma confined to the mucosa, whereas LN metastases were detected in 23.4%, 48.2%, and 69.8% of patients with carcinoma invading the submucosa, muscularis propria and serous layer, respectively^[14]. Gertler *et al.*^[15] showed that not only infiltration of the submucosa but also lymphatic vessel invasion, multifocal tumor growth, younger patient age and poor tumor differentiation were associated with nodal disease. Besides T stage, LN involvement can also be influenced by tumor size. The overall accuracy of tumor size for preoperative N staging was 82.13%^[16]. The incidence of LN metastasis in patients with a cancer size of 3-5 cm is 64.9%, 80% in patients with a cancer size of 5-7 cm and 84.3% in patients with a cancer size of > 7 cm^[14]. Additionally, early gastric cancer (EGC) has nodal metastases in 38.9% in poorly differentiated or undifferentiated types of tumor, in 41.7% with Lauren diffuse type and in 33.3% with a size larger than 3 cm^[17]. Yang *et al.*^[18] found that venous invasion, submucosal invasion or antral tumor location were independent predictors for LN metastasis in multivariate analysis. The rates of LN metastasis were 1.1% for patients with one or no predictor and 17.8% for those with two or more predictors^[18].

While the prognostic significance of macrometastasis in the LNs is obvious, the role of lymphovascular invasion (LVI) or micrometastasis (MM) is controversial. Lee *et al.*^[19] confirmed that the recurrence-free survival is lower in N0/LVI(+) patients than in N0/LVI(-) patients, however they did not find any effect of LVI+ on overall survival. The incidence of LN MM is lower than 10% in patients with node negative EGC^[20] but it is higher in histologically diffuse type tumors^[21]. The presence of MM influenced DFS, although the OS analysis revealed no significant difference between MM-positive and MM-negative patients^[19]. The reverse transcriptase polymerase chain reaction proved to be the most sensitive method in the detection of MM^[22].

Meanwhile, a multivariate survival analysis concluded that the number of examined LN (eLN) was an independent predictor of overall survival of patients with node-negative gastric cancer. According to the cut-point analysis, T2-T4 patients with 11-15 eLN had a significantly longer mean OS than those with 4-10 eLN or 1-3 eLN. Patients with ≤ 15 eLN were more likely to experience locoregional and peritoneal recurrence than those with > 15 eLN^[23]. However, this trend was not observed when the number of examined LN exceeded 30^[24].

These results are potentially associated with the elimination of MM in negative LNs^[25]. Based on these findings, LVI and MM should be considered in postoperative management of gastric cancer^[19].

Table 1 The primary and revised results of prospective randomized trials comparing D1 to D2 dissection

Ref.	Morbidity (%)		Mortality (%)		Splenectomy (%)		Pancreatectomy (%)		RR (%)		OS (%)	
	D1	D2	D1	D2	D1	D2	D1	D2	D1	D2	D1	D2
Dent ^[32]	13.6 ^a	38 ^a	0	0	0	0	0	0			81	76
British ^[33,36]	28 ^a	46 ^a	6.5 ^a	13 ^a	27	9	4 ¹	57 ¹	NS	NS	35	33
Dutch ^[34,37]	24 ^a	43 ^a	4 ^a	10 ^a	11	37	3	30	43	47	45	47
Dutch - 15 yr ^[38]									22 ^a	12 ^a	21	29
Taiwanese ^[39,44]	7.3 ^a	17.1 ^a	0	0	3	1	1 ¹	13 ¹	50.6	40.3	53.6 ^a	59.5 ^a
Italian ^[35,43]	12	17.9	3	2.2	6.8	9.0	1.5	1.5			66.5	64.2

^a*P* < 0.05. ¹Pancreato-splenectomy. RR: Relapse risk; OS: Overall survival; NS: Non-significant.

LYMPHADENECTOMY

D1 vs D2 lymphadenectomy

The adequate extension of lymphadenectomy differs significantly between East Asian and Western countries. Extended lymphadenectomy (D2) is the standard of care in Japan and South Korea, while for example, the majority of United States patients receive at most a limited lymphadenectomy (D1)^[26,27]. This controversy may originate from different factors. First, the incidence of gastric cancer is significantly higher in Asia than in European Union, or in the United States^[27,28].

Second, centralization of treatment has not yet been solved in the latter regions; 80% of Medicare patients with gastric cancer in the United States go through surgery in centers performing less than 20 procedures per year^[29] and there is a significant number of low-volume surgeons performing less than two cases annually^[30,31].

Table 1 shows the primary and revised results of prospective randomized trials (RCT) comparing D1 to D2 lymphadenectomy in association with postoperative morbidity, mortality, frequency of splenectomy and pancreatectomy and long term oncological outcomes such as relapse risk and overall survival (OS). The three earliest studies found a higher morbidity and mortality rate following extended LN dissection of patients with gastric cancer when compared to those undergoing D1 dissection only^[11,32-34]. These higher rates were related mostly to splenectomy and pancreatectomy. Although Dent *et al.*^[32] did not perform resection of these organs, this study should be evaluated with reservations because of the small series size. Furthermore, limited surgical experience could explain these results. The quality control of lymphadenectomy was inadequate, as the non-compliance rate (absence of LNs from more than two LN stations that were supposed to be harvested) was 51% in the D2 group in the Dutch trial^[34,35] and, in the extended group of the British trial, the dissection of LN station no.7 was 63.5%, and was less than 50% in station no.8 and no.9^[36].

Moreover, extended LN dissection did not have any effect on oncological outcomes. The relapse risk and survival were similar in these studies. Only the revision of the Dutch trial showed better survival in advanced disease in the D2 group, after 11-year follow-up^[37]. The 15-year follow-up results revealed that cancer-related death rates were lower (37% vs 48%) with a lower rate

of local recurrence in the D2 lymphadenectomy group (Table 1)^[38]. Subgroup analysis of this trial demonstrated significantly higher survival for females (35% vs 21%) and in stage II disease (33% vs 15%) in the D2 arm. The 15-year survival in patients without pancreatico-splenectomy was significantly higher with D2 than D1 dissection (35% vs 22%)^[38].

The two latest randomized trials from the 21st century did not present significant differences in postoperative mortality between the D1 and D2 group^[35,39]. The morbidity rate was higher with D2 lymphadenectomy in the Taiwanese trial (which compared D1 to D3 dissection; however their D3 lymphadenectomy is similar to the current definition of D2 dissection). The Italian study did not show this difference and proved that D2 dissection could be performed safely without splenectomy and distal pancreatectomy, with comparable mortality and morbidity to those with D1 dissection in specialized centers^[39,40]. These rates are comparable to the Japan Clinical Oncology Group (JCOG) 9501 trial and the nationwide Japanese registry where the mortality was less than 2% after D2 dissection^[41,42]. Neither did the latter study find any survival benefit from the extended lymphadenectomy^[43]. Subgroup analyses showed a 5-year disease-specific survival benefit for patients with pathological tumor 1 (pT1) disease in the D1 group (9% vs 83% for the D2 group; *P* = 0.015), and for patients with pT2-4 status and positive LNs in the D2 group (59% vs 38% for the D1 group; *P* = 0.055). However, the non-compliance rate was 33.6%^[43]. It was concluded that the contamination (over-extensive nodal dissection) (18%) and the higher rate of stage IA disease in the D1 group and of stage IV in the D2 arm, apparently nullified the effect of correct extended dissection^[41,43]. The other randomized trial from Taiwan proved a better (*P* = 0.041) survival with D2 dissection^[44].

The results of these recent studies call attention to the importance of the learning curve and the necessity of standardized procedures with routine preservation of the spleen and pancreas in experienced centers^[40].

Besides the RCT, the latest meta-analysis found significant differences in morbidity, anastomotic leakage, pancreatic leakage, reoperation rates, wound infection, pulmonary complications and postoperative mortality, all of which favoured D1 dissection. The conclusion was that there is no difference in OS when comparing the D1 and D2 arm. Subgroup analysis of patients

with T3 tumor shows a survival difference favoring D2 lymphadenectomy (25.9% vs 11.5%), and there is a trend towards a lower risk of gastric cancer-related death among patients having a D2 dissection with preservation of the spleen or pancreas and non-statistically significant improvement of survival for node-positive patients^[40,45]. Unfortunately, the main problem of meta-analysis was that it was not possible to match patient groups for treatment with age, sex, type of gastrectomy, pathological stage, tumor location, co-morbidity, treatment strategies, surgeon experience, hospital case volume and extent of LN dissection, all of which affect postoperative complications and overall survival rates^[40].

Keeping this in mind, the comparison of oncological outcomes of D1 and D2 dissections in association with different T and N stages could be problematic due to the concept of stage migration. The reason for this is that a limited lymphadenectomy can not represent the adequate staging of LN involvement. Conversely, extended lymphadenectomy could improve stage-specific survival due to the stage migration phenomenon. Furthermore, Xu *et al.*^[46] demonstrated that it is necessary to examine at least 16 LNs for accurate pathological examination of gastric cancer, even in node-negative gastric cancer patients^[25], and Datta *et al.*^[47], who analyzed the data of more than 22000 patients found that the examination of 15 or more LN is a reproducible prognostic factor for gastric cancer outcomes in the United States and should continue to serve as a benchmark for the quality of care.

In addition to the quality of surgery, the pathologist plays a large role in the proper identification and examination of the extracted LN^[48].

EXTENSION OF LYMPHADENECTOMY BEYOND SUGGESTED LIMITS

The latest issue of the Japanese Gastric Cancer Association treatment guideline contains the standard lymphadenectomies regarding the type of gastric resection: Total gastrectomy with D2: D1 (Nos.: 1-7) + Nos. 8a, 9, 10, 11p, 11d, 12a; distal gastrectomy with D2: D1 (Nos. 1, 3, 4sb, 4d, 5, 6, 7) + Nos. 8a, 9, 11p, 12a; pylorus-preserving gastrectomy with D1+: D1 (Nos. 1, 3, 4sb, 4d, 6, 7) + Nos. 8a, 9; and in proximal gastrectomy with D1+: D1(Nos. 1, 2, 3a, 4sa, 4sb, 7) + Nos. 8a, 9, 11p^[49].

In the field of tumor-location specific LN involvement recent studies can be divided into 2 cohorts depend on the position of the gastric tumor (proximal vs middle and distal).

Proximal gastric cancer

The frequency of metastasis in station no.4d, 5 and 6 LNs in patients with proximal gastric cancer is more than 10%^[14,50]. The incidence of station no.10 LN metastasis is 11.82% in upper third advanced gastric cancer (AGC). The estimated OS were 46% and 37% regarding station

no.10 dissection or not, which was not statistically significant. Authors suggest high-quality studies with larger sample sizes to determine the clinical significance of no.10 LN removal^[51]. Following an 18 mo follow-up of 108 patients Li *et al.*^[52] concluded that routine no.10 lymphadenectomy may be unnecessary for advanced, upper third gastric cancer without serosal invasion, unless T3 tumors are located in the greater curvature.

Middle and distal gastric cancer

LN metastasis in station no.2 LNs from distal gastric cancer is only 1.0%, while the metastasis in station no.4 LNs is more than 20%. Since station no.11p is immediately adjacent to stations no.7 and no.9, in the case of distal gastric cancer, station no.11d should be preserved; however, both no.11p and no.11d stations should be removed in cases of proximal gastric cancer^[14]. According to Japanese gastric cancer treatment, as station no.14v is closely adjacent to station no.6, station no.14v LNs should also be removed if suspicion of metastasis to the LNs in station no.6 arises^[14,49].

As the LN metastasis rate in station no.7 was similar to that of perigastric LNs in 570 patients with advanced distal gastric tumor it is reasonable to include LNs in the no.7 station in the D1 LN dissection^[53]. Evaluating LN involvement after total gastrectomy, Galizia found that the incidence of nodal involvement of stations no.10, no.11d, and no.12a was 5%, and the 5-year DFS rate was zero; they concluded that modified D2 lymphadenectomy confers the same oncologic adequacy as standard D2 lymphadenectomy, with a significant reduction of postoperative morbidity^[54].

During investigation of LN involvement of the hepato-duodenal ligament (HDLN) a logistic regression analysis showed that no.5 and no.12a LN metastases were associated with a 6.9 and 11.3 fold increase respectively, for risk of no.12p and no.12b LN metastases. In addition, significant differences in 5-year OS of patients with and without no.12p and no.12b LN metastases were observed^[55]. However, the clinical significance of removing these LN was not evaluated. Analyzing the data of 1872 patients, LN involvement in station no.12 was 3.6% whereas HDLN metastasis was not a significant factor for survival in multivariate analysis and the 5-year survival rate of 41 patients with HDLN metastasis without distant metastasis at any other site was significantly higher than that among 120 patients with stage IV disease without HDLN metastasis. It is suggested that the inclusion of HDLN in the distant metastatic LN group in gastric cancer is inappropriate and that the seventh American Joint Committee on Cancer criteria for node grouping should be revised^[56].

The incidence of no. 14v LN metastasis was 5.0% in 1661 patients who underwent curative resection for middle or lower third gastric cancer. In clinical stages I and II, no.14v LN dissection did not affect overall survival; in contrast, no.14v LN dissection was an independent prognostic factor in patients with clinical stage III/IV

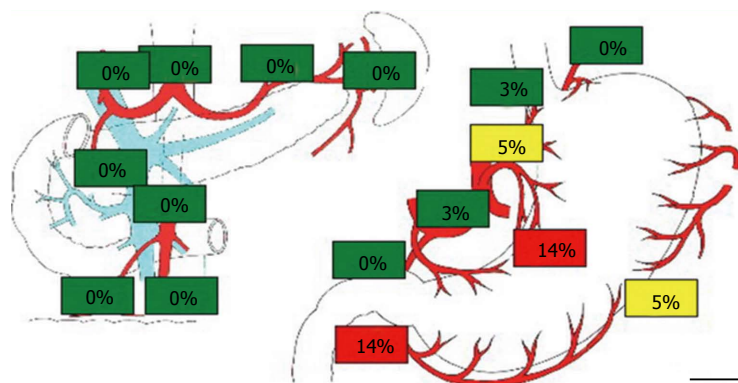


Figure 1 Prediction of lymph node involvement by the Maruyama computer program in a 65-year-old male patient. The tumor histology was well differentiated adenocarcinoma, showing muscular mucosa involvement, early cancer type 2B. The lesion was found in the anterior wall in the lower third of the stomach and had a maximal diameter of 30 mm.

Involvement of no.13 nodes is defined as M1 in the current version of the Japanese classification. However, excision of this LN may be an option in a potentially curative gastrectomy for tumors invading the duodenum^[49].

Para-aortic nodal dissection

In overall 5-year survival Zhang *et al*.^[62] could not demonstrate a significant difference between patients underwent D2 plus PAND surgery and those underwent D2 surgery. He suggests that this "over-extended" dissection should only be recommended for T3-4 and N2 stage

gastric tumor and should not be utilized for EGC and total gastrectomy^[62].

So, the D2 lymphadenectomy is the gold standard in R0 resection by the Japanese^[49] and European guidelines^[27].

The American NCCN guidelines recommend a D1+ or a modified D2 LN dissection, the latter performed by experienced surgeons in high-volume centers^[27,63]. To support this, the deployment of centralization and implementation of national clinical guidelines in Denmark resulted in a decrease in mortality from 8.2% to 2.4% and the proportion of patients with at least 15 LNs removed has increased from 19% to 76%^[64].

MARUYAMA COMPUTER PROGRAM

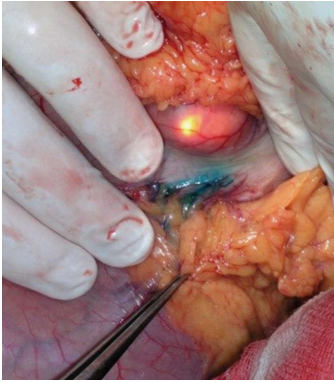


Figure 2 Sentinel lymph node mapping following submucosal marking by an endoscopist.

93%^[72].

Our study demonstrated a similar degree of reliability of MCP to those cited above, with 90.2% of sensitivity, 63.3% of specificity and 78.4% of accuracy. The rate of false negatives was 9.8%^[73]. These studies demonstrate that the results of the computerized prediction of LN metastases are superior to those of the standard pre-operative imaging techniques.

Another advantage of the MCP is that it can determine long term oncological results. Hundahl defined the Maruyama Index (MI) at first in 2002 as a measure of unresected regional nodal disease in gastric cancer using the data of the Intergroup 0116 trial and he proved it is an independent predictor of survival^[74,75]. Peeters *et al*^[76] reanalyzed the data of the Dutch D1-D2 trial using univariate and multivariate analyses and showed that the MI is an independent predictor of overall survival ($P = 0.016$, HR = 1.45) and relapse risk ($P = 0.010$, HR = 1.72). It was concluded that the MI is a quantitative yardstick for assessing the adequacy of lymphadenectomy in gastric cancer patients^[75,76]. Later, Hundahl evaluated autopsy findings from the Dutch D1-D2 trial and showed that MI < 5 or a low MI for surgery is associated with enhanced regional control and survival^[76,77]. Dikken *et al*^[78] proved the prognostic significance of low MI in a 2-year survival rate (82% vs 59%), as did Sachdev, who demonstrated that lower MI correlated with better survival, as a continuous ($P < 0.02$) and categorical ($P < 0.04$) variable^[79].

Overall these results suggest that a Maruyama Index less than 5 has a better impact on survival, than D-level guided surgery. For these reasons, the preoperative application of MCP is recommended routinely, with an aim to perform "low Maruyama Index surgery". In addition, the application of MCP to predict LN involvement can influence the indication for neoadjuvant chemo-therapy, and furthermore a "high Maruyama Index" could indicate the necessity for postoperative oncological treatment.

SENTINEL LYMPH NODE BIOPSY

While the MCP calculates the probability of LN involvement preoperatively, the concept of sentinel lymph

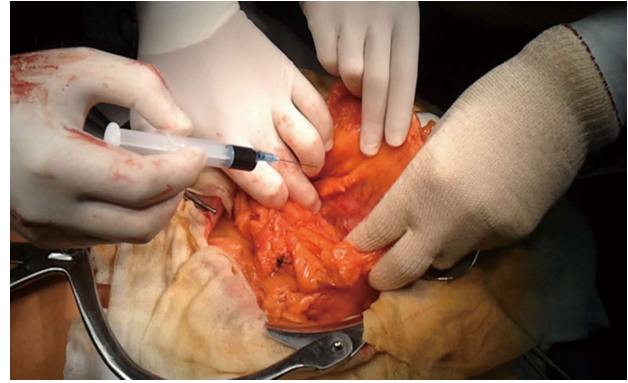


Figure 3 Subserosal marking by a surgeon.

node biopsy (SNB) can determine the existence of LN metastases intraoperatively. The first potentially affected LN, the sentinel lymph node (SLN), reliably reflects the status of the nodes in the second and third line, which is supported by data of numerous publications. If the SLN contains tumor deposit(s), extended dissection is warranted, but if findings are negative, the patient could be spared additional complications associated with extended dissection. However, the method of dye/tracer injection and the tracer's selection is controversial. Some authors use dye alone (patent blue, indocyanine green, isosulfan blue)^[11,80-82], Kitagawa *et al*^[83] handle 99m Tc colloid, and Aikou *et al*^[84] uses the combination of these tracers. The latest systematic review concluded that the SLN's identification rate is the same with the dual or single mapping method^[85]. It is eminent that body-mass index (BMI) affects the sentinel LN detection rate^[86]. The Hungarian study proved that the identification of sentinel LNs in obese patients can be difficult owing to the feathering of blue dye in the fatty tissues^[11]. This was concluded as the only patient in whom marking did not occur had a BMI significantly higher than average (26.8 vs 22.8)^[11]. Then again, the application of blue dye for SNB has a beneficial side effect, as it significantly increased the number of harvested LN and the ratio of the number of the harvested LN per time^[87]. To avoid quick dispersal to multiple LNs Kong applied ICG/poly- γ -glutamic acid complex, which remained longer than diluted ICG in animal models^[88].

Yaguchi, Lee and Tóth have compared the subserosal to the submucosal labeling method (Figures 2 and 3) without any significant difference and they suggest the endoscopic injection of a tracer in cases of non-palpable tumors and/or laparoscopic procedures^[89-91].

The cardinal problem in the SNB concept is the intraoperative false negative rate. The JCOG 0302 trial called attention to the importance of the learning curve and the inadequacy of the pick-up method. The demand for only five patients per institute provided an insufficient learning period which presented a 46% false negative rate^[92].

Lee *et al*^[93] proved that the removal of entire nodal basins can significantly decrease this rate against the pick-up method, and Kumagai *et al*^[94] called attention to the opportunity of introducing the one-step nucleic acid

amplification test for the intraoperative diagnosis of LN metastasis with similar results to postoperative 2-mm-interval histological examination.

Miyashiro *et al.*^[95] demonstrated that an extensive surgical experience is necessary for application of SNB concept and standardization of SLN mapping technique, using improved tracer, and guideline to evaluate the positiveness of SLN specimen should be planned to incorporate SNB in routine practice^[96]. Recent studies and the latest meta-analysis of SLN mapping have shown a high detection rate (93.7%) and an accuracy of 92%^[97] and suggest that the SNB concept could be suitable for tumors following endoscopic resection^[98] and could represent a new era of sentinel node navigation surgery in EGC^[99,100]. Moreover, its success rate did not correlate to tumor grade^[101].

Based on the results of the largest prospective multicenter trial from Japan with an identification rate of 97.5% and an accuracy of 99%^[102], a phase III multicenter trial for individualized surgery for EGC based on SLN mapping has been commenced in the Eastern Asian countries. The long-term results of these studies will be available between 2018 and 2020.

CONCLUSION

The latest RCT comparing D1 and D2 dissections represents a higher surgical quality (more contamination, less non-compliance, low morbidity and mortality rate) than previous trials^[43]. This could lead to a trend towards the execution of the less limited D1 lymphadenectomy for more experienced and well-trained surgeons, and hopefully the results of western surgeons will achieve a level similar to those of the Asian surgical outcomes in the near future.

On the other hand, the era of multimodal treatment and the increase in elderly patients with serious comorbidities indicates the necessity of a stage- and patient-adapted, individualized surgery in gastric cancer. It was conceived at an expert panel, also: "A D2 lymphadenectomy is preferred for curative-intent resection in advanced, non-metastatic gastric cancer; in patients with EGC or substantial comorbidities, a D1 lymphadenectomy is more appropriate"^[103]. The Japanese guidelines enclose that the AGC should be treated with D2 lymphadenectomy. D1 or D1+ should be recommended as a choice for EGC. D1+ can be an alternate for D2 in high-risk patients^[104]. Inokuchi *et al.*^[105] suggested that the presence of heart or liver disease is a significant risk factor for postoperative morbidity in patients who undergo laparoscopic gastrectomy. Although it did not reduce complications, insufficient LN dissection (for example, D1+ for advanced gastric cancer) might be permissible in high-risk patients as it had no negative impact on gastric cancer-specific survival. More accurate stage-adapted surgery could be performed using the MCP and SNB in parallel fashion.

It is generally accepted that metastases in the SLNs warrant a D2 lymphadenectomy. The authors analyzed

the relevance of MCP in sentinel node positive patients in an earlier study^[73]; while the efficiency of SNB method is superior to MCP, the positive predictive value of MCP and SNB was proven equivalent in the sentinel node positive group and the accuracy of MCP in these cohort of patients was 10% higher. For these reasons it would be interesting to find the appropriate combination of these techniques in the future and we suggest using them simultaneously in the operating room.

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2016 Gastric Cancer: Global view

Role of *Helicobacter pylori* in gastric cancer: Updates

Jahanarah Khatoon, Ravi Prakash Rai, Kashi Nath Prasad

Jahanarah Khatoon, Ravi Prakash Rai, Kashi Nath Prasad, Department of Microbiology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, Uttar Pradesh 226014, India

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Correspondence to: Dr. Kashi Nath Prasad, Professor, Department of Microbiology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Rae Bareilly Road, Lucknow, Uttar Pradesh 226014, India. kashinprasad@gmail.com
Telephone: +91-522-2668631
Fax: +91-522-2668017

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Abstract

Helicobacter pylori (*H. pylori*) infection is highly prevalent in human, affecting nearly half of the world's population; however, infection remains asymptomatic in majority of population. During its co-existence with humans, *H. pylori* has evolved various strategies to maintain a mild gastritis and limit the immune response of host. On the other side, presence of *H. pylori* is also

associated with increased risk for the development of various gastric pathologies including gastric cancer (GC). A complex combination of host genetics, environmental agents, and bacterial virulence factors are considered to determine the susceptibility as well as the severity of outcome in a subset of individuals. GC is one of the most common cancers and considered as the third most common cause of cancer related death worldwide. Many studies had proved *H. pylori* as an important risk factor in the development of non-cardia GC. Although both *H. pylori* infection and GC are showing decreasing trends in the developed world, they still remain a major threat to human population in the developing countries. The current review attempts to highlight recent progress in the field of research on *H. pylori* induced GC and aims to provide brief insight into *H. pylori* pathogenesis, the role of major virulence factors of *H. pylori* that modulates the host environment and transform the normal gastric epithelium to neoplastic one. This review also emphasizes on the mechanistic understanding of how colonization and various virulence attributes of *H. pylori* as well as the host innate and adaptive immune responses modulate the diverse signaling pathways that leads to different disease outcomes including GC.

Key words: Cag pathogenicity island; Gastric cancer; Gastric mucosa; *Helicobacter pylori*; Type IV secretion system

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Core tip: Although the incidence and mortality of gastric cancer (GC) is declining in recent decades but it still remains a major threat in developing countries as compared to developed one. Among various etiological agents, *Helicobacter pylori* (*H. pylori*) play a detrimental role in development of GC. Through this review we focus on the recent progress in the field of research on *H. pylori* induced GC and providing the brief insight into *H. pylori* pathogenesis, the role of major virulence factors of *H. pylori* that modulates the host environment

and transform the normal gastric epithelium to neoplastic one.

Khatoon J, Rai RP, Prasad KN. Role of *Helicobacter pylori* in gastric cancer: Updates. *World J Gastrointest Oncol* 2016; 8(2): 147-158 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v8/i2/147.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v8.i2.147>

INTRODUCTION

In 1984 Marshall and Warren^[1] identified *Helicobacter pylori* (*H. pylori*) from gastric biopsy culture. In 1994, *H. pylori* was recognized as definite carcinogen by International agency for research on cancer. *H. pylori* induced gastric cancer (GC) is accountable for 5.5% of global cancer burden^[2].

H. pylori is spiral shaped, gram-negative, microaerophilic, flagellated human pathogen that successfully colonizes gastric mucosa of majority of individuals^[3]. Epidemiologically, the *H. pylori* infection exists all over the world, but colonization rates vary considerably; high in developing compared to the developed world^[4]. *H. pylori* acquisition thought to occur in early childhood. Fecal-oral or oral-oral were considered as possible route of *H. pylori* transmission^[4,5]. *H. pylori* urease is among the various virulence factors that aids in colonizing the highly acidic environment of stomach via breakdown of urea into ammonia, generating hospitable locale for its colonization^[6] (Figure 1). Among the majority of *H. pylori* infected individuals only a small percentage of colonized individuals develop severe clinical disease such as GC. Determining factors responsible for variation in clinical outcomes of *H. pylori* infection are still not well studied. For a longer period of time association between *H. pylori* and GC was debatable. A study from Japan on 1526 patients gives a clear evidence that *H. pylori* infection is significantly associated with risk of developing GC^[6]. Proof that *H. pylori* has an influence on early stages of gastric carcinogenesis is demonstrated by randomized prospective studies which shows association between *H. pylori* eradication and reduction of premalignant tumors^[7,8]. Research on experimentally challenged Mongolian gerbils, provide evidence concerning *H. pylori* eradication with attenuation of developmental process related to GC progression^[9,10]. Together these studies authenticate that *H. pylori* plays a key role in development of GC and indicate that *H. pylori* eradication provide protection against *H. pylori*-induced GC. Interaction among environmental factors, host genetic polymorphism and bacterial virulence attributes collectively influence the clinical outcome of *H. pylori* infections^[11].

This review aims to highlight recent progress in *H. pylori* pathogenesis, especially the bacterial and host factors that are involved in the host-pathogen interaction during persistent colonization. It also highlights the host immune response towards *H. pylori* colonization

and its effect on diverse clinical outcomes, especially on advancement leading to GC.

EPIDEMIOLOGY OF GC

GC is a multifactorial disease. Correa's model describes array of event beginning from chronic active gastritis, atrophic gastritis, intestinal metaplasia, dysplasia and eventually leads to GC^[12] (Figure 2). Risk factors for the development of the GC include interaction among the pathogen, environmental and host-related factors^[13]. World Health Organization recognized *H. pylori* as class I carcinogen in 1994. GC is identified as the fifth most common malignancy and third leading cause of cancer-related morbidity globally, constituting 9.7% of all cancer-related mortality^[14]. Highest age-standardized mortality rate (ASMR) is predicated for Eastern Asia (28.1 per 100000 in men, 13.0 per 100000 in women), the lowest ASMR in North America (2.8 and 1.5 per 100000, respectively)^[15]. Studies reported high mortality rates are from East Asia, Central and Eastern Europe, Central and South America^[15]. Developing countries have high burden of GC compared to the developed world and GC accounts for approximately 70% of both new cases and deaths^[16]. Categorizing on basis of gender, 466900 cases of males were reported from developing as compared to 173700 cases from developed countries and for females the corresponding disease load was 247000 and 102000 cases, respectively. GC is associated with age incidence; commonly occurs in age group of 55 to 80 years, rare among young individual. Frequency of GC rates are two fold higher in males than females^[17].

Over past decades in western nations, GC has considerably declined. The possible reasons behind this reduction include fall in *H. pylori* prevalence accompanied by better hygienic practices and innovative medical diagnostic facility. Despite the decline in GC incidence in developed world, the scenario of developing world is diverse. GC incidence and mortality rate remain very high in the developing nations, particularly in regions of East Asia and South America^[17]. It is expected that if appropriate measures are not implemented the number of estimated GC cases are likely to increase in future.

PATHOLOGICAL DIFFERENTIATION

Majority of gastric malignant tumors are adenocarcinomas. Histologically Lauren categorized gastric adenocarcinoma in intestinal and diffuse subtypes. Intestinal type adenocarcinoma is event dependent, start from chronic atrophic gastritis to intestinal metaplasia to dysplasia and finally carcinoma. Intestinal type adenocarcinoma is more frequent in developing world, common in male, and associated with age incidence, whereas diffuse type occurs more often in younger patients having family history of cancers, more frequent in females, background of atrophic gastritis is not prerequisite condition for its occurrence^[18,19]. Anatomical site of origin is another way of differentiation of gastric adenocarcinoma. Tumors

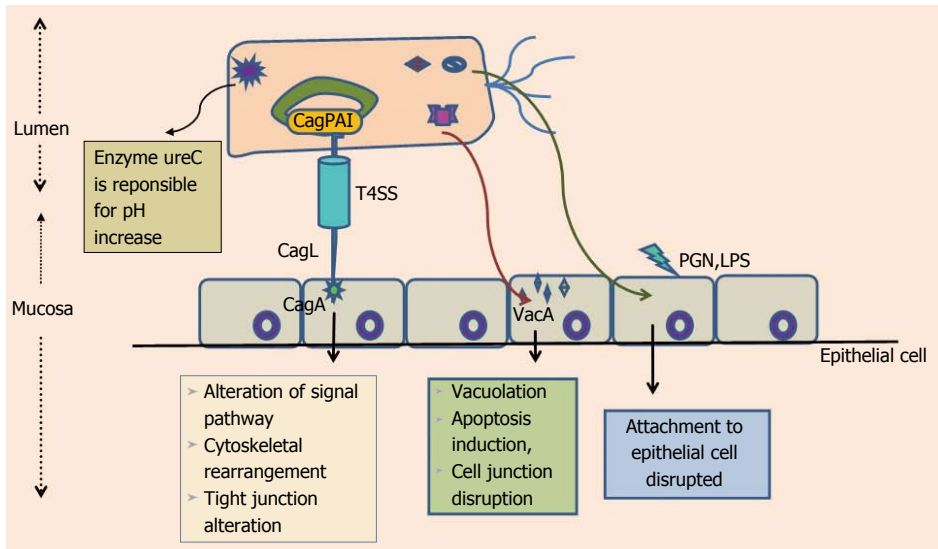


Figure 1 Interaction between *Helicobacter pylori* type IV secretion system and virulence determinants such as CagA, CagL, lipopolysaccharides, peptidoglycan and vacuolating cytotoxin gene, with mucosal epithelial cells, resulting in alteration of signal pathways, cell polarity disruption and vacuolation, which ultimately leads to death. T4SS: Type IV secretion system; LPS: Lipopolysaccharides; PGN: Peptidoglycan; VacA: Vacuolating cytotoxin; CagPAI: Cag pathogenicity island.

arising in the cardia region of the stomach are said to be proximal, and those from body and antrum (non-cardia region) as distal. Histological subtypes represent etiological and epidemiological differences between the two tumor sub sites. Globally GC incidence is declining. However, studies show rise in incidence of cardia carcinoma which may be partly due to more accurate reporting and fall in incidence of distal cancers^[20].

H. PYLORI AS A RISK FACTOR FOR GC

Colonization of the stomach by *H. pylori* causes development of gastritis. *H. pylori* is truly an “opportunistic” bacterium that uses various well defined virulence factors as tool for attachment and persistent colonization of human gastric mucosa. The possible transmission route is fecal-oral, but contaminated food or water are also reported^[21,22]. The most likely sources are person-to-person contact in families and/or exposure to a common source of infection such as contaminated water or food as supported by majority of data^[23]. This notion is supported by studies of children in custodial care where the prevalence of infection is higher than expected and from studies of crowded families in which there is at least one infected child^[24].

Before attachment of *H. pylori* to gastric epithelium, it has to first cross the thick mucus layer by adhering to the mucosal surface. This is aided by the presence of unipolar sheathed flagella, which allows *H. pylori* to quickly move from inhospitable low pH of gastric lumen to surface epithelium where pH is high and favorable for its successful colonization despite efforts made by the host to get rid of this bacterium. Non-motile mutant *H. pylori* strains fail to colonize the stomach of gnotobiotic piglets^[25,26]. In majority of infected individuals

colonization results in development of inflammatory and immune responses against *H. pylori*, but in some subjects *H. pylori* infection becomes chronic and leads to induction of gastric inflammation which can eventually lead to destruction of normal gastric glands and their replacement by intestinal-type epithelium resulting in atrophy of gastric mucosa.

The risk for atrophic gastritis depends on pattern as well as extent of distribution of chronic active inflammation. The individuals with lower acid output show a higher tendency towards atrophy^[27]. Reduction in gland size and level of intestinal metaplasia were associated with rise in GC risk by 5- to 90-folds depending on the extent and severity of atrophy^[28].

Increased odds ratios were evident from case-control studies that aimed to seriously study the signs of earlier *H. pylori* infection in GC patients and controls for development of non-cardia GC in presence of *H. pylori* infection^[29]. This fact is supported by data from animal models including Mongolian gerbil model, in which *H. pylori* infection induces atrophic gastritis and GC^[30-32]. A small number of subjects for research purposes were deliberately infected with pathogenic *H. pylori* strain and individuals developed acute inflammation of gastric mucosa with neutrophilic infiltration^[33,34]. Volunteers after several decades when exposed repeatedly to intragastric pH-electrodes contaminated with *H. pylori* developed conditions called “epidemic hypochlorhydria”^[34]. Such hypochlorhydric gastritis can either resolve spontaneously or change into chronic gastritis.

ROLE OF HOST GENETICS

H. pylori infection results in three possible outcomes. First is corpus-predominant gastritis beginning from

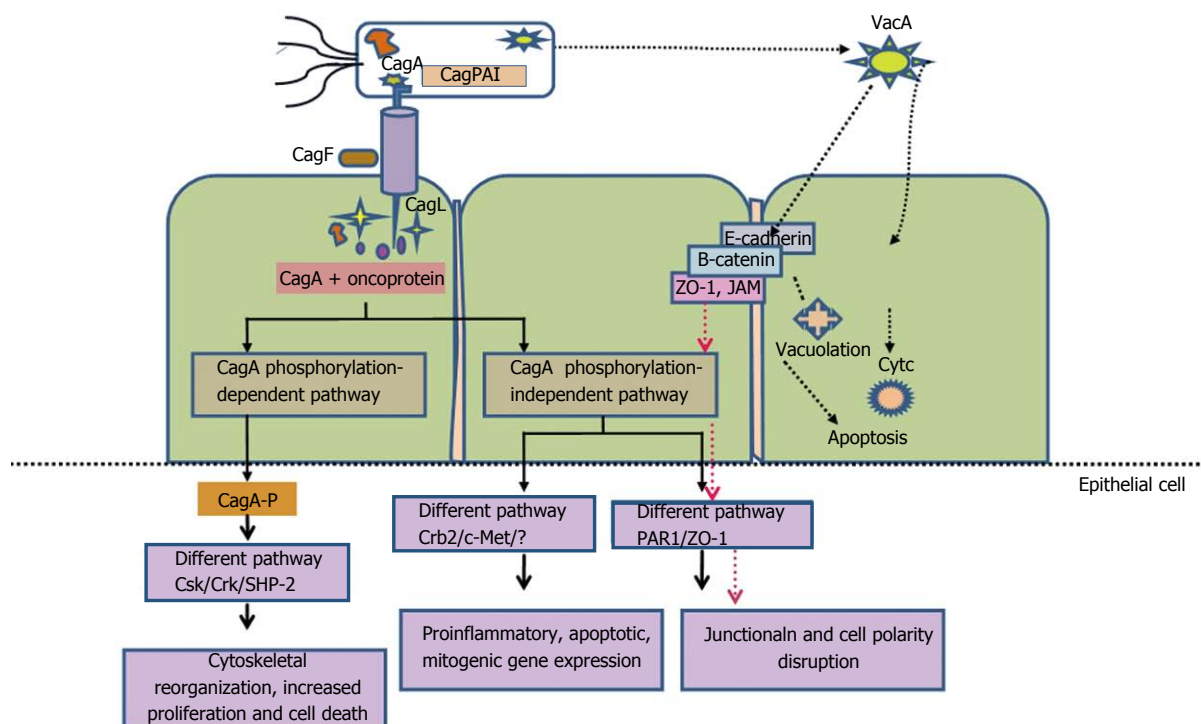


Figure 2 Combination of host, bacterial and environmental factors, which act in a synergetic way, resulting in development of precancerous cascade that ultimately leads to development of gastric cancer. ZO-1: Zonula occludens 1; CagPAI: Cag pathogenicity island; JAM: Junctional adhesion molecule; VacA: Vacuolating cytotoxin; CytC: Cytochrome.

atrophic gastritis to hypochlorhydria and finally to GC. Second type results in a pangastritis having slightest impact on the host gastric acid production. Duodenal ulcer is third outcome, where an antrum-predominant gastritis leads to hyperchlorhydria. There arises controversy that infections of *H. pylori* can predispose to two equally exclusive situations. The possible explanation why some people are more expected to develop GC phenotype when compared with others may be due to disparity among individual host response to *H. pylori* infections (Figure 1). Initial evidence for the importance of host genetic polymorphisms was reflected in the study where a rise in incidence of atrophic gastritis and hypochlorhydria was evident from relatives of *H. pylori* induced GC patients than controls^[35].

Pro-inflammatory cytokine like interleukin-1 β (IL-1 β) act as a powerful negative regulator of acid secretion. IL-1 β gene is now considered as a potential contender for host genetic polymorphisms that may elevates GC risk. Individuals possessing IL-1 β gene cluster polymorphisms have 2–3-folds increased risk of non-cardia cancer^[36,37] (Figure 2). Elevated levels of TNF- α in gastric mucosa of *H. pylori* infected individuals were evident from numerous studies. However, down regulation of anti-inflammatory cytokine IL-10, that suppresses the level of pro-inflammatory cytokines including IL-1 β , TNF- α and interferon- γ (IFN- γ) is also reported^[36].

The risk associated with GC development in *H. pylori* infected individuals upsurges 27-folds in individuals with three or four polymorphisms^[38]. This evidently illustrates that interaction between host genetics and environment

plays a key role in progression of GC, by regulating hosts adaptive immune response resulting in transformation of normal gastric mucosa to neoplastic one.

IL-8

Higher expression of chemokine IL-8 and polymorphism (promoter region) has been reported in studies and linked with increased risk for GC^[39]. Study on Caucasian populations proved that relationship among functional polymorphism within Toll like receptor 4, risk of GC and decrease in production of anti-inflammatory cytokine IL-10^[40]. These studies reflect that host genetic polymorphisms are capable of modulating the innate immune response which results, severe inflammation and premalignant lesions in *H. pylori* infected individuals (Figure 2). These studies raises a query that whether *H. pylori* strain characteristics are responsible for increasing cancer risk employed by host genotypes, needs to be studied further. Odds ratios for non-cardia GC were highest for individuals with elevated IL-1 β expression, colonized by *H. pylori* vacAs1-type strains^[41].

It is evident from case-control studies that *H. pylori* successfully form a vital equations with host by its ability to send and receive signals from its hosts^[42,43]. Only certain *H. pylori* strains enhance the possibility of carcinogenesis because the equilibrium is likely different for each colonized individual. For example, individual infected with CagA strains leads to severe gastritis, which results in rise of proinflammatory cytokines levels that are responsible for both amplifying the mucosal inflammatory response as well as reducing the acid

production. This creates a milieu encouraging growth of *H. pylori* that promote inflammation and continually produce oxidative stress, thus augmenting risk for transformation of normal mucosa to neoplastic through series of events (Figure 2).

Cyclooxygenase

H. pylori triggers numerous forms of proinflammatory cyclooxygenase (COX) enzymes. Production of endoperoxide from arachidonic acid is brought by COX enzymes. Enzymes prostaglandin synthases produces prostaglandins and various eicosanoids from endoperoxide^[44]. Important role is played by prostaglandins in regulating physiologic processes for instance immunity and development. Two COX isoforms (COX-1 and COX-2) have been categorized on the basis of variances in expression characteristics and inhibition profiles for nonsteroidal anti-inflammatory drugs (NSAIDs). COX-2 expression is inducible while COX-1 is constitutively expressed in cells and tissues^[45-47]. Expression of COX-2 can be stimulated by proinflammatory cytokines, growth factors such as TNF- α , IFN- γ and IL-1. COX-2 expression are raised in *H. pylori* infected human gastric mucosa, gastric premalignant and malignant lesions^[47-49]. Inhibitors of COX (aspirin and NSAIDs) are associated with reduced risk of non-cardia GC^[50]. Numerous studies demonstrate substantial role of COX-2-generated products involved in promoting neoplasia. Mechanisms like apoptosis inhibition, regulation of expression of cell surface adhesion, and production of promoting factors of neoplasia leads to malignancy^[51,52] (Figure 2).

H. PYLORI VIRULENCE FACTORS

Cag pathogenicity island

H. pylori have genetically heterogeneous genome. A number of *H. pylori* virulence factors are supposed to play an essential role in diverse clinical outcome of *H. pylori* infections. The Cag pathogenicity island (CagPAI) is a 40-kb region, consisting of 32 genes, flanked by 31-bp direct repeats. CagPAI is an island consisting of virulence genes, which are acquired by horizontal transfer. CagPAI encodes a type IV secretion system (T4SS) that is responsible for the entrance of a most remarkably investigated *H. pylori* virulence determinant effector protein CagA^[53-55] (Figure 1). Positive association of CagA was found with peptic ulcer disease^[56,57]. Due to its association with several gastroduodenal pathologies, initially CagA was considered as an indicator for presence of the entire CagPAI but as research speeded up, studies demonstrated that despite its presence, CagPAI intactness and clinical outcome varied.

More or less 70% of *H. pylori* strains from western world and nearly 100% of East Asian strains express virulent protein CagA^[54,58,59]. Majority of *H. pylori* strains induces superficial gastritis but the risk for chronic gastritis, atrophic gastritis, metaplasia, and non-cardia GC with intact CagPAI is much higher compared to those

that lacked it^[56,57,60-66]. Among 32 genes of CagPAI, 18 genes are thought to code for structural parts of a T4SS, this system is responsible for exporting peptidoglycans and cagA into host gastric epithelial cells, *via* forming a pilus like assembly connecting bacterial and host epithelial membrane (Figure 1).

CagA

CagA is terminal gene product of the CagPAI. Classifying *H. pylori* strains on the basis of presence and absence of cagA into cagA-positive and cagA-negative strains. After the *H. pylori* attachment to epithelial cell, CagA is internalized through T4SS apparatus. After translocation, CagA is tyrosine phosphorylated at glutamate-proline-isoleucine-tyrosine-alanine (EPIYA) motif, *i.e.*, EPIYA motif which is associated with cell morphological changes known as "the hummingbird phenotype," which results in increased cellular migration^[67-71].

Polymorphic region of CagA, has been identified within the carboxy-terminal and distinguished by different amino acid sequences. Till date, four distinct EPIYA motifs (EPIYA-A, -B, -C and D) are known^[72,73]. EPIYA-A and -B motifs are present in strains all over the world, whereas EPIYA-C is specific to western world (Europe, North America, and Australia). Variation in number of EPIYA-C sites occurs, while majority of CagA proteins contain a single EPIYA-C site (A-B-C type). The level of phosphorylation of EPIYA-C sites is greater than EPIYA-A and EPIYA-B sites. Risk for development of GC is found to be associated with the number of cagA EPIYA-C in western strains^[74]. EPIYA-D motif is exclusive to East Asian strains (from Japan, South Korea, and China), and strains possessing this motif produces higher level of IL-8 from gastric epithelial cells as compared to strains harboring western A-B-C-type CagA^[72,75].

CagA phosphorylation-dependent host cell signaling

Kinase families of Abl and Src are responsible for phosphorylation of CagA into phospho-CagA. Interaction between phosphorylated CagA and various intracellular effectors, triggers an eukaryotic tyrosine phosphatase (SHP-2), which results in continuous stimulation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), Crk adaptor^[76] and C-terminal Src kinase in a tyrosine phosphorylation-dependent manner. In East Asian A-B-D types, negative response is induced by interactions of phospho-CagA with C-terminal Src kinase resulting down regulation of Src signaling^[77] (Figure 3).

Experimental studies on cell lines revealed that CagA internalization give rise to "hummingbird phenotype". These alterations are characterized by cell elongation and cell scattering^[69,78]. Additional study also indicates that interplay among phosphorylated CagA, dephosphorylation of SHP-2 and down-regulation of focal adhesion kinase, causes cell elongation^[69,79]. A different mechanism of cell elongation by phosphorylated CagA is by making a defect in cell retraction; yet the signaling molecules prerequisite for this phenotype remain vague^[80].

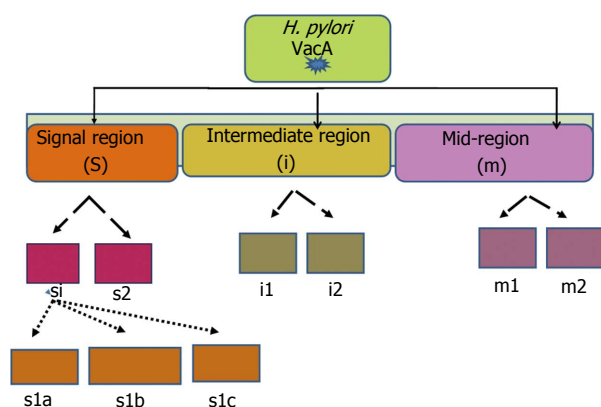


Figure 3 Schematic representation of multiple pathways of *Helicobacter pylori* pathogenesis involved type IV secretion system and internalization of virulence determinants like CagA and oncoproteins; CagA-phosphorylation dependent and CagA-phosphorylation independent pathways leads to cytoskeletal reorganization, increase proinflammatory and mitogenic gene expression. Another major virulent factor, VacA is responsible for alteration of junction and cell polarity by binding with tight junction molecules such as E-cadherin, ZO. VacA also causes mitochondrial membranes depolarization, Cytochrome release from mitochondria to cytosol and caspase-3 activation followed by cell apoptosis. T4SS: Type IV secretion system; VacA: Vacuolating cytotoxin; ZO: Zonoccludins; Cyto: Cytochrome; *H. pylori*: *Helicobacter pylori*.

Phosphorylated CagA obstructs the enzymatic activity of c-Src, which leads to tyrosine dephosphorylation of actin binding proteins such as cortactin, ezrin, and vinculin, ultimately results in cell elongation^[81-83] (Figure 3).

CagA phosphorylation-independent host cell signaling

Non-phosphorylated CagA have a different way of exerting effects within the cell. CagA translocation without phosphorylation leads to aberrant catenin activation, apical-junctional complex disruption and cellular polarity loss^[84-89]. Relation between non-phosphorylated CagA and epithelial tight junction scaffolding proteins, zonula occludens 1 and junctional adhesion molecule A, results in imperfect association of tight junctions at located sites of bacterial attachment. Additional molecules includes E-cadherin, hepatocyte growth factor receptor c-Met, phospholipase C gamma (PL), adaptor protein Grb2, and kinase partitioning defective 1b/microtubule affinity-regulating kinase 2 (PAR1b/MARK2) resulting in mitogenic responses, interruption of cell-cell junctions and cell polarity destruction^[84,87,88,90] (Figure 3). Recent study revealed that CagA directly binds to the cell polarity regulator such as PAR1b/MARK2. This binding prevents kinase PAR1b/MARK2 activity and deregulates the formation of mitotic spindle by cells which affects cell polarity^[88,91].

Studies on transgenic mice revealed the correlation between CagA and oncogenesis by showing that CagA expression led to gastric epithelial cell proliferation and neoplastic changes. However, the following modifications were not detected in mice expressing phosphorylation-resistant CagA^[52].

Presence of contradictory documentation on functionality of CagA as a bacterial oncoprotein in mammals exists besides solid proof provided by animal models.

Pathological alterations described for transgenic CagA mice followed by absence of inflammation, which reflects disparity to what is seen in humans^[52]. Although CagA act as oncoprotein, it remains to be explored why only few individuals inhabited by CagA-positive *H. pylori* develop GC. Recent study demonstrate that *H. pylori* prompts the presence of a host phospholipid, phosphatidylserine where CagA can explicitly interact and gain entry into the cells^[92]. Focus of future research should be to define the exact mechanism of CagA internalization in gastric epithelial, factor responsible for regulation of this process and when during chronic infection CagA delivery in human epithelial cells.

VacA

Another important *H. pylori* virulence gene is vacuolating cytotoxin (VacA), which encodes a bacterial toxin (VacA) that induces series of cascades leading apoptosis of epithelial cells *via* induction of cytoplasmic vacuoles (Figure 4). VacA is found throughout the *H. pylori* strains. The diverse polymorphic form of VacA are related with clinical outcomes^[93]. Considerable genetic variations are found in: The s (signal) region with alleles s1a, s1b, s1c, or s2; the m (middle) region with m1 or m2 alleles; and the i (intermediate) region with type i1 or i2 alleles (Figure 4).

H. pylori strains having combination VacAs1/m1 or vacAs1/m1/i1 are associated with increased risk of progression to premalignant lesion and GC than vacA s2/m2 or vacAs2/m2/i2 strains^[94] (Figure 5).

OTHER RISK FACTORS

Besides *H. pylori*, the following other environmental factors are considered to contribute in the pathogenesis of GC.

Diet

The variations in GC incidence are due to environmental inputs, particular in dietary pattern. Previous accumulating studies have been indicated that downward trend in GC occurrence. This may be due to the advent of widespread refrigeration of foodstuff and reduction in dependency on food preservation. In addition, other studies have suggested that a preventive role of diet containing fresh vegetables and fruits. However, data from European prospective study failed to show an overall association between fresh fruits and vegetables intake and GC risk^[95]. Recent studies being conducted on this field revealed that a significant association between total dietary vegetables contents (onion and garlic intake) and intestinal GC subtypes.

Additional studies are required for demonstration of positive association between *H. pylori* eradication and prevention of cancer. The controversy related to point of no return in case of atrophy and metaplasia is still debatable. Proposed studies on side effects and expenses of such preventive measures are required in future for proper management and treatment of GC, therefore GC prevention remains a key part of research on *H. pylori*.

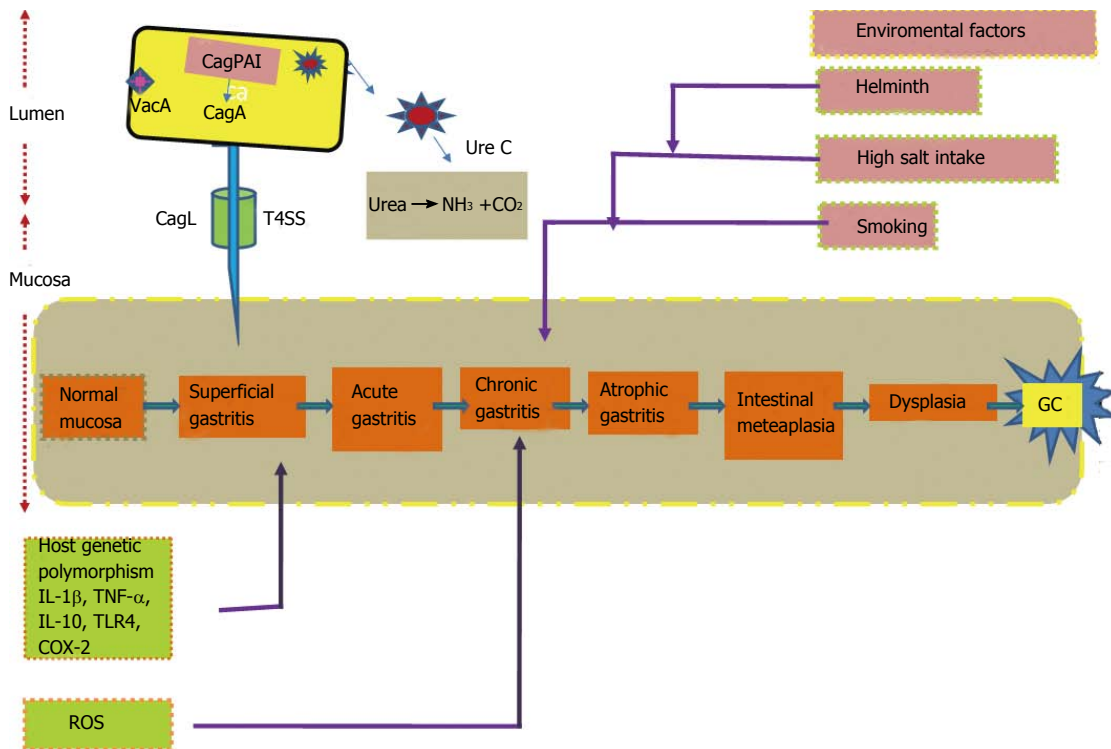


Figure 4 Representation of *Helicobacter pylori* major virulence factor, vacuolating cytotoxin containing three domain 1: signal sequence(S) 2: middle region (m) 3: recently identified intermediate region (i) s, m and i region are further stratified into the subtypes s1, s2, m1, m2 and i1, i2 respectively. TLR4: Toll like receptor 4; T4SS: Type IV secretion system; VacA: Vacuolating cytotoxin; CagPAI: Cag pathogenicity island; GC: Gastric cancer; ROS: Reactive oxygen species.

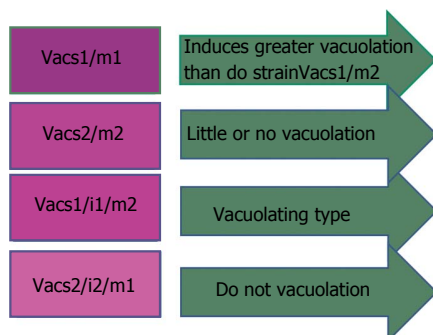


Figure 5 Vacuolating cytotoxin of *Helicobacter pylori* may have any combination of signal sequence and mid region with different virulence activities as stated above. Vac: Vacuolating cytotoxin.

Salt

H. pylori is not the only the culprit for the development of GC; other influential causes include host polymorphisms and environmental elements (Figure 2). High dietary salt intake was found to be uniformly been associated with an increased risk of GC^[12,96]. Two studies, one study from Japan and other a case-control study from South Korea stated that *H. pylori*-infected subjects taking high-salt diet had an greater risk of GC than those with lower levels of salt^[97,98]. Association between the frequency of *H. pylori* infection and amount of dietary salt intake is reported in another study^[99].

Research on Mongolian gerbils had shown that the *H. pylori* presence and usage of a more salt containing

diet applied concerted effects on development of precancerous satge^[100,101]. Additional study on *H. pylori*-infected gerbils demonstrates that there is a positive association between level of severity of gastric inflammation and rate of proliferation of epithelial cells in gerbils consuming high-salt diet than those consuming a normal diet^[100]. Similar studies on gerbils infected with *H. pylori*, when treated with carcinogen (N-methyl-N-nitroso urea) shows that higher frequency of GC related with animals consuming high-salt diet as compared to animals with a normal diet^[101,102].

Mechanisms behind the high-salt diet increases the risk of development of GC in humans remains unclear. Among various explanations, one plausible hypothesis is that salt may lower the threshold for malignant transformation by altering the physiology of gastric epithelium thus allowing entry of carcinogens into gastric tissue and resulting in damage to gastric mucosa. Another possibility is that high salt intake might be regulating the gene expression in *H. pylori*. Two independent studies suggested that consumption of excess amount of salts in diet leads to higher expression of *H. pylori* virulence factors^[103,104].

Dietary antioxidants

Many studies had proved the antioxidants present in food in green vegetables and fruits plays a preventive role against progression of GC^[105]. There is scarcity on studies on association of *H. pylori* infection with nutritive elements in gastric carcinoma. A case-control study

recommended that consistent excessive consumption of vitamin C and carotene might be able to curtail the casual for developing GC in subjects having infection of *H. pylori*^[106].

A randomized study on population susceptible for GC development demonstrated that combination of vitamin C and carotene dietary supplements and *H. pylori* eradication increases the preneoplastic lesions regression at 6 years of follow-up; at another 6 years follow-up lacking dietary supplements, the protective role of vitamin C and carotene gradually end up^[7]. These results were also validated by other studies^[95,106]. A similar study from Hawaii proof that consumption of fresh vegetable among *H. pylori* infected individuals provided a little protection against GC occurrence^[107]. On the contrary, other studies fail to provide a positive association between *H. pylori* infection and plasma vitamin C level, with risk of GC incidence^[108]. Additional research is required to determine whether antioxidants are capable of providing protection against GC among *H. pylori* infected patients.

Cigarette smoking

It is evident from various studies that cigarette smoking is associated with risk of developing GC in *H. pylori* infected subjects. In Japan, cigarette smoking and *H. pylori* infection together are considered as potential threat for developing GC^[109]. Swedish and German population-based case control studies also demonstrated combination of cigarette smoking and infection by CagA positive *H. pylori* strains increased the risk of developing GC (Figure 2). Los Angeles study also reported a tendency toward increased risk of GC in smokers^[59,110]. On collectively analyzing studies, it emerges that there exists relationship between *H. pylori* infection and smoking with increased risk of developing GC.

Helminth infection

H. pylori co-infection with helminths may have some impact in disease pathogenesis. Reduced Th1 response associated with higher levels of Th2 cytokines was reported in one study^[111]. Another study on Colombian children from a coastal region having infection of both helminths and *H. pylori*, showed a higher Th2 associated IgG1 response^[112]. Further studies are needed to assess the impact of *H. pylori* and helminthes co-infection in disease pathogenesis.

FOCUS OF FUTURE ENDEAVORS

Gastric cancer remains a major threat to mankind. Improvement in living standards, increase awareness in sanitation and hygiene practices, reduction in intake of salted food products and advent of refrigeration in households resulted in measurable decline both in incidence of *H. pylori* infection and GC. Although both *H. pylori* infection and GC are showing decreasing trends in the developed world, they still remain a major threat to

human population in the developing countries. Therefore, there is a need for improvement in early diagnosis, identification of risk factors, and development of preventive strategies and initiation of timely therapeutic interventions, especially focused for the developing countries. Further, it remains to be investigated why a small fraction of individuals colonized by *H. pylori* develop GC, and future research should focus on bacterial, host genetics, environmental and dietary factors.

There is need to formulate clear cut recommendation for screening and timely intervention of high risk population with family history of GC. Whether all high-risk areas should undergo routine screening of *H. pylori* infection is still questionable. Since the patients having atrophic gastritis or dysplasia in the gastric mucosa are at increased risk of developing GC, there is a need for special recommendations including endoscopic surveillance for such patients.

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2016 Pancreatic Cancer: Global view

Endoscopic approach to the diagnosis of pancreatic cystic tumor

Yoshiaki Kawaguchi, Tetsuya Mine

Yoshiaki Kawaguchi, Tetsuya Mine, Department of Gastroenterology, Tokai University School of Medicine, Isehara 259-1193, Japan

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Correspondence to: Yoshiaki Kawaguchi, MD, PhD, Department of Gastroenterology, Tokai University School of Medicine, 143 Shimokasuya, Isehara 259-1193, Japan. y711kawa@is.icc.u-tokai.ac.jp
 Telephone: +81-463-931121
 Fax: +81-463-937134

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Abstract

Because of the aging of the population, prevalence of

medical checkups, and advances in imaging studies, the number of pancreatic cystic lesions detected has increased. Once these lesions are detected, neoplastic cysts should be differentiated from non-neoplastic cysts. Furthermore, because of the malignant potential of some neoplastic pancreatic cysts, further differentiation between benign and malignant cysts should be made regardless of their size. Although endoscopic ultrasound (EUS) has a very high diagnostic performance for pancreatic cystic lesions among the various imaging modalities, EUS findings alone are insufficient for the differentiation of pancreatic cysts and diagnosis of malignancy. In addition, cytology by EUS-guided fine-needle aspiration (FNA) has a high specificity but a low sensitivity for diagnosing malignancy in pancreatic cystic tumors. The levels of amylase, lipase, and tumor markers in pancreatic cystic fluid are considered auxiliary parameters for diagnosis of benign and malignant cysts, and a definitive diagnosis of malignancy using these parameters is difficult. Thus, in addition to EUS, cytology by EUS-FNA, and cystic fluid analysis, new techniques based on EUS-guided through-the-needle imaging, such as confocal laser endomicroscopy and cystoscopy, have been explored in recent years.

Key words: Endoscopic ultrasound; Endoscopic retrograde cholangiopancreatography; Endoscopic ultrasound-needle aspiration; Pancreatic cystic tumor; Cytology

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Core tip: The number of pancreatic cystic lesions detected has increased. Neoplastic cysts should be differentiated from non-neoplastic cysts. Further differentiation between benign and malignant cysts should be made regardless of their size. In addition to endoscopic ultrasound (EUS), cytology by EUS-fine-needle aspiration, and cystic fluid analysis, new techniques based on EUS-guided through-the-needle imaging, such as confocal laser endomicroscopy and

cystoscopy, have been explored in recent years. We reviewed an endoscopic approach to the diagnosis of pancreatic cystic tumor.

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INTRODUCTION

Because of the aging of the population, prevalence of medical checkups, and advances in imaging studies, the number of incidentally detected pancreatic cystic lesions has increased. Pancreatic cystic lesions include a variety of entities, including non-neoplastic pancreatic pseudocysts, such as those resulting from pancreatitis, and retention cysts, as well as neoplastic pancreatic cysts and solid tumors with cystic degeneration. As differential diagnosis of these lesions is important in the consideration of therapeutic strategies^[1], it is essential to differentiate between neoplastic pancreatic cysts, including intraductal papillary mucinous neoplasm (IPMN), mucinous cystic neoplasm (MCN), and serous cystic neoplasm (SCN), and to further determine whether they are benign or malignant^[1].

Diagnostic imaging modalities used in the evaluation of pancreatic cystic lesions include abdominal ultrasound (US), contrast-enhanced computed tomography (CT), magnetic resonance cholangiopancreatography (MRCP), endoscopic ultrasound (EUS), and endoscopic retrograde pancreatography (ERP). US is a non-invasive method but is affected by the presence of gastrointestinal gas, making the evaluation of the entire pancreas difficult. Although CT is superior in depicting solid lesions, radiation exposure and allergic reactions to contrast media, limit its application. MRCP is superior in depicting pancreatic cystic lesions, while EUS is highly valued, as it provides high image resolution despite the presence of gastrointestinal gas, allowing close observation of the entire pancreas. Although ERP is superior in depicting details of the pancreatic duct and allows a pathologic diagnosis by cytology of the pancreatic juice at same time, attention should be paid to pancreatitis as a potential complication of endoscopic retrograde cholangiopancreatography (ERCP). At present, the lesions are comprehensively diagnosed by a combination of these methods. In recent years, EUS, EUS-guided fine-needle aspiration (FNA), contrast-enhanced EUS, and other modalities of interventional EUS, have been especially useful in the accurate differentiation of pancreatic cystic tumors^[1,2].

TRANSPAPILLARY DIAGNOSIS

A transpapillary approach is significant for the diagnosis

of either, main-duct or branch-duct type of IPMNs formed in the pancreatic duct^[3]. This approach allows to demonstrate the presence of mucus, and is also effective in the diagnosis of concurrent pancreatic ductal carcinoma. However, for the diagnosis of SCNs and MCNs, which generally do not communicate with the pancreatic duct, the transpapillary diagnostic approach not only lacks significance but may also causes pancreatitis after ERCP. IPMN are pancreatic cystic tumors in which transpapillary diagnosis is significant.

Pancreatic juice cytology

As the pancreatic juice in IPMNs is viscous and often difficult to aspirate, pancreatic juice cytology is used to improve the diagnostic performance of ERCP by allowing the collection of pancreatic juice *via* an implanted endoscopic naso-pancreatic drainage tube. Branch-duct type IPMN, which communicates with the main pancreatic duct, is well indicated for this technique because mucus-containing abundant tumor cells are found in the main pancreatic duct.

IPMNs are high mucous-producing and often well-differentiated adenocarcinomas, even when they are cancerous. Therefore, the diagnosis of this type of tumors using pancreatic juice cytology is difficult. To overcome these limitations, the genetic analysis of pancreatic juice is being studied to aid the objective evaluation of malignancy. Such studies show that tumor markers, including carcinoembryonic antigen (CEA), telomerase activity, matrix metalloproteinase activity, human telomerase reverse transcriptase, mRNA, sonic hedgehog, K-ras, and p-53, present in pancreatic juice may be useful in the assessment of cancer risk in patients undergoing ERP, while complementing pancreatic juice cytology findings^[4-10].

EUS DIAGNOSIS

The differential diagnosis of pancreatic cystic lesions can be made by focusing on EUS findings, *i.e.*, size, number, overall cyst shape, state of cyst walls, and features of cystic contents, as well as the presence of underlying lesions^[11]. Sedlack *et al*^[12] classified 34 resected pancreatic cystic lesions into two groups: A group of benign pancreatic cysts, including simple cysts, pseudocysts, and SCNs, and a group of malignant or malignant potential lesions including MCNs, IPMNs, neuroendocrine tumors with necrotic lesions, and cystic adenocarcinomas. Comparison of the diagnostic performance between the 2 groups showed that EUS had a sensitivity, specificity, and diagnostic accuracy of 91%, 60% and 72%, respectively. Song *et al*^[13] evaluated 75 pancreatic cysts (58 neoplastic pancreatic cysts and 17 pancreatic pseudocysts) using EUS, and showed that, while intracystic debris and pancreatic parenchymal changes were characteristic EUS findings of pancreatic pseudocysts, the presence of septa and nodes were typical of neoplastic pancreatic cysts. Song *et al*^[13] reported that although EUS is useful in the diffe-

rential diagnosis of pancreatic cystic lesions, it might be insufficient on its own, to completely differentiate pancreatic cysts. In addition, in a multicenter study conducted by Brugge^[14] to evaluate the performance of EUS in the diagnosis of pancreatic cyst malignancy, low sensitivity, specificity, and diagnostic accuracy values of 56%, 45% and 51%, respectively, were observed. Moreover, Ahamad *et al.*^[15] demonstrated that the diagnostic accuracy of EUS for pancreatic cysts and non-cystic lesions varied from 40% to 93% among 8 endoscopists, indicating that experience and skills influence the diagnostic performance of this method.

DIFFERENTIATION OF PANCREATIC CYSTIC LESIONS USING CONTRAST-HARMONIC EUS

Differentiation between neoplastic (IPMNs, MCNs, and SCNs) and non-neoplastic pancreatic cystic lesions is important. Although there are sporadic reports on the use of B-mode imaging for pancreatic cystic lesions diagnosis^[16,17], reports on similar studies using contrast-harmonic (CH)-EUS are limited. However, because CH-EUS clearly depicts the internal structure and shape of lesions, it appears to be useful for picking up the characteristic imaging findings of each lesion. Compared to conventional B-mode imaging, CH-EUS facilitates pancreatic duct observation by depicting it as a structure without blood flow. In consequence, communication between a lesion and the pancreatic duct, an important aspect for differentiation of pancreatic cystic lesions, can be easily confirmed. In cases of IPMN in which a structure is observed in the dilated pancreatic duct, differentiation between a mucinous mass or tumor resulting from papillary growth by B-mode imaging, is often difficult. However, the CH mode allows their differentiation according to the presence or absence of blood flow.

EUS-FNA DIAGNOSIS

In Japan, because of a reported incident of peritoneal metastasis caused by EUS-FNA for IPMN^[18], doctors have become reluctant to perform the procedure. However, EUS-FNA is commonly used for the diagnosis of pancreatic cystic tumors worldwide, as well as for the evaluation of pancreatic cystic fluid, in terms of its nature (mucinous or serous), cytology, and measurement of CEA/amylose levels^[19].

The nature of the cystic fluid collected by EUS-FNA is important for differentiation of pancreatic cystic tumors. IPMNs and MCNs, or SCNs should be suspected if the fluid is mucinous, or serous, respectively.

The cytology of pancreatic cystic tumors by EUS-FNA, has a high specificity for diagnosis of malignancy similar to that of ERP, albeit with a low sensitivity. Moreover, in cases of multilocular cysts, sufficient specimens may not be collected due to the small diameter of each cyst or

high viscosity of the cystic fluid, which limits its aspiration with a puncture needle. The inability to collect sufficient amounts of cells seems to be the cause of the low sensitivity. The rate of successful collection of specimens required for cytology is reported to be approximately 80%, and the differential diagnostic accuracy for pancreatic cysts ranges from 13%-96%^[12,15,20-26]. In addition, the diagnosis of malignancy has a specificity of 86%-100% and a sensitivity of 25%-88%. The international guidelines for the differential diagnosis between benign and malignant lesions, therapeutic strategies, and follow-up procedures of main-duct and branch-duct type IPMNs were revised in 2012. According to the revised guidelines, the cytological assessment of especially worrisome features (main pancreatic duct diameter of 5-9 mm and absence of either nodes or growth in main-duct and branch-duct type, respectively) is important. The results of a meta-analysis showed that, despite the high specificity and diagnostic accuracy of cytology, its sensitivity is low, with a possibility of misdiagnosing malignant lesions as benign, concluding that cytology needs to be complemented by the additional measurement of CEA, carbohydrate antigen (CA) 19-9, micro-RNA, *etc.*^[27].

Amylase, CEA, and CA19-9 levels in cystic fluid are highly useful for IPMNs, MCN, and SCN differentiation. Amylase levels in cystic fluid are high in IPMNs because they communicate with the pancreatic duct. By contrast, as MCNs and SCNs do not communicate with the pancreatic duct, their amylase levels are typically low. In addition, a cut-off amylase value in cystic fluid set at 250 U/L, has a sensitivity and specificity of 44% and 98%, respectively, for excluding pancreatic pseudocysts from the diagnosis of pancreatic cystic lesions^[28].

CEA levels in cystic fluid are useful for differentiation between MCN (including IPMN) and SCN. A CEA cut-off value in cystic fluid of 192 ng/mL, had a 79% diagnostic accuracy for MCN, which was higher than that of 59% using diagnostic imaging by EUS^[22]. In a cyst containing ≥ 800 ng/mL of CEA in cystic fluid, or diagnosed as malignant by cytology, the specificity for diagnosing the cyst as MCN was 98%-100%. Moreover, a CEA level in cystic fluid ≤ 5 ng/mL had a 95% specificity for the diagnosis of a pancreatic cyst as benign, of which, 6% were, however, MCNs^[28].

A CA19-9 level in cystic fluid ≤ 37 U/mL has an accuracy and specificity for diagnosing a pancreatic cystic tumor as benign of 46% and 94%, respectively. CA19-9 is useful for complementing diagnosis of benign and malignant pancreatic cystic tumors^[28].

Thus, analysis of amylase, CEA, and CA19-9 levels in cystic fluid improves the ability to differentiate mucinous from serous pancreatic cystic tumors. Because malignant SCN is rare, its reliably diagnosis is important. However, levels of amylase, CEA, and CA19-9 in cystic fluid are reportedly not helpful for differentiation of cancer among MCN^[29].

Various attempts have been made to improve the diagnosis of malignancy in pancreatic cystic tumors. As

a reason for the low sensitivity of cystic fluid cytology is the scarcity of cell components in cystic fluid, attempts to collect more cells have been reported. These include, abrasion of cystic wall by brushing^[30]. Abrasion/puncture of cystic wall with the tip of a puncture needle while cystic fluid is aspirated^[31], and direct biopsy of cystic wall with miniature biopsy forceps that can be passed through a puncture needle^[32]. Although of cystic fluid specimens collected by all of these techniques contain more cell components than those collected by conventional aspiration, they have failed to improve the diagnostic performance for malignancy. This is attributed to the fact that the grade of atypism is not always consistent in the cystic wall itself. If target biopsy of nodular lesions can be performed, diagnostic performance may be improved.

Procedural accidents

While serious complications or procedural accidents associated with EUS-FNA for pancreatic cystic lesions have not been reported, pancreatitis (0.5%-4%)^[33], cyst infection (< 1%)^[20,33,34], and intracystic hemorrhage (< 1%)^[15,20,35], rarely occur. Cyst infections can be prevented by infusion of antibiotics before EUS-FNA or oral administration of antibiotics for 2 to 5 d after puncture, while EUS-FNA can be safely performed using a 22-gauge puncture needle^[15,33].

EUS-GUIDED THROUGH-THE-NEEDLE IMAGING OF PANCREATIC CYSTIC TUMORS

Confocal laser endomicroscopy

In many reports, confocal laser endomicroscopy (CLE) has been described as useful for virtual biopsy and provides images similar to pathological images during endoscopic observation^[36]. There are a CLE device that incorporates an endoscope and probe-based CLE (pCLE) in which a probe is inserted through the forceps channel of the endoscope for observation. These devices are reported to be useful for detailed examination of the gastrointestinal tract before therapeutic endoscopy.

Needle-based CLE

A prototype device (Cellvizio AQ-Flex-19®, Mauna Kea Technologies, Paris, France) with a diameter smaller than that of pCLE has been developed. This device can be inserted in an EUS-FNA 19-gauge needle and used to perform EUS-guided needle-based CLE (nCLE) for the diagnosis of pancreatic cysts.

The *in vivo* CLE Study in the Pancreas With Endosonography of Cystic Tumors trial^[37], compared the findings of EUS-guided nCLE with those of pathological analysis. When the findings of nCLE were classified into 3 categories, *i.e.*, epithelial structure, non-epithelial structure, and intracystic floating components, an abnormal epithelial structure, mainly including papillary projections, was a characteristic finding of mucinous

tumors. In addition, nCLE of IPMNs revealed dark aggregates with high cell density in areas suspected of dysplasia, while blood vessels, which are non-epithelial structures, were seen as white bands in other areas. SCNs, only showed non-epithelial structures, whereas no epithelial structure was observed. Although the specificity of the findings of EUS-guided nCLE was 100%, the sensitivity was low, with a value of 57.9%. According to a report indicating that findings reflecting hypervascular patterns of cystic walls and septa of SCNs are useful, there was no technical problem, whereas it was difficult to puncture lesions of the pancreatic head with a 19-gauge needle^[38].

Cystoscopy

Cystoscopy is a diagnostic procedure in which a pancreatic cystic tumor is punctured with a 19-gauge FNA needle, and a SpyGlass probe made of optic fiber directly is inserted into the pancreatic cyst to observe cystic contents and the nature of the cystic wall. According to cystoscopy, the cystic fluid in IPMNs and MCNs is mucus. Regarding the cystic wall, IPMNs have papillary projections or communicate with the pancreatic duct, while MCNs have a smooth cystic wall. However, the cystic fluid of SCNs is clear, while the cystic wall is smooth and has abundant blood vessels.

Combination of cystoscopy and nCLE

In the Diagnosis of Pancreatic Cysts: EUS-guided Through-the-needle Confocal Laser-induced Endomicroscopy and Cystoscopy Trial (DETECT study)^[39], the contribution of the cystoscopy and nCLE combination to further improve diagnostic performance, was evaluated. For the diagnosis of mucinous cysts, the specificity of both cystoscopy and nCLE was 100%, whereas their sensitivity was also relatively favorable with values of 71% and 77%, respectively. Furthermore, when these 2 modalities were combined, the specificity remained at 100%, and the sensitivity was elevated to 88%, indicating an improved diagnostic performance. However, in terms of diagnosis of malignancy, the image quality of cystoscopy and nCLE decreased as the diameter of a probe reduced. Therefore, the image quality of this technique is insufficient at present.

CONCLUSION

We have described the endoscopic diagnosis of pancreatic cystic tumors. While the diagnosis of benign and malignant cysts is especially important, the diagnostic performance of endoscopy is still insufficient. Further advances, mainly in EUS technology are thus awaited in the future.

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2016 Pancreatic Cancer: Global view

Advancement in treatment and diagnosis of pancreatic cancer with radiopharmaceuticals

Yu-Ping Xu, Min Yang

Yu-Ping Xu, Min Yang, Key Laboratory of Nuclear Medicine, Ministry of Health, Jiangsu Key Laboratory of Molecular Nuclear Medicine, Jiangsu Institute of Nuclear Medicine, Wuxi 214063, Jiangsu Province, China

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Correspondence to: Min Yang, Professor, Key Laboratory of Nuclear Medicine, Ministry of Health, Jiangsu Key Laboratory of Molecular Nuclear Medicine, Jiangsu Institute of Nuclear Medicine, 20 Qianrong Road, Wuxi 214063, Jiangsu Province, China. yangmin@jsinm.org
Telephone: +86-510-85508862
Fax: +86-510-85513113

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Abstract

Pancreatic cancer (PC) is a major health problem. Conventional imaging modalities show limited accuracy for reliable assessment of the tumor. Recent researches suggest that molecular imaging techniques with tracers provide more biologically relevant information and are benefit for the diagnosis of the cancer. In addition, radiopharmaceuticals also play more important roles in treatment of the disease. This review summaries the advancement of the radiolabeled compounds in the theranostics of PC.

Key words: Pancreatic cancer; Diagnosis; Therapy; Radiopharmaceuticals; Positron emission tomography

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Core tip: This review describes the development of radiopharmaceuticals in diagnosis and therapy of pancreatic cancer. We herein discuss the role of the radiolabeled compounds in the preoperative diagnosis, staging, post-therapeutic monitoring, prognosis and the treatment of the disease.

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INTRODUCTION

Pancreatic cancer (PC) is a major health problem due to low 5-year survival rate^[1-3]. Surgery is the only curative treatment but less than 20% of cases are suitable to

be respectable during diagnosis for the late onset of the symptoms^[4-6]. Therefore, suitable diagnosis and staging is essential for management of the disease.

Computed tomography (CT), magnetic resonance imaging (MRI), and endoscopic ultrasound (EUS), *etc.*, provide information regarding tumor size, location, and morphology, which can be used for initial staging, tumor evaluation and follow-up. However, it also remain suboptimal in the preoperative diagnosis and may hamper the treatment. The discrimination between benign and malignant lesions are still challenging with these methods^[7,8].

Molecular imaging techniques are important tool capable of providing high sensitive non invasive and quantitative images of various cancer^[9-11]. Radiopharmaceuticals is a key factor in the non-invasive molecular imaging technique which enables specific cellular and molecular processes to be functionally visualized. The development of molecular imaging agents target for specific biomarkers could provide more sensitive and specific cancer detection.

Meanwhile, a number of compounds labeled with therapy radionuclides have been employed for cancer treatment through intratumoral administration^[12-15]. Compared with traditional high-dose external radiation, intratumoral administration delivers more radioactivity to the tumor than the normal structure^[16].

Here, we review the pertinent literatures and the advancement in treatment and diagnosis of PC with radiopharmaceuticals was discussed.

SMALL MOLECULE TRACERS FOR TUMOR IMAGING

¹⁸F-fluorodeoxyglucose

Over the past decade, positron emission tomography (PET) is an important molecular imaging methods in various malignancies^[17-20]. ¹⁸F-fluorodeoxyglucose (¹⁸F-FDG) is an analogue of glucose. After injected into the body, it is actively transported *via* glucose transporters (GLUT) into cells, then phosphorylated by hexokinase in the same pathway as glucose. However, unlike normal glucose, the reactions of ¹⁸F-FDG do not proceed further and the corresponding product remains in the cells^[21,22]. Overexpression of GLUT-1 and hexokinase-II has been reported in PC^[23]. In patients with PC, several studies have demonstrated that ¹⁸F-FDG PET/CT was an important key factor for in staging, detecting postoperative recurrence, and evaluating the response to treatment^[24-28]. The recent typical researches and interest findings were listed in the follow.

Preoperative diagnosis: Ergul *et al.*^[29] compared the values of ¹⁸F-FDG PET/CT, multidetector row computed tomography (MDCT), MRI and EUS in the diagnosis and management of the tumor. It revealed that sensitivity of PET/CT were equal to EUS (100%) and higher than those of MDCT and MRI. Meanwhile, Specificity of MDCT

was significantly lower than PET/CT. It suggested that ¹⁸F-FDG PET/CT is an useful imaging techniques for management of the disease^[29].

Maximum standardized uptake value (SUVmax) reflects tumor aggressiveness as a marker of tumor glucose metabolism. Hu *et al.*^[30] found that the SUVmax of benign lesions significantly lower than that of malignant tumors (2.9 ± 2.0 vs 6.3 ± 2.4 respectively). A positive correlation between the SUVmax and Ki-67 was existed. It suggested that the SUVmax of ¹⁸F-FDG can be applied in the differential diagnosis and can also benefit for monitoring the proliferative status of PC^[30].

Nagamachi *et al.*^[31] compared ¹⁸F-FDG PET/CT and ¹⁸F-FDG PET/MRI fusion image in diagnosing tumor. ¹⁸F-FDG PET/MRI fusion image significantly improved accuracy. Results showed that this image technique was useful in differentiating diagnosis^[31].

Zhang *et al.*^[32] reviewed 116 patients with pancreatic cystic tumors who had been treated with different imaging modalities. Compared with CT and EUS, PET had the best sensitivity, specificity and accuracy for detecting malignant cystic tumors^[32].

When the conventional imaging modalities or biopsies are unavailable, PET also plays an important role in diagnosis of PC. Based on the ¹⁸F-FDG uptake pattern, sensitivity, specificity, positive predictive value, negative predictive value, and accuracy for FDG-PET/CT in differentiating benign and malignant lesions were all greater than 85% respectively^[33].

Diagnostic performance of diffusion-weighted MRI and ¹⁸F-FDG PET/CT in the detection of pancreatic malignancy was also obtained by Wu *et al.*^[34]. When diagnosing patients with pancreatic malignancy, the sensitivity of PET/CT was higher than MRI but the specificity of the former was lower than the latter^[34].

Staging: Wang *et al.*^[35] evaluate the value of ¹⁸F-FDG PET/CT on the pre-operative staging of the disease. The sensitivity and accuracy of the imaging modality to detect distant metastasis especially metastatic lymph nodes are significantly higher than those of MDCT. It showed that the extra staging information PET/CT provided could be helpful for screen of surgery^[35].

¹⁸F-FDG PET/CT scans were performed at 17 patients in baseline and six weeks post-CRT. SUVmax significantly decreased during CRT (median pre- 8.0 and post- 3.6). It revealed that the baseline ¹⁸F-FDG PET was benefit for definition of the biological target volume for non-uniform dose prescriptions^[36].

Topkan *et al.*^[37] evaluated the impact of ¹⁸F-FDG PET/CT restaging on management decisions and outcomes in patients with LAPC scheduled for concurrent CRT. According with PET/CT before therapy, these individuals were classified into non-metastatic (M0) and metastatic (M1) groups then received different treatment. Twenty-six point eight percent of distant metastases were detected *via* PET/CT not by conventional staging. Three additional regional lymph nodes were found by PET/CT restaging and the volumes of the tumors were larger

than CT-defined borders. The initial management decisions of 26 patients were changed through PET/CT.

Median overall survival (OS) and progression-free survival (PFS) of M0 patients were greater than those of M1 patients. These findings conformed that PET/CT-based restaging may benefit for screening patients suitable for CRT^[37].

Post-therapeutic monitoring: Picchio *et al.*^[38] evaluated the role of ¹⁸F-FDG PET/CT in screening patients with locally advanced PC for suitable treatment and monitoring the efficacy. Results showed that PET/CT play more important factors in designing the treatment plans for individual patient than conventional CT^[38].

Kittaka *et al.*^[39] performed ¹⁸F-FDG PET in patients classified as responders and nonresponders before and after preoperative CRT. A pre-CRT SUV > 4.7 was seen in 15 (71%) of 21 responders and in 6 (32%) of 19 nonresponders. A regression index > 0.46 was observed in 15 (71%) responders and 5 (26%) nonresponders. It showed that the SUV based on FDG-PET/CT is a useful implement for predicting the response of treatment^[39].

To study whether FDG-PET parameters can predict relatively long-term survival in patients, Chang *et al.*^[40] assess the effect of coregistered ¹⁸F-FDG PET in monitoring radiographically occult distant metastasis (DM) in patients with LAPC. Patients with a baseline standardized uptake value (SUV) < 3.5 and/or SUV decline ≥ 60% had significantly better OS and PFS than those having none, even after adjustment for all potential confounding variables. ¹⁸F-FDG PET can spare one-third of patients with occult DM from the potentially toxic therapy. ¹⁸F-FDG PET parameters including baseline SUV and SUV changes may serve as useful clinical markers for predicting the prognosis in LAPC patients^[40].

Prognosis: Several prognostic factors for PC recurrence have previously been reported including tumor size, T stage, lymph node metastasis, tumor differentiation, lymphovascular invasion, involvement of the surgical margin, and serum carbohydrate antigen 19-9 (CA19-9) level. Yamamoto *et al.*^[41] evaluated whether preoperative ¹⁸F-FDG PET can predict the resectable PC. Among the patients, 34 cases with an SUVmax ≥ 6.0 developed recurrence within half year, however only 3 patients with an SUVmax < 6.0 exhibited early recurrence. The median OS time of patients with a SUVmax < 6.0 was significantly greater than those of patients with an SUVmax ≥ 6.0. Therefore, an SUVmax ≥ 6.0 maybe a significant predictor of recurrence of PC^[41].

The histopathological grade of differentiation is also one of the significant prognostic factors in the disease, especially in the patients with unresectable PC. It was found that a significant correlation of SUVs and pathologic grades existed by ¹⁸F-FDG PET scans in 102 patients with histologically proven pancreas adenocarcinoma. It showed that ¹⁸F-FDG SUV is related with histologic grade and might be competitive predictor for survival^[42].

Xi *et al.*^[43] determined ¹⁸F-FDG SUVmax in patients

with PC at 1 h and 2 h post injection, and the retention index (RI) was defined as the percentage change between the values of two time points. It was found that there existed a significant positive correlation among RI and the tumor, node, and metastasis stage^[43].

Shinoto *et al.*^[44] evaluated whether ¹⁸F-FDG PET can be used as an indicator of preoperative carbon-ion radiotherapy (CIRT) for PC patients. SUVmax was significantly correlated with DMFS and OS. The DMFS and OS in high-SUVmax group were significantly lower than those in low SUVmax group. ¹⁸F-FDG PET might be suitable for determining the indication of preoperative short-course CIRT for patients with resectable PC^[44].

The prognostic role of ¹⁸F-FDG PET/CT in the prediction of PFS and chemotherapeutic response in patients with locally advanced or metastatic PC was also investigated by Moon *et al.*^[45] PFS of the low SUVmax (< 6.8) group was significantly longer than those of the high SUVmax (≥ 6.8) group. Resulted showed that SUVmax may be useful in independent predicting PFS of PC^[45].

The prognostic value of volumetric parameters on preoperative ¹⁸F-FDG PET/CT was assessed. Results revealed that metabolic tumor volume and total lesion glycolysis are independent prognostic factors for predicting RFS and OS. Thus, ¹⁸F-FDG PET/CT can provide useful prognostic information for patients undergoing resection of PC with curative intent irrespective of neoadjuvant treatment^[46].

Choi *et al.*^[47] evaluated the prognostic value of ¹⁸F-FDG PET in patients with resectable PC. The OS and DFS were significantly longer in the low SUVmax group than those of high SUVmax group^[47].

Hwang *et al.*^[48] reviewed retrospectively the medical records of 165 patients with a diagnosis of PC. Patients were allocated to high (> 4.1) and low (≤ 4.1) SUV groups, and median survivals of these patients were 229 d and 610 d, respectively. Furthermore, SUVmax was found to be significantly related to survival in each stage. The median survival was also found to be significantly related to tumor size, site, serum level of CA19-9, distant metastasis, and type of treatment^[48].

Epelbaum *et al.*^[49] evaluated the possibility of dynamic ¹⁸F-FDG PET/CT parameters used as an indicator in the tumor. The OS of patients with a high ¹⁸F-FDG influx was significantly lower than that of patients with a low ¹⁸F-FDG influx (5 and 6 mo vs 15 and 19 mo respectively). Quantitative ¹⁸F-FDG kinetic parameters in newly diagnosed PC correlated with the aggressiveness of disease^[49].

Limitation: Although significant advances have been achieved in ¹⁸F-FDG PET diagnostic technologies, it has some limitations in detecting cancer. Due to increased glycolytic metabolism, ¹⁸F-FDG can also accumulate in the inflammatory cells^[50]. As a result, it often yields false positive interpretations for PET. Kato *et al.*^[51] evaluated the efficacy of ¹⁸F-FDG PET/CT for the differential diagnosis in 47 individuals. It showed that differentiation is difficult by ¹⁸F-FDG PET/CT due to overlapping in

SUVmax between the two diseases. In addition, elevated serum glucose levels may decrease the uptake in tumors for competitive inhibition, which decreased the sensitivity of ^{18}F -FDG PET in hyperglycemic patients^[51]. Therefore, a numbers of other small molecule-based tracers were designed and developed for PET imaging of PC.

3-Deoxy-3-18F-fluorothymidine

A surrogate marker of DNA synthesis, 3-Deoxy-3-18F-fluorothymidine (^{18}F -FLT), is another potential tracer for visualization of proliferating tissues^[52-55]. For differentiation of pancreatic tumors, ^{18}F -FLT PET showed a lower sensitivity but higher specificity than ^{18}F -FDG PET/CT (70% vs 91% and 75% vs 50% respectively)^[56].

RADIOLABELED PEPTIDES FOR PC IMAGING

Peptides and their derivatives have been successfully developed for the tracer due to favorable characteristics such as low antigenicity, high specificity, fast clearance from blood and rapid tissue penetration. Radiolabelled receptor-binding peptides have become important radiopharmaceuticals for diagnosis and therapy in tumor^[57-61]. Recently, a few radiolabeled peptides have been successfully used for PC imaging. It may be a promising imaging strategy for PC diagnosis and treatment.

Radiolabeled RGD analogs

Angiogenesis is necessary for tumor growth and metastasis, and the integrin $\alpha\text{v}\beta 3$ receptor plays an important role in promoting, sustaining, and regulating the angiogenesis^[62]. *In vitro* analysis demonstrated that integrin $\alpha\text{v}\beta 3$ receptor was expressed in 60% of invasive pancreatic ductal carcinomas and would be an excellent target for the early detection of malignant PC^[63]. Radiolabeled Arg-Gly-Asp (RGD) peptides are widely used as integrin $\alpha\text{v}\beta 3$ receptor imaging agents in various types of tumors^[63]. Yoshimoto *et al*^[64] employed ^{111}In -DOTA-c(RGDfK) for the early detection of PC in pancreatic carcinogenesis model. PC lesions as small as 3 mm in diameter as clearly were visualized after injection with the tracer. High tumor-to-normal pancreatic tissue radioactivity ratios were found by ARG analysis. There existed a significant relationship between the uptake of ^{111}In -DOTA-c(RGDfK) and $\alpha\text{v}\beta 3$ -integrin expression. It also found that the false-positive rate of ^{111}In -DOTA-c(RGDfK) was lower than that of ^{18}F -FDG. It revealed that SPECT with ^{111}In -DOTA-c(RGDfK) was benefit for the early accurate diagnosis of PC^[64].

Trajkovic-Arsic *et al*^[65] used ^{68}Ga -NODAGA-RGD PET for $\alpha\text{v}\beta 3$ integrin receptor *in vivo* imaging of spontaneous pancreatic ductal adenocarcinoma (PDAC) occurring in mice. It showed that $\alpha\text{v}\beta 3$ integrin is expressed in human and murine PDAC and can be detected by molecular imaging technologies in PDAC. This strategy can further be exploited for identification of patients with $\alpha\text{v}\beta 3$ integrin positive and application of $\alpha\text{v}\beta 3$ targeted

therapies^[65].

Aung *et al*^[66] performed a preclinical evaluation of ^{64}Cu -RAFT-RGD in a clinically relevant orthotopic xenotransplantation model of PC. It was confirmed that the uptakes of ^{64}Cu -RAFT-RGD in tumor was greater than those of normal tissues. Meanwhile, the tumor to background uptake ratios of the tracers was higher than those of ^{18}F -FDG. It suggested that ^{64}Cu -RAFT-RGD PET imaging might be useful in the diagnosis of PC^[66].

Radiolabeled exendin-4 analogs

Insulinomas are the most frequent hormone-active tumors of the pancreas arising from pancreatic β cells^[67-69]. Recently, glucagon-like peptide-1 receptor (GLP-1R) was found to be massively overexpressed in gut and lung neuroendocrine tumors, especially insulinomas. It provides an attractive target for the cancers^[70-72].

Several radioligands towards GLP-1 receptor have been developed for GLP-1R-positive tumor imaging. At first, the analog of native receptor ligand, GLP-1(7-36) amide, was labeled with ^{123}I and used for GLP-1R imaging. Although preclinical data showed ^{123}I -GLP-1(7-36) amide possessed high accumulation in a RINm5F insulinoma tumor, the low stability of the peptide due to rapid degrading of GLP-1 by the enzyme dipeptidyl peptidase IV (DPIV) limited its clinical use^[73].

Exendin-4 arised from the salivary gland of the gila monster lizard and has a 53% amino acid homology with GLP-1. It is more resistant to the DPIV digestion and binds with great affinity to the GLP-1R^[73]. ^{111}In - and $^{99\text{m}}\text{Tc}$ -labeled exendin-4 analogs have been evaluated for SPECT imaging of GLP-1R in rodents and humans, respectively, and promising results were obtained^[74-77].

The sensitivity, imaging contrast and spatial resolution of PET was significantly higher than SPECT. In the past few years, exendin-4 analogs have been labeled with PET radionuclides for preclinical insulinomas imaging. Exendin-4 labeled with radio metals (^{68}Ga , ^{64}Cu) showed significant uptake in INS-1 insulinoma xenografts^[78,79]. However, the substantial kidney uptake may limit their use in clinical practice due to high radiation exposure to the organs.

^{18}F is the commonly used isotope. It has nearly optimal nuclear decay characteristics and chemical properties for peptide-based receptor imaging studies. In the past few years, exendin-4 analogs have been modified with either a C-terminal or N-terminal cysteine to allow site-specific labeling with a maleimide-selective prosthetic reagent, ^{18}F -FBEM^[80]. *In vivo* study showed that the INS-1 tumor uptake of ^{18}F -FBEM-Cys⁴⁰-exendin-4 was higher than that of ^{18}F -FBEM-Cys⁰-exendin-4^[80]. Based on the above results, other Cys⁴⁰-exendin-4 analogs were developed for GLP-1R imaging^[81,82].

In vitro receptor competitive binding study confirmed that the nine amino acid sequence at C-terminal of exendin-4 was not key for the biological activity or binding to the receptor. Meanwhile, serine is almost same as cysteine except for the difference in hydroxy and sulfhydryl group. Thus, replacing Ser³⁹ with Cys³⁹ could

provide a unique site for attachment of a radiolabeling thiol-reactive group (such as ^{18}F -FBEM) and may have less impact on the binding affinity of the peptide to the receptor^[83]. Xu *et al.*^[83] synthesized a novel ^{18}F -labeled exendin-4 analog, ^{18}F -FBEM-Cys³⁹-exendin-4. The tracer showed specific binding to GLP-1R and had better tumor to background radioactivity ratio and lower abdominal backgrounds than those of ^{18}F -FBEM-Cys⁴⁰-exendin-4^[83]. It suggested that ^{18}F -FBEM-Cys³⁹-exendin-4 may be a potential probe for insulinomas imaging^[83].

Despite the encouraging results, the tedious radio-synthesis would hinder the tracer to widespread use. Recently, a one-step simple procedure for preparing ^{18}F -labeled peptides *via* chelating ^{18}F FAI with NOTA has been reported^[84]. Xu *et al.*^[84] conjugated Cys³⁹-exendin-4 with NOTA-MAL and obtained NOTA-MAL-Cys³⁹-exendin-4. The compound was simply radiolabeled with ^{18}F FAI complex by one step in 30 min^[85]. ^{18}F FAI-NOTA-MAL-Cys³⁹-exendin-4 shows favorable characteristics for insulinoma imaging in mice bearing INS-1 tumor and may be translated to clinical studies^[85].

THERAPY WITH RADIOPHARMACEUTICALS

Recently, only few patients have resectable disease. High-dose external radiation to the pancreas may damage the surrounding organs. The intratumoral administration of radiopharmaceuticals delivers the maximum amount of radioactivity to the tumor with limiting side effects^[86-88].

During the past several decades, implantation of radioactive isotopes for the treatment has been used. Some basic research indicated that ^{125}I seed with continuous low dose rate irradiation may be beneficial to PC^[86-88]. Zhongmin *et al.*^[89] implanted ^{125}I seeds into PC under CT guidance in thirty-one patients with inoperable PC. It was found that overall responding rate was greater than 60% and median survival time was about 10 mo^[89]. The efficacy of intraoperative ultrasound-guided implantation of ^{125}I seeds was also assessed for the treatment of unresectable PC by Wang *et al.*^[90]. Most of the patients achieved favorable pain relief. These studies revealed that ^{125}I seeds implantation was benefit for the treatment of PC patients^[90].

Phosphorus 32 is another ideal unsealed therapeutic radionuclide. Colloid ^{32}P has been applied for the treatment of intracavitary malignancies^[91-93]. Preclinical study showed that ^{32}P -chromic phosphate colloid (^{32}P -CP) through intratumoral injection mainly accumulated in the BxPC-3 human tumor and retained for a long time^[94]. The safety and efficacy of the therapy to PC was also confirmed^[94].

Poly (L-lactic acid) (PLLA) has been widely used as a drug delivery system due to excellent biocompatibility and biodegradability^[95-99]. ^{32}P -CP-PLLA microparticle was successfully prepared and used for brachytherapy in several tumor models^[95-99]. Yang *et al.*^[100] evaluated

its biodistribution, bioelimination, and therapeutic effect in mice bearing BxPC-3 human PC. Results showed that ^{32}P -CP-PLLA was mostly remained at the tumor (> 95% ID) and almost no radioactivity excretion was observed in urine and feces. As compared, some radioactivity (over 5% ID) of ^{32}P -CP colloid was found in the normal organs^[100]. Meanwhile, the tumor volumes was significantly decreased after treatment with ^{32}P -CP-PLLA microparticle^[100]. It showed that ^{32}P -CP-PLLA microparticle might be benefit for the management of PC^[100].

CONCLUSION

Radiopharmaceuticals are favorable diagnostic and therapy facility for PC. The development of new tracers may be beneficial to personalized management of the disease.

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Molecularly targeted therapy for advanced hepatocellular carcinoma - a drug development crisis?

Kiruthikah Thillai, Paul Ross, Debashis Sarker

Kiruthikah Thillai, Paul Ross, Debashis Sarker, Department of Medical Oncology, Guy's and St Thomas NHS Trust, London SE1 9RT, United Kingdom

Kiruthikah Thillai, Debashis Sarker, Division of Cancer Studies, King's College London, Guy's Hospital, London SE1 9RT, United Kingdom

Paul Ross, Debashis Sarker, Institute of Liver Studies, King's College Hospital NHS Trust, London SE5 9RS, United Kingdom

Author contributions: All authors equally contributed to this paper with conception and design of the manuscript, literature review and analysis, drafting and critical revision and editing, and final approval of the final version.

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Correspondence to: Dr. Debashis Sarker, Division of Cancer Studies, King's College London, Guy's Hospital Campus, Great Maze Pond, London Bridge, London SE1 9RT, United Kingdom. debashis.sarker@kcl.ac.uk
Telephone: +44-20-71884260
Fax: +44-20-71880919

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Abstract

Hepatocellular carcinoma is the fastest growing cause of cancer related death globally. Sorafenib, a multi-targeted kinase inhibitor, is the only drug proven to improve outcomes in patients with advanced disease offering modest survival benefit. Although comprehensive genomic mapping has improved understanding of the genetic aberrations in hepatocellular cancer (HCC), this knowledge has not yet impacted clinical care. The last few years have seen the failure of several first and second line phase III clinical trials of novel molecularly targeted therapies, warranting a change in the way new therapies are investigated in HCC. Potential reasons for these failures include clinical and molecular heterogeneity, trial design and a lack of biomarkers. This review discusses the current crisis in HCC drug development and how we should learn from recent trial failures to develop a more effective personalised treatment paradigm for patients with HCC.

Key words: Hepatocellular carcinoma; Molecular targets; Genomics; Sorafenib; Tyrosine kinase inhibitors

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Core tip: This review discusses the current drug therapy landscape for advanced hepatocellular carcinoma, in particular the reasons for failure of several clinical trials of molecularly targeted therapy and future directions of research to address these problems.

Thillai K, Ross P, Sarker D. Molecularly targeted therapy for advanced hepatocellular carcinoma - a drug development crisis?

INTRODUCTION

Hepatocellular cancer (HCC) is the sixth most prevalent cancer worldwide and accounts for over 745000 deaths a year^[1]. Despite the implementation of screening programs for high-risk individuals, the majority of patients present with incurable disease. Median overall survival for advanced disease remains poor at less than 12 mo and there is an urgent need for more effective treatments^[2]. Global epidemiological patterns vary depending on the prevalence of risk factor. Incidence rates are highest in East Asia in areas where hepatitis B and C are endemic^[3]. However, improved management of early viral hepatitis in Japan has seen a reduction in new HCC cases^[4]. By contrast the upward trends of HCV, obesity and metabolic syndrome in North America and Europe contribute to HCC being the fastest growing cause of cancer related mortality in these regions^[5]. Resection, radiofrequency or microwave ablation, and liver transplantation comprise the mainstay of treatment for early disease offering the only chance of cure, but only one third of patients present with disease suitable for these treatments^[6]. Loco-regional therapy with trans-arterial chemoembolization (TACE) can lead to sustained disease control for intermediate stage HCC^[7,8]. Sorafenib, a multi-targeted tyrosine kinase inhibitor (TKI), remains the only systemic therapy that is effective in advanced disease offering marginal survival benefit without significant improvement in cancer related symptoms or quality of life^[2]. After many years of disappointing results with chemotherapy, sorafenib was thought to herald a new era in HCC treatment with great optimism for molecularly targeted therapies. Disappointingly, several negative first and second line phase III clinical trials ensued. However, the combination of recent extensive genomic studies and biomarker based clinical trials, provide hope for the development of a more personalised treatment paradigm. This review discusses the current concepts and management of advanced HCC with a particular focus on the failure of molecular targeted therapy beyond sorafenib and outlines how this should be addressed.

Current therapy for advanced disease

Despite only marginal benefits with chemotherapy reported in single arm studies, lack of alternative treatments meant its use was routine prior to the advent of sorafenib. Challenges with toxicities (especially in patients with underlying liver disease) led to chemotherapy being reserved for patients with good performance status and preserved hepatic function. Single agents such as doxorubicin, cisplatin and fluorouracil offer response rates of 10%^[9-11]. This increases to 20% with combination regimens, none of which impact survival^[9,12]. The recently

reported EACH trial, a phase III study conducted in China, Taiwan, Korea and Thailand randomly assigned 371 patients with advanced disease to receive either combined oxaliplatin and fluorouracil/leucovorin (FOLFOX4) or doxorubicin^[13]. The trial failed to demonstrate a significant survival difference between each arm, although a trend towards improved outcomes with FOLFOX4 was noted (median overall survival was 6.4 mo for FOLFOX4 and 4.97 mo for doxorubicin; $P = 0.7$; HR = 0.8; 95%CI: 0.63-1.02).

The search for more efficacious treatments eventually led to two large randomised phase III trials that reported a significant survival benefit with sorafenib in close succession. The first, conducted in a European, Australian and American population, demonstrated a median overall survival (OS) of 10.7 mo for patients treated with sorafenib (400 mg BD) compared with 7.9 mo for placebo (HR = 0.69; 95%CI: 0.55-0.87; $P < 0.001$)^[2]. The latter, conducted in the Asian-Pacific region reported that patients treated with sorafenib led to a median overall survival of 6.5 mo compared with 4.2 mo (HR = 0.68; 95%CI: 0.50-0.93; $P = 0.014$)^[14]. The survival advantages in both trials were modest and neither study established any improvement in cancer symptoms or quality of life. Yet this benefit was sufficient for sorafenib to become the new standard of care for patients with advanced disease. Data extracted from the prospectively maintained GIDEON database (Global Investigation of Therapeutic Decisions in Hepatocellular Carcinoma and of its Treatment with Sorafenib) showed that in 3202 patients treated with HCC, adverse events were comparable between patients with Child-Pugh A and Child-Pugh B cirrhosis^[15]. Yet the frequency of serious adverse events was higher in the Child-Pugh B group (60.4% for Child-Pugh B and 36.0% for Child-Pugh A) and median overall survival was shorter 5.2 mo (4.6-6.3) for Child-Pugh B and 13.6 mo (12.8-14.7) for Child-Pugh A (Table 1).

Four separate phase III trials exploring different multi-targeted TKIs have now failed to show superior outcomes to sorafenib. HCCs are vascular tumours and both VEGF and angiopoietin-2 (Ang2) were independent prognostic markers during the SHARP trial and have been associated with tumour growth and metastatic spread^[16]. The success of sorafenib was thought to be predominantly related to its anti-angiogenic properties and subsequent studies aimed to identify more potent anti-angiogenic drugs. Sunitinib, a multi-kinase inhibitor targeting VEGFR, PDGFR, c-KIT and FLT-3 has been approved for use in gastro-intestinal stromal tumours and renal cell carcinomas and was more potent than sorafenib in preclinical models^[17,18]. Phase II studies showed modest benefit in HCC at best although did highlight potential biomarkers such as interleukin-6, stromal-derived factor1alpha and soluble c-KIT, as changes in tumour vascular permeability and circulating inflammatory molecules were associated with poorer outcome^[19-21]. Adverse events in these phase II studies were concerning with liver related toxicities including encephalopathy and hepato-renal syndrome and 5%-10%

Table 1 First line trials with molecular targeted therapies in advanced hepatocellular cancer

Trial	Drugs	Design	n	Median survival	HR	P value	Ref.
ASIA-PACIFIC	Sorafenib <i>vs</i> placebo	Superiority	150	6.5	0.68	0.01	[14]
			76	4.2			
SHARP	Sorafenib <i>vs</i> placebo	Superiority	229	10.7	0.69	0.001	[2]
			303	7.9			
SUNITINIB	Sunitinib <i>vs</i> sorafenib	Superiority	530	7.9	1.3	0.001	[22]
			544	10.2			
BRISK-FL	Brivanib <i>vs</i> sorafenib	Non-inferiority	577	9.5	1.06	0.31	[28]
			578	9.9			
LIGHT	Linifanib <i>vs</i> sorafenib	Non-inferiority	514	9.1	1.04	0.52	[24]
			521	9.8			
SEARCH	Sorafenib/erlotinib <i>vs</i> sorafenib/placebo	Superiority	362	9.5	0.92	0.48	[29]
			358	8.5			

of patients died from treatment related causes. The daily dose of 50 mg that is routinely used in other tumour types was deemed too high for patients with HCC where it precipitated liver toxicities including portal hypertension, encephalopathy, oesophageal variceal bleeding, ascites and thrombocytopenia. A subsequent head-to-head phase III study of 1074 patients randomised to either sunitinib or sorafenib patients terminated early due to both futility and safety concerns^[22]. The most frequent grade 3/4 adverse events in the sunitinib group were thrombocytopenia (29.7%) and neutropenia (25.7%) and in the sorafenib group were hand-foot syndrome (21.2%). Overall survival was also significantly lower in the sunitinib arm (7.9 mo *vs* 10.2 mo $P = 0.0014$). Temporary treatment discontinuation was more frequent with sunitinib (76.6% *vs* 58.7%). The failure of sunitinib was likely related to a combination of inadequate dosing, toxicities and trial design, and highlights the need for caution in over-interpretation of phase II data and decision to move to Phase III trials.

Pre-clinical studies identified linifanib as a more potent dual vascular epidermal growth factor receptor (VEGFR) and platelet derived growth factor receptor (PDGFR) inhibitor than sorafenib ($IC_{50} = 25$ nmol for linifanib and $IC_{50} = 57$ nmol sorafenib) and VEGFR ($IC_{50} = 8$ nmol for linifanib and $IC_{50} = 90$ nmmol for sorafenib)^[23]. A single arm phase II trial in the first line setting resulted in a median overall survival of 9.7 mo (10.4 mo in patients with Child-Pugh-A status), which led to a non-inferiority phase III trial with sorafenib^[24]. The study of 1035 patients failed to reach its end-point with an overall survival of 9.1 mo for linifanib and 9.8 mo for sorafenib (HR = 1.04; 95%CI: 0.89-1.22; $P = 0.001$)^[25]. Toxicities of hypertension and hepatic toxicities including encephalopathy were also higher in the linifanib arm.

A single arm first line phase II study of 55 patients treated with brivanib, an ATP competitive inhibitor of several kinases including VEGFR2 ($IC_{50} = 25$ nmol), FGFR-1 (148 nm) and VEGFR1 (380 nmol), resulted in a median overall survival of 10.0 mo^[26,27]. Phase II studies confirmed that brivanib was well tolerated and one patient had a completed response, three had a partial response and twenty-two had stable disease. Yet BRISK-FL, the subsequent phase III direct comparison

trial of brivanib and sorafenib, failed to establish a significant survival benefit (9.5 mo for brivanib *vs* 9.9 mo for sorafenib; HR = 1.06; $P = 0.31$)^[28]. Due to the trial design, in order to demonstrate non-inferiority, brivanib needed to produce a hazard ratio between 1 and 1.08, which it narrowly failed to reach. The BRISK-FL trial highlighted the difficulties in extracting comprehensive survival data from non-randomised phase II trials. Grade 3/4 toxicities for sorafenib and brivanib were hyponatraemia (9% and 23% respectively), elevated liver enzymes (17% and 14%), fatigue (7% and 15%) and hand-foot reaction (15% and 2%). Even if this trial had met its end-point of non-inferiority, the significant toxicity and economic profiles were not more favourable than sorafenib, and thus would have been of little meaningful clinical benefit.

Erlotinib, an epidermal growth factor receptor (EGFR) TKI was tested in a first-line phase III trial in combination with sorafenib compared to placebo/sorafenib in a study of 720 patients with advanced disease^[29]. The combination had not previously been tested in phase II trials, with two single arm phase II studies demonstrating modest disease control^[29-31]. The combined treatment did not improve overall survival (9.5 mo compared with 8.5 mo for sorafenib alone HR = 0.92; $P = 0.2$). Toxicities in the combination arm were also higher resulting in a reduced median treatment duration that may have contributed to its diminished efficacy. This trial demonstrates both the danger of proceeding to large-scale phase III trials without a clear signal of efficacy from earlier phase studies and the difficulties in combining therapies for HCC (especially for drugs that have overlapping toxicities). Robust HCC-specific phase I / II studies are needed to identify optimal dosing of combination regimens (Table 2).

FGF has been pursued as a potential target in HCC and recent data suggests the FGF signalling pathway may play a key role in the development of resistance to anti-VEGF therapies by activating alternative proangiogenic signalling pathways^[32]. Forty-six patients who had not responded to prior anti-angiogenic therapies were treated with brivanib in a single arm phase II study^[33]. The results were promising with a median overall survival of 9.7 mo. A subsequent phase III trial that was conducted in parallel to the BRISK-FL trial compared brivanib with placebo

Table 2 Second line trials with molecular targeted therapies in advanced hepatocellular cancer

Trial	Drugs	Design	n	Median survival	HR	P value
BRISK-PS	Brivanib <i>vs</i> placebo	Superiority	263	9.4	0.89	0.33
			132	8.2		
EVOLVE-1	Everolimus <i>vs</i> placebo	Superiority	362	7.6	1.05	0.68
			184	7.3		
REACH	Ramucirumab <i>vs</i> placebo	Superiority	277	9.2	0.87	0.14
			276	7.6		

as second line treatment failed to meet its end point^[34]. Patients treated with brivanib had a median overall survival of 9.7 mo compared with 8.2 mo in the placebo arm ($P = 0.3$). Yet significant improvements were seen in the secondary end points of overall response rate (10% for brivanib *vs* 2% for placebo $P = 0.003$), disease control rate (61% *vs* 40% $P \leq 0.001$) and alpha-feto protein reduction in 74% of patients with elevated baseline levels ($> 50\%$ reduction seen in 54% *vs* 7%). These indicate that brivanib has anti-tumour activity despite the negative primary outcome. Furthermore, despite stratification the placebo cohort had fewer patients with macro-vessel invasion and a numerically lower median AFP level. The unexpectedly long survival of patients in the placebo cohort has been cited as one of the reasons for treatment failure. As expected, there were also higher rates of treatment discontinuation and elective patient withdrawal from the brivanib arm, which may have reduced efficacy in this group.

Mammalian target of rapamycin (mTOR) is upregulated in many solid tumours including HCC and appears to have a critical role in pathogenesis^[35,36]. A second line study with the mTOR inhibitor everolimus, offered no survival advantage over placebo (7.6 mo for everolimus *vs* 7.3 mo; HR = 1.05; $P = 0.68$)^[37]. Ramucirumab is a fully human monoclonal antibody against vascular endothelial growth factor receptor 2 (VEGFR2), which also failed to improve survival compared with placebo (median overall survival for ramucirumab was 9.2 mo compared with 7.6 mo; HR = 0.86, $P = 0.13$) in the REACH trial^[38]. However, a pre-planned sub-group analysis revealed that in patients with elevated baseline alpha-feto protein (AFP) of more than 400 ng/mL, ramucirumab extended both overall and progression free survival. Grade 3 toxicities that occurred more frequently in the ramucirumab arm included hypertension (12% compared with 4%) and fatigue (5% compared with 2%), but its toxicity profile is otherwise favourable compared to the multi-targeted TKIs. Due to this data, a phase III trial with second line ramucirumab in a select population with AFP > 400 ng/mL is ongoing.

In the majority of patients with HCC, the cancer arises predominantly as a consequence of liver injury secondary to a variety of causes. It is clear that underlying liver pathology affects both outcome and treatment response, suggesting trials need to be stratified according to aetiology as well as Child-Pugh status, histological grade and stage^[39]. Whilst patients with hepatitis B had longer overall survival and shorter time to progression following treatment with sorafenib in the SHARP trial, these results may have been confounded by the imbalance in numbers between patients with hepatitis B and C^[2]. Without prior stratification, it is difficult to analyse the survival between sub-groups, highlighting the need for careful trial design.

Limited understanding of oncogenic drivers mean all recent negative phase III trials were for “all comers”, yet there is marked molecular heterogeneity amongst HCC tumours. Extensive genomic studies have revealed multiple genetic aberrations with more than 30 somatic mutations per tumour^[40,41]. The challenge lies in distinguishing which are oncogenic drivers and which are bystander passenger mutations. Once drivers are identified, trials can be tailored to pertinent pathways. However, several studies have challenged the idea that single biopsies can represent the mutational landscape of the whole cancer. With highly mutated tumours such as HCC, the key is finding the so-called “trunk” mutations that exist in all tumour sites^[42]. Even if a driver is found, inhibiting pathways may induce resistant mutations. Whilst “liquid” biopsies evaluating circulating DNA are under evaluation, further research is needed to validate these techniques before their use in the clinical setting^[43]. One of the barriers to drug development is that many previous HCC trials did not mandate a tissue diagnosis, relying on clinical criteria alone. Several studies have now highlighted histological changes following treatment with loco-regional therapy such as TACE. In a prospective analysis of 80 nodules found in explant livers following transplantation for HCC, 14 cases of mixed hepatocholangiocellular tumours were found in patients who had received TACE whilst none were seen in the treatment-naïve group, implying differentiation into a cholangiocellular phenotype for some patients^[44]. Furthermore, the lack of histology arguably impedes both predictive and prognostic biomarker development. For example, a phase II trial with the selective non ATP competitive c-MET inhibitor tivantinib, did not offer a survival advantage in patients with advanced HCC but a post study sub-group analysis revealed that the overall survival was longer in patients

REASONS FOR THE FAILURE OF PHASE

III TRIALS

Clinical and molecular heterogeneity

So far all phase III trials have unexpectedly failed to reach their end-points. There are several reasons for this.

with high baseline expression of c-MET (overall survival was 7.2 mo for tivantinib and 3.8 mo for placebo HR = 0.38, $P = 0.01$)^[45]. A phase III trial for patients with tumours over-expressing c-MET in the second line setting is on going (NCT01755767). Therefore, several agents that have failed in phase III trials may still be efficacious in sub-groups of patients, emphasising the urgent need for tissue collection and more sophisticated trial designs that accommodate molecular stratification.

Underlying liver cirrhosis

Another challenge when treating patients with HCC is the presence of underlying liver cirrhosis. Historically, clinical trials were reserved for patients with good hepatic reserve so that competing liver morbidity does not overshadow outcomes from malignancy. Yet even in patients with preserved baseline hepatic function, reaching the optimal maximum tolerated dose in patients can be limited by hepatotoxicity. Treatment duration in these trials may have been insufficient to elicit a response. Liver dysfunction and co-existing cirrhosis may affect drug metabolism and due to the consequent changes in the pharmacokinetic and pharmacodynamics profiles of drugs, there is now a trend to conduct HCC-specific phase trials rather than extrapolate results from "all-comer" phase 1 studies conducted in patients with normal or near normal liver function.

There are no approved therapies in patients who progress on sorafenib and who retain well preserved liver function and good performance status. Many centres use cytotoxic chemotherapy (usually with FOLFOX due to results of the EACH trial) despite the lack of clear evidence supporting its use. Due to the lack of effective second-line therapy, patients are encouraged to enter clinical trials of novel agents. By definition, patients suitable for second line trials are more likely to have less aggressive disease than the wider HCC population in whom performance status often deteriorates rapidly on progression and is associated with decompensation of liver function. In a number of the recent second-line phase III trials comparing novel therapies to placebo, there has been unexpected prolonged survival in the placebo cohort, potentially diminishing the survival differences between groups. Although the trend for overall survival favoured brivanib in the second line BRISK-PS trial, the results were non-significant suggesting the study was not sufficiently powered to detect benefits with brivanib against a placebo controlled population in whom survival was unexpectedly long^[34].

Novel direct-acting antivirals (DAA) that target HCV-encoded proteins necessary for viral replication, can offer patients with hepatitis C sustained virological responses (SVR). The increasing use of these novel agents are expected to have a future impact on the incidence of HCV related HCC. Yet the presence of advanced fibrosis will continue to pose a risk for oncogenesis, even in the absence of a detectable viral load, and screening high risk individuals is still required^[46]. The development

of molecular predictive biomarkers could help identify patients that require ongoing surveillance. Furthermore, biomarker based stratification could be used to enrich HCC chemoprevention trials^[47].

Response evaluation

Finally, response criteria in trials must be chosen carefully. Traditional endpoints such as tumour shrinkage relate to chemotherapy treatments and may not be applicable when assessing the benefits of targeted treatments, which can be cytostatic rather than cytoreductive^[48]. Drugs that have been deemed failures in phase III studies may have therapeutic activity in HCC, but insufficient potency to improve conventional end-points in phase III trials^[49]. Furthermore liver disease can elicit an inflammatory response, which can be mistaken for progression resulting in premature cessation of treatment. Thus the use of traditional imaging has been highlighted as insufficient in assessing response in HCC whereby functional imaging provides more useful information. RECIST criteria that is routinely used to measure disease response in many solid tumours, has been recognised as insensitive in HCC. In the SHARP trial, despite an improvement in overall survival, only 2% of patients treated with sorafenib underwent a response by RECIST criteria. The RECIST response criteria were amended to incorporate tumour necrosis induced by treatment. The modified RECIST (mRECIST) measures arterially enhancing lesions that are more representative of residual viable tumour^[50,51]. Large multi-centre clinical trials in patients with HCC pose unique challenges and future study designs must accommodate these in order to exploit the true potential of novel agents in this disease^[52,53].

THE GENETIC BACKGROUND OF HCC

In malignancies such as melanoma, key driver mutations have now been identified, leading to the use of effective targeted therapy that directly translates to improved patient survival^[54]. Despite the presence of more than 40 somatic mutations, there does not appear to be solitary frequent genetic defects in the majority of HCC tumours^[40,41,55,56]. Polydonality has been noted in patients with HCC reflecting a complex genetic landscape. The recently proposed concept of "trunk vs branch" heterogeneity can be applied to HCC, whereby key mutations that drive tumorigenesis exist in both primary and secondary lesions (trunk) and need to be distinguished from those that are only present in a minority of tissue (branch)^[42]. The question remains as to whether the vast number of genetic alterations in HCC reflect multiple "trunk" mutations that would each require inhibition, or if the majority are mere passenger alterations that do not need treating. Recent advances in high throughput sequencing have uncovered several mechanisms of genetic changes, including somatic mutations, copy number alterations, HBV integration and somatic changes of retrotransposons^[55,57]. Whole genome sequencing of 88 primary HCC tumours with

matched adjacent liver tissue revealed the predominant oncogenic mutation was beta catenin (15.9%) which is mutually exclusive with the most frequently mutated tumour suppressor gene Tp53 (35.2%) echoing results from previous genomic studies^[41,55,58,59]. Further mutations have been found in ARID1 and 2 (both of which regulate chromatin remodelling pathways) and rare mutations in RPS6KA3 which codes for RSK2 (a serine threonine kinase of the MAPK pathway)^[60]. A larger study of 503 HCC liver genomes revealed 30 driver genes implicating 11 core pathways in tumorigenesis. Recurrent focal amplifications were seen in 25% of cases, including telomerase reverse-transcriptase (TERT) and CCND1-FGF19. Key oncogenic pathways included TP53-RB, Wnt and mTOR-PIK3CA^[61]. Frequently altered in HCC, somatic TERT mutations have also been found in pre-cancerous cirrhotic nodules and hepatic adenomas, suggesting they play a pivotal role in malignant transformation. Sequencing of the promoter region of tissue taken from 305 HCCs revealed recurrent TERT mutations in 179 samples (59%) at two common mutually exclusive hot spots^[62]. Yet despite a greater understanding of the role of TERT in HCC, its potential as a druggable target remains unknown. A small early phase II study of a telomerase derived peptide, GV1001, failed to elicit any responses, although the trial was not enriched for TERT mutated tumours^[63].

HCC can be classified into two distinct sub-groups based on genetic aberrations^[64-67]. The proliferative subclass is characterised by activation of RAS, mTOR and IGF signalling and has been associated with poor outcomes. This group can be further divided into those with Wnt/transforming growth factor (TGF)- β activation and the progenitor cell group that have higher progenitor cell, epithelial cell adhesion molecules and type 1 cytoskeletal 19 markers. By comparison, the non-proliferative group is more heterogeneous with less shared mutations. The Wnt/beta catenin and JAK/STAT signalling pathways are the most frequently affected pathways, with alterations in as many as 50%-62.5% and 45% of cases respectively^[66,68,69]. Several distinct protein-altering JAK1 mutations have been identified, the majority of which affect the kinase domain^[55,70]. HCC development is often attributed to chronic inflammation triggered by both viral infection and cell necrosis and the JAK/STAT pathway has been identified as a promoter of carcinogenesis in a sub-set of HCC *via* cytokine-induced JAK/STAT pathway activation^[55,71].

Copy number analyses using array based comparative genomic hybridization (aCGH) have revealed recurrent amplifications in genes for p53, Wnt signalling, proliferation pathways with recurrent deletions of genes involved in the immune response, chromatin remodelling and NF- κ B pathways^[72,73]. Furthermore, the DNA virus hepatitis B (HBV), a leading cause of HCC, integrates into the host genome affecting gene expression. Deep sequencing of HCC samples on a background HBV found direct genetic disruption, aberrations of viral promoter-driven transcription, viral-human transcription and copy number changes confirming theories that alternate aetiologies

lead to distinct genetic alterations^[74,75]. Whole exome sequencing of 243 liver tumours revealed mutational signatures that appeared to correlate with specific risk factors for HCC development including CTNNB1 (alcohol) and TP53(HBV)^[76]. In addition, different mutations were associated with varying clinical outcomes. Early stage disease harboured TERT promoter mutations whereas FGF, CCND1, TP53 were associated with more aggressive pathology.

Conclusions from these extensive genetic studies have highlighted not only the heterogeneity of HCC tumours but also the significant differences in key oncogenic drivers of HCC compared with many other solid malignancies. In breast, colorectal and lung for example, MAPK and PI3K as well as EGFR activated pathways dominate progression in distinct cohorts^[77-79]. However, for HCC Wnt/ β -catenin and JAK/STAT pathways have consistently been identified as responsible for key oncogenic signalling. These differences are likely to explain the failures of therapies in HCC that have provided benefit in other malignancies. Comprehensive genetic mapping will undoubtedly aid drug development for HCC but a major challenge is that the majority of pathways found remain "undruggable" and interacting protein kinases must be targeted instead (Figure 1). A selection of key pathways and novel agents recently or currently under investigation are discussed below.

EMERGING TARGETS IN DRUG DEVELOPMENT

MEK inhibition

The RAF/MEK/ERK pathway plays a pivotal role in several cellular process including proliferation, apoptosis and migration^[80,81]. Although RAS and RAF mutations are uncommon in HCC, there is evidence that this pathway is activated in the majority of HCC tumours. Selumetinib, a potent selective MEK 1/2 inhibitor, was assessed in a single arm phase 2 trial in 19 patients who had not received prior systemic therapy. There were no responses and time to progression was short (8 wk). The trial was subsequently terminated at the interim analysis^[82]. Examination of pre and post treatment tissue revealed that four out of five patients achieved significant inhibition of phospho-ERK1/2 in tumours suggesting the failure of selumetinib was not due to lack of target inhibition. A small study assessing in combination with sorafenib resulted in three partial responses and six with stable disease. Whilst these numbers were small and therefore difficult to interpret, it suggests that perhaps this combination should be assessed further^[83]. A phase II study assessing the efficacy and safety of combination inhibition using sorafenib and the MEK inhibitor refametinib, resulted in a median time to progression of 122 d and median OS of 290 d^[84]. Toxicities however were significant with rash, diarrhoea, elevated liver enzymes and vomiting and the majority of patients required dose reductions. Interestingly the best responders harboured a RAS mutation and a proof of concept phase II trial using this combination for

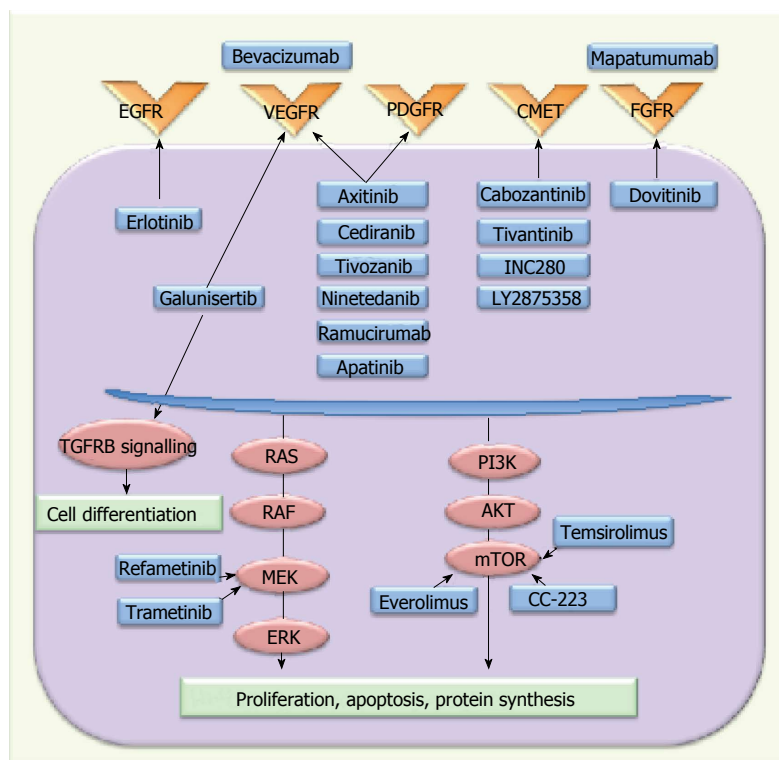


Figure 1 Novel compounds under investigation and their predominant targets. EGFR: Epidermal growth factor receptor; VEGFR: Vascular endothelial growth factor receptor; PDGFR: Platelet derived growth factor receptor; FGFR: Fibroblast growth factor receptor; PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase; mTOR: Mammalian target of rapamycin.

patients with RAS mutations is on going (NCT01915602). Crucially, this study is one of the first attempts to select a specific cohort of HCC patients based on molecular genotype utilising cfDNA to detect mutations in RAS. The study raises a number of important issues regarding feasibility and cost given the incidence of RAS mutation is approximately 3%-5%, requiring a large cohort of patients to be prescreened to identify the small group with aberrant genotype (Table 3).

Anti-angiogenic therapy

HCC is a hyper vascular tumour enriched with high levels of angiogenesis due to the presence of growth factors such as vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF)^[85]. A meta-analysis assessing the prognostic value of VEGF expression confirmed that tissue and serum VEGF levels seemed to predict poor disease free and overall survival^[86]. Biomarker data from the SHARP trial also demonstrated that VEGF and angiopoietin-2 [(Ang2) a further critical molecule in angiogenesis] were independent prognostic markers but not predictive of response^[16]. Sorafenib has anti-angiogenic properties and its success fuelled the search for more potent, selective anti-angiogenics. Yet several negative clinical trials have questioned the emphasis on VEGF inhibition in HCC, supporting theories that multiple mechanisms may be in play. As discussed the VEGF inhibitors, sunitinib, linifanib and brivanib failed to prove non-inferiority compared with sorafenib. Some commentators have therefore argued that an antiangiogenic monotherapy "ceiling" has been reached, and combination strategies will be required to extend survival beyond this^[87]. Trials of sorafenib

in combination with other antiangiogenic therapy (bevacizumab), chemotherapy (doxorubicin or FOLFOX) or other molecularly targeted therapy (e.g., everolimus and temsirolimus) are on-going. In order to ensure optimal results with these agents, the development of predictive biomarkers is needed to select patients who are most likely to benefit.

HGF/c-MET pathway

In vitro studies suggest that c-Met may play a role in proliferation, angiogenesis and metastatic spread in HCC and the hepatocyte growth factor (HGF)-cMET axis is therefore an attractive target. Whilst HGF expression in HCC tumours is low compared with surrounding liver tissue, over-expression of cMET has been observed in nearly a quarter of HCC cases and there is some evidence to suggest c-MET expression is a poor prognostic marker^[88-90]. Biomarker data from the SHARP trial revealed that HGF levels correlated with tumour size^[16]. There is also evidence of an interaction between c-MET and both EGFR and VEGF^[91]. Preliminary data from c-MET inhibition with cabozantinib is promising and as previously discussed a phase III trial with tivantinib in patients with high levels of MET expression is on going^[92].

FGFR inhibition

Fibroblast growth factors are trans membrane receptor kinases that signal downstream pathways including the RAS-RAF-MAPK. FGF3/4 is expressed in normal tissue including benign hepatocytes^[93]. Gene array studies and Immunohistochemical expression assays have shown overexpression of FGF3 and FGF4 in HCC tumours that mediate proliferation, cell death and alpha

Table 3 Novel agents currently under evaluation in clinical trials

Drug	Phase	Target	Enriched population	Trial identifier	Study location
Tivantinib	III	MET/tubulin	High MET expression	NCT01755767	North America, Europe
Axitinib	II	VEGFR/c-KIT/PDGFR	No	NCT01334112	North America
Tivozanib	I / II	VEGFR	No	NCT01835223	North America
Nintedanib	I / II	VEGFR/FGFR/PDGFR	No	NCT00987935	Asia
Ramucirumab	III	VEGFR2	AFP > 400	NCT02435433	North America, Asia, Europe
Apatinib	III	VEGFR2	No	NCT02329860	Asia
Cabozantinib	III	MET	No	NCT01908426	North America, Asia, Europe
INC280	II	MET	MET aberration	NCT01737827	Asia
LY2875358	I / II	MET/VEGFR	No	NCT01287546	North America
Refametinib	II	MEK	RAS mutations	NCT01915602	North America, Asia, Europe
Trametinib	I / II	MEK1/2	No	NCT02292173	North America
Dovitinib	II	VEGFR, FGFR	No	NCT01232296	Asia
Temsirolimus	I, II	mTOR	No	NCT01687673	North America
Cc-223	I, II	mTOR	No	NCT01177397	North America, Europe
Galunisertib	II	TGFR β	No	NCT02423343	North America
Mapatumumab	I / II	TRAIL-R1	No	NCT01258608	North America, Europe
Nivolumab	I	PD1	No	NCT01658878	North America, Europe, Asia
Lenvatinib	III	VEGF	No	NCT01761266	North America, Europe, Asia
Enzalutamide	II	Androgen receptors	No	NCT02528643	TBC
OMP-54F28	I	Wnt signalling	No	NCT02069145	North America

feto protein (AFP) levels^[94]. Brivanib, in addition to its anti-angiogenic properties as discussed above, is an ATP competitive inhibitor of FGF1-3. Although it failed to improve survival in the first and second line setting, further multi-kinase inhibitors that also target FGFR are currently underway. The lack of response to brivanib may be partly explained by its use in an unspecified population and biomarkers may aid selection of patients likely to respond to inhibition. Lenvatinib, an oral multi-targeted tyrosine kinase inhibitor of VEGFR-1, FGFR1-4, PDGFR β , RET and KIT is currently under evaluation in a non-inferiority study with sorafenib following a phase II trial which resulted in a median time to progression of 12.8 mo (95%CI: 7.23-14.7) and median OS of 18.7 mo NCT01761266^[95]. The REFLECT phase III trial comparing sorafenib to lenvatinib has recently been completed. This trial has attempted to learn the lessons from the previous high profile failures described in this article by utilising stricter criteria for trial entry, excluding poor prognosis groups such as those patients with greater than 50% liver involvement, bile duct invasion, or main branch portal venous infiltration.

Dovitinib, an FGFR, VEGFR and PDGFR TKI demonstrated efficacy in xenograft mouse models and is currently under investigation in a phase II trial^[96,97]. FGF19, located on chromosome 11q13, a region amplified in 10%-15% of HCC tumours, is a potential predictive biomarker for FGF inhibitors and FGF19 targeted antibodies are under investigation in *in vitro* models^[97]. *In vivo* studies with murine models suggest that dual targeting with FGFR and mTOR inhibition impaired tumour growth unlike treatment with the FGFR inhibitor alone providing support for combination trials^[98].

TGF- β signalling

TGF- β signalling plays a role in the micro-tumour en-

vironment promoting epithelial-mesenchymal transition (EMT), dysplastic nodule formation and subsequent HCC development^[99-101]. Patients with higher levels of TGF- β signalling are associated with larger less differentiated tumours with higher levels of AFP^[102]. It remains unclear whether TGF- β plays a role in a sub-group of patients, or in the carcinogenesis of all HCCs due to its dual role in tumour suppression in normal tissue and tumour promotion in HCC. TGF- β inhibitors modulate EMT leading to reduced tumour growth in pre-clinical models. Galunisertib, a selective TGF- β TKI is currently under investigation in a phase II trial (NCT02178358).

Immunotherapy

Recent years have seen a resurgence in the use of immunotherapy, led partly by the success of anti-CTL4 antibodies in solid tumours such as melanoma and more recently antibodies targeting the programmed death (PD) receptor and its ligand^[103,104]. Immunotherapy works by enhancing anti-tumour response, an important mechanism in HCC as the surrounding micro-tumour environment is rich in immune cells. Tremelimumab, a fully human IgG2 monoclonal anti-CTL4 antibody was assessed in a phase II study of 24 patients with HCC on a background of HCV. The drug had a good safety profile and a partial response of 17.6% and disease control rate of 76.4%. Time to progression was 6.48 m (95%CI: 3.95-9.14). Changes were also seen in the predominant variants of HCV as well as a reduction in viral loads. These early reports are promising and suggest that immunotherapy may have the dual benefit of treating both HCC and underlying viral hepatitis. Anti-programmed death ligand 1 (PDL1) inhibitors are checkpoint inhibitors that block T cell activation when bound by PD ligands 1 and 2. Patients with tumours that over-express PD-L1 are associated with a poorer prognosis. In a recently reported phase I / II dose

escalation study, patients received 0.1 to 10.0 mg/kg of the anti-PDL1 agent nivolumab intravenously for up to 2 years. 2 patients had a complete response (CR) and a further 7 patients had a partial response (PR)^[105]. The overall survival rate at 6 mo was 72%. Although these results are from a very small early phase trial, they are highly encouraging and a number of trials using checkpoint inhibitors are now planned in both first and second line settings.

CONCLUSION

The era of personalised medicine and treatment stratification has yet to impact clinical practice of HCC and the failure of several clinical trials has been disappointing. Nevertheless our understanding of this unique disease has improved significantly with the benefit of genomic sequencing and biomarker data from clinical trials. Proof of concept studies such as the ongoing phase II trial with refametinib for RAS mutated cancers and tivantinib for c-MET positive tumours are a step forward in designing adequate trials to maximise potential benefit of novel agents in pre-determined sub groups. Molecular testing, improved clinical trial design and the development of predictive biomarkers should finally see an improvement in survival for this global disease.

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Neoadjuvant radiotherapeutic strategies in pancreatic cancer

Falk Roeder

Falk Roeder, Department of Radiation Oncology, University Hospital of Munich (LMU), 81377 Munich, Germany

Falk Roeder, Clinical Cooperation Unit Molecular Radiation Oncology, German Cancer Research Center (DKFZ), 69120 Heidelberg, Germany

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Correspondence to: Dr. Falk Roeder, PhD, Department of Radiation Oncology, University Hospital of Munich (LMU), Marchioninistr 15, 81377 Munich, Germany. falk.roeder@med.uni-muenchen.de
Telephone: +49-89-440073729
Fax: +49-89-440076770

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Abstract

This review summarizes the current status of neoadjuvant radiation approaches in the treatment of pancreatic cancer, including a description of modern radiation techniques, and an overview on the literature regarding neoadjuvant

radio- or radiochemotherapeutic strategies both for resectable and irresectable pancreatic cancer. Neoadjuvant chemoradiation for locally-advanced, primarily non- or borderline resectable pancreas cancer results in secondary resectability in a substantial proportion of patients with consecutively markedly improved overall prognosis and should be considered as possible alternative in pretreatment multidisciplinary evaluations. In resectable pancreatic cancer, outstanding results in terms of response, local control and overall survival have been observed with neoadjuvant radio- or radiochemotherapy in several phase I / II trials, which justify further evaluation of this strategy. Further investigation of neoadjuvant chemoradiation strategies should be performed preferentially in randomized trials in order to improve comparability of the current results with other treatment modalities. This should include the evaluation of optimal sequencing with newer and more potent systemic induction therapy approaches. Advances in patient selection based on new molecular markers might be of crucial interest in this context. Finally modern external beam radiation techniques (intensity-modulated radiation therapy, image-guided radiation therapy and stereotactic body radiation therapy), new radiation qualities (protons, heavy ions) or combinations with alternative boosting techniques widen the therapeutic window and contribute to the reduction of toxicity.

Key words: Pancreatic cancer; Neoadjuvant; Radiation therapy; Review; Radiation techniques

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Core tip: This review summarizes the current status of neoadjuvant radiation approaches for pancreatic cancer. Neoadjuvant chemoradiation for locally-advanced cases results in secondary resectability in a substantial proportion of patients with consecutively improved overall prognosis. In resectable pancreatic cancer, outstanding results in terms of response, local control and overall survival have

been observed in several phase I/II trials. Further investigation of neoadjuvant chemoradiation strategies should be performed preferentially in randomized trials and included evaluation of optimal sequencing with chemotherapy and patient selection based on molecular markers. Modern radiation techniques widen the therapeutic window and contribute to the reduction of toxicity.

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INTRODUCTION

Multimodal treatment of patients with pancreatic cancer remains one of the largest challenges in gastrointestinal oncology. Surgery is the cornerstone of curative intent treatment^[1], however only 10%-20% of the patients are deemed resectable at presentation while 30%-40% already suffer from locally-advanced, irresectable disease and the remaining group shows distant metastases^[2]. Given a median survival of approximately 24 mo and a 5-year overall survival rate of roughly 10%-20% even in the most favourable group with primarily resectable, locally confined disease, pancreatic cancer remains a disease with one of the most dismal prognosis in oncology^[3].

While neoadjuvant strategies are already part of the standard approaches in most other gastrointestinal tumors (e.g., rectal cancer, esophageal cancer)^[4,5], surgery followed by adjuvant treatment still represents the standard of care for resectable pancreatic cancer. Adjuvant chemotherapy seems the preferred approach in Europe based on the Conko-001 trial^[6], while adjuvant chemoradiation is frequently used in the US based on the GITSG trial^[7] and several non-randomized single-center studies with excellent results^[8-10]. In primarily non-resectable locoregionally confined tumors, mainly definitive-palliative strategies have been used so far, which either consist of systemic therapy alone, combined chemoradiation or various combinations of both^[11-13]. However, the mentioned strategies show limited success in terms of overall prognosis. On the other hand, the high rates of microscopically incomplete resections^[14] with consecutively significant local recurrence rates^[15] and the high frequency of locoregionally confined but primarily non-resectable tumors in combination with the clear advantages of neoadjuvant treatment strategies shown in other gastrointestinal tumor diseases despite clearly more favourable resectability, may form a strong rationale for the use of neoadjuvant strategies both for locally-advanced non-resectable as well as for primarily resectable pancreatic cancer patients.

Such strategies have different aims and include different possible advantages dependent on the resectability of the primary lesion: (1) in primarily non-resectable locoregionally confined pancreatic cancer, the main aim of neoadjuvant chemoradiation consists of tumor shrinkage including a drawback of the tumor from the major vessels to achieve secondary resectability; and (2) in primarily resectable cases, the main aim consists in enhanced local control either by increased probability of microscopic complete resection (R0) due to tumor shrinkage or by sterilization of microscopic tumor remnants in case of a microscopically incomplete (R1) resection. Substantial potential benefits further exist independent of the resection margin: (1) neoadjuvant treatment allows a local response evaluation which may reflect the overall disease prognosis; (2) neoadjuvant chemoradiation usually requires several weeks, which enables a stratification of patients with response or stable disease vs patients with rapid systemic progress. This may allow a potential omission of major surgery in those patients who are unlikely to benefit. The radiation therapy component thereby prevents patients without rapid systemic progression from a worsened overall prognosis due to local progression caused by locally insufficient effects of systemic therapy alone; (3) efficacy of radiation is enhanced in the neoadjuvant setting in comparison to postoperative radiotherapy because of the increased oxygenation of the untreated tissue; (4) the probability that additional therapy must be cancelled due to postoperative complications is reduced; and (5) target volume definition is simplified, resulting in smaller safety margins with consecutively lower dose to organs at risk and reduced toxicities.

Due to the complexity of the disease and the different aims in distinct stages, a variety of neoadjuvant concepts exist. They include chemotherapy, radiation therapy, chemoradiation or combinations like induction-chemotherapy followed by chemoradiation. This review focuses mainly on neoadjuvant radiotherapeutic strategies (radiation alone, radiation with concurrent chemotherapy) and advances in radiation technique rather than neoadjuvant concepts using chemotherapy alone or induction chemotherapy.

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NEOADJUVANT RADIOTHERAPEUTIC TECHNIQUES AND CONCEPTS

3D-conformal radiation therapy

3D-conformal radiation therapy has been the standard radiation technique for the treatment of pancreatic cancer for the last two decades. This technique includes three-dimensional treatment planning based on computed tomography as first step. In the neoadjuvant setting, the target volume includes the primary tumor and the regional lymph nodes with a safety margin for daily repositioning error and tumor motion. If and to what extent the regional lymph node areas have to be included into the target volume is indeed part of an ongoing discussion. Multiple radiation fields are arranged in a way to ensure sufficient coverage of the target volume with best possible sparing of organs at risk at the same time (so called forward treatment

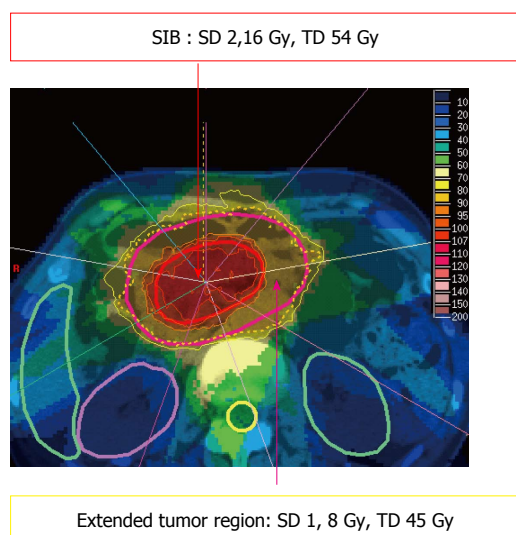


Figure 1 Example for a 9-beam intensity modulated radiation therapy treatment plan in a pancreatic cancer patient with simultaneously integrated boost. SD: Single dose; TD: Total dose in 25 fractions; SIB: Simultaneously integrated boost.

planning) with small bowel and kidneys representing the main dose-limiting structures. Usually total doses of 45-54 Gy are applied in conventional fractionation (5-6 wk overall treatment time) in combination with simultaneous 5-FU or gemcitabine based chemotherapy.

Intensity-modulated radiation therapy

Treatment of irregularly shaped target volumes directly adjacent to radiation sensitive organs at risk can generally be improved by the use of so called "complex" photon irradiation techniques like intensity-modulated radiation therapy (IMRT). In contrast to 3D-conformal therapy, IMRT allows the delivery of different doses to certain segments of the same radiation field, creating a so called "fluence matrix" for every beam. By addition of multiple segments within several beams, superior coverage of irregularly shaped target volumes can be achieved. At the same time, steep dose gradients are possible, allowing improved sparing of directly adjacent organs at risk remain possible. Treatment planning is called "inverse" planning, because in contrast to 3D-conformal therapy the field geometry is usually not directly adjusted by the planner. Instead, doses are prescribed to the target volume(s) and to the outlined organs at risk (so called "dose constraints") with a prespecified arrangement of beams. The treatment plan is then generated by an iterative computer-aided process by adjusting the fluence matrix and/or the constraints. This technique further allows the treatment of regions inside the target volume (for example gross tumor) with a higher dose, and other regions (for example elective nodal areas) with a lower dose within the same fraction. This enables dose escalation in certain areas within an unchanged number of fractions (so called "SIB = simultaneously integrated boost", Figure 1).

Numerous dosimetric studies showed clear advantages

for intensity-modulated techniques compared to 3D-conformal treatments. In particular, lower doses in small bowel and liver could be achieved^[16] and the possibility of a dose escalation up to 65 Gy was suggested^[17]. Further on, several clinical studies have clearly confirmed, that these dosimetric advantages translate into reductions of acute and late side effects^[18].

Image-guided radiation therapy

In general, several sources of uncertainty must be addressed in external beam radiation therapy regarding the coverage of target volumes with the prescribed dose. Intrafractional motion is mainly caused by respiration. On the other hand interfractional variations are the result of a combination of different factors. One major source is the displacement of the target by different filling of adjacent distensible structures like stomach or small bowel. Another source is the so-called "set-up error", i.e., the uncertainty due to variation in daily positioning of the patient. All these variations must be compensated by safety margins. However, if directly adjacent organs at risk are present, every increase of safety margins consequently leads to increased side effects, which builds up the rationale for image-guided radiation therapy (IGRT). In doing so, three-dimensional datasets in treatment position are generated with imaging devices directly mounted on the linear accelerators (so called "on-board imaging"). These allow a comparison of the actual situation with the one during treatment planning and a real time correction of the position prior to irradiation. The increased precision of treatment application consequently allows a reduction of the safety margin. Several analyses have shown, that the safety margins needed to compensate for set-up error in the upper abdominal region can be reduced from 10 to 5 mm if IGRT is used^[19]. In a tumor with 5 cm diameter, this margin reduction would lead to a 30% decrease of irradiated volume^[18] with a significant dose reduction in small bowel, kidneys and liver^[20].

Intrafractional respiratory motion differs from patient to patient, but can reach several centimeters^[21]. Different strategies have been used to reduce the safety margins needed to account for such large variations. First, the individual respiratory motion can be measured for example by 4-dimensional computed tomography and allow definition of individualized anisotropic margins. This strategy can result in a mean reduction of the target volume by one third compared to the use conventional margins^[20]. Some modern linear accelerators also allow gating, i.e., on board detection of tumor motion and application of radiation only at distinct positions of the tumor in its motion cycle. Another technique supported by some accelerators is a continuous adjustment of the beams to the particular tumor position (so-called "tracking"). Especially for these methods, the implantation of fiducials into the tumor may further increase the precision of dose application^[22].

Adaptive radiation therapy strategies

In contrast to the mentioned techniques, adaptive

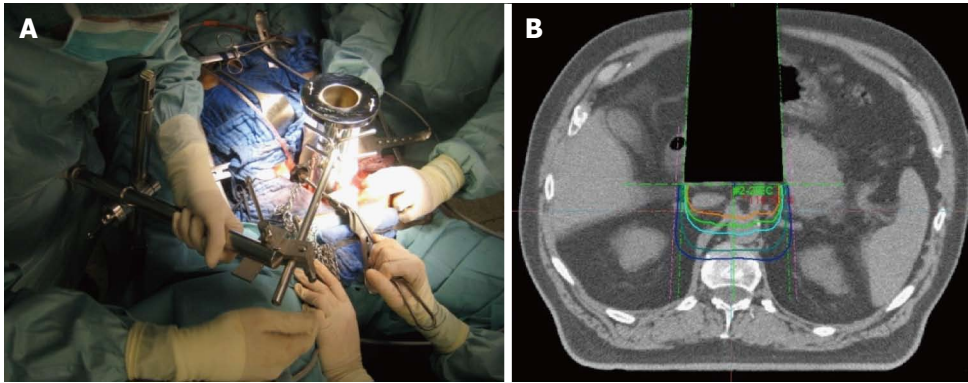


Figure 2 Intraoperative electron radiation therapy. A: Placement of the applicator after resection; B: Schematic dose distribution.

strategies use regular imaging to adapt the radiation treatment plan semi-automatically to anatomical changes during the radiation therapy series, for example due to tumor shrinkage. Although the models for routine use of these techniques are currently still under development, theoretical studies suggest marked reductions in dose to several organs at risk, for example duodenum^[23].

Intraoperative radiation therapy

Although modern radiation techniques allow an improved sparing of surrounding organs at risk, dose-limitations in external beam therapy still exist, mainly due to directly adjacent structures with low radiation tolerance. Intraoperative electron radiation therapy (IOERT) offers an elegant possibility to overcome these dose-limitations after neoadjuvant radio (chemo)therapy. Due to its unique opportunity to guide a high single dose directly to the tumor bed or residual tumor during surgery, while adjacent organs at risk can be manually removed, IOERT can effectively prevent adjacent organs at risk from radiation exposure. Further advantages of IOERT in comparison to an external beam boost include at least theoretically smaller field sizes (because safety margins for daily positioning errors can be omitted) and the higher biological effectiveness of a single dose compared to the same amount of fractionated radiation therapy^[24-29]. If typical dose concepts for the combination are used (10-15 Gy IOERT + 45-54 Gy EBRT), total doses can be reached which are biologically equivalent to 70-90 Gy of conventionally fractionated external beam radiation therapy without markedly increased toxicity^[24-29]. Practically, an applicator of appropriate size is placed inside the abdominal cavity under visual control to cover the tumor bed/residual tumor after performed/attempted resection and manual removal of adjacent structures at risk. After moving and adjusting the patient below the accelerator, the irradiation itself is performed inside the operation theater, lasting about 1-2 min. Adequate depth coverage is achieved by appropriate selection of the electron energy (Figure 2). Unfortunately, this technique is only available at a limited number of centers so far (although numbers are heavily increasing in the recent years). Efficacy of IOERT remains difficult to assess, since many series report

only single-center experiences covering large observation periods. Regarding primarily resectable pancreatic cancer, several Italian series reported significantly decreased local recurrence rates with the addition of IOERT^[30,31]. Reni *et al*^[32] confirmed these results in a larger comparison vs surgery alone. Beside an increased local control rate, they also found a significantly improved median overall survival in the subgroup of patients with early stages without increased perioperative morbidity. A multi-institutional series from Japan described a local recurrence rate of only 15% in 210 patients after gross complete resection with IOERT^[33] and a European pooled analysis reported a very encouraging median overall survival of 30 mo for the combination of neoadjuvant chemoradiation, surgery and IOERT in a series of 270 patients^[34]. Regarding primarily unresectable pancreatic cancer, IOERT can be used for dose escalation after neoadjuvant chemoradiation both in case of achieved secondary resectability as well as in case of further irresectability, resulting not only in improved local control but in the achievement of durable pain control in 75%-90% of the patients^[35]. The Mayo group reported a series of 115 patients with unresectable pancreatic cancer and found that the addition of IOERT during explorative laparotomy after neoadjuvant irradiation resulted in a significantly increased 1-year local control rate (48% vs 82%)^[36]. Shibamoto *et al*^[37] compared EBRT, EBRT + IOERT and IOERT alone in a cohort of 150 patients and described an improved survival for the combination in the subgroup of patients with an initial CA 19-9 < 1000. The MGH group reported a median overall survival of 12 mo for the combination of EBRT and IOERT in their series of 194 patients with irresectable pancreatic cancer^[38]. If the combined local treatment was further enhanced by a systemic treatment component, several series consistently reported median overall survival times of 16-18 mo with 2-year local control rates around 70%^[39,40]. Even in patients with isolated local pancreatic cancer recurrences, the combination of EBRT, surgery and IOERT resulted in high local control and encouraging overall survival rates^[26].

In summary, IOERT provides an elegant possibility to escalate dose allowing total doses which could not be achieved by EBRT alone even with the use of the most sophisticated techniques. In resectable pancreatic

cancer, IOERT seems clearly to improve local control, while its influence on overall survival cannot be finally assessed at present. In primarily irresectable pancreatic cancer, it can be suggested based on large single-center experiences, that especially the combination of EBRT, IOERT and chemotherapy achieves improved quality of life due to durable pain control, high local control rates and encouraging overall survival rates compared to other treatment approaches, although no phase III data currently exists to confirm these results.

Stereotactic body (ablative) radiation therapy

Stereotactic radiation therapy was primarily developed to treat small intracranial tumor lesions (for example brain metastases) and was successfully used in these situations for several decades. Initially it was defined as a treatment with a high single dose, which was guided precisely to the target using a stereotactic frame as external coordinate system for target localisation and very rigid patient fixation systems to reduce safety margins to a minimum (also known as radiosurgery). After expansion of the technique to extracranial sites and introduction of image guided radiation therapy, the definition of stereotactic body radiation therapy (SBRT) has widened. Today it summarizes different methods, which consistently apply so called "ablative" doses (biologically equivalent doses far beyond those achievable by conventional fractionation) in one to few fractions with optimal precision aiming at durable control of macroscopic tumor lesions. This technique has for example emerged as the standard of care in medically inoperable patients with early stage non-small cell lung cancer^[41]. Currently it is used also in pancreatic cancer, although due to the lower contrast to the surrounding tissue (compared with lung cancer) implantation of fiducials is usually needed to achieve a safe detection of tumor position and motion with simple imaging modalities. Fiducial based approaches can additionally be combined with motion compensating radiation techniques (gating or tracking) to reduce safety margins to a minimum.

The present clinical experience for SBRT in pancreatic cancer is based mainly on small series of patients with irresectable locally-advanced pancreatic cancer^[42]. Although very different dose schemes ($1 \times 15\text{-}25$ Gy, $3 \times 8\text{-}15$ Gy, 5×6.5 Gy) have been used^[42], these series consistently report very high local control rates of 80%-100% with partially very encouraging overall survival especially if combined with sequential chemotherapy^[43-45]. However, the therapeutic window of this technique is narrow and therefore dose to directly adjacent organs at risk (like duodenum) must be strictly limited to avoid major complications^[46], as shown by the range of gastrointestinal grade 3 complications reaching from 14% to 79% in the major series, and depending mainly on target volume size and dose to the duodenum^[45,47]. Adaptive dose prescriptions depending for example on the distance between tumor and duodenum seem to be beneficial^[44].

Recently, SBRT has also been introduced into

neoadjuvant treatment approaches. One series describes the use of SBRT in 73 patients of whom 56 were deemed borderline resectable^[48]. Treatment consisted of 3 cycles induction-chemotherapy followed by SBRT which guided 35-50 Gy to the vessel-approaching tumor parts and 25-30 Gy to the remaining tumor parts in 5 fractions. Seventy-seven percent of the borderline resectable patients responded and were surgically explored. Resection was possible in 56% of the patients (97% R0), showing a significantly improved survival. Severe gastrointestinal toxicity (grade 3) was observed only in 5% of the patients^[48].

In summary, SBRT yields high local control rates, which seem so be superior to the results of conventionally fractionated RT. However, SBRT in the upper abdomen remains a demanding technique with a narrow therapeutic window and has been so far investigated mainly in irresectable pancreatic cancer. Nevertheless, it seems to be a promising approach also in the neoadjuvant setting especially if combined with systemic therapy.

Particle therapy

A least theoretically, more advantages could be exploited by the use of radiation qualities like protons or heavy ions. In contrast to photons, particle beams deposit most of the dose in a narrow range of tissue depth depending on the beam energy. This so-called "Bragg-peak" can be used to focus the dose very precisely to the target volume, while adjacent tissues can be safely spared (Figure 3). Especially heavy ions further show an enhanced biological effectiveness, because they generate a different pattern of DNA-damage in the tumor cells which is less easily repaired by cellular DNA-repair mechanisms in comparison to damages set by photon therapy. Some drawbacks remain in the upper abdomen due to difficulties to account for bowel gas movement during treatment planning. These can lead to large dosimetric uncertainties compared to photons^[49]. Nevertheless several encouraging preliminary results have been reported by several centers. For example, the MGH group showed a very low severe gastrointestinal toxicity rate of 4% during chemoradiation in a phase I / II trial, where neoadjuvant proton radiotherapy with 5×5 Gy combined with simultaneous capecitabine and followed by resection and adjuvant gemcitabine was evaluated in primarily resectable pancreatic cancer. With a median follow-up of 38 mo, they reported a local recurrence rate of 16% and a median overall survival of 17 mo^[50]. Investigators from Chiba (Japan) launched a phase I trial including 26 patients with resectable pancreatic cancer, treated with increasing doses of 30-36.8 Gy in 8 fractions with carbon ions of whom 81% proceeded to surgery. They reported a local control rate of 100% with 1- and 5-year survival rates of 89% and 52% in resected patients^[51]. Irresectable pancreatic cancer patients were also included into a dose escalation trial with doses of 38.4-52.8 Gy in 12 fractions at the same center resulting in 81% local control and 60% overall survival after one year^[52].

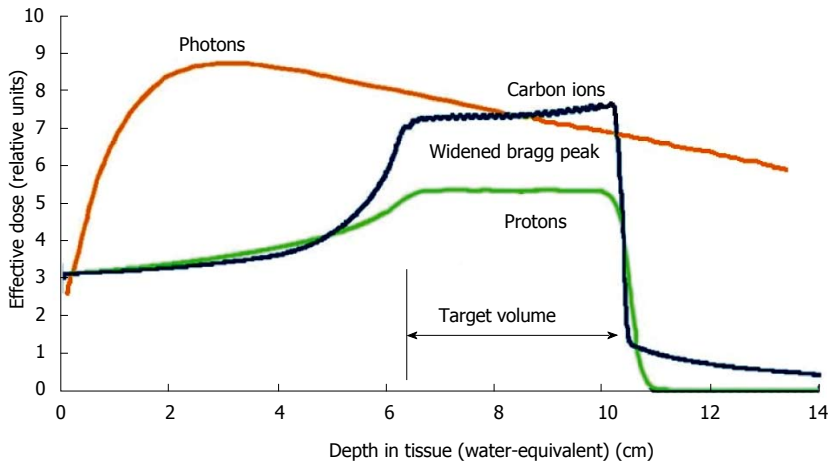


Figure 3 Schematic comparison of the depth dose curves of photons and particles. Lower dose distribution in the radiation path before and after the target with particles by exploiting the Bragg-peak.

In summary, particle radiation therapy seems to be a promising modality with regard to high local control rates with low toxicity. However, the current knowledge is based only on a few studies with low patient numbers and short follow-up and has therefore to be regarded as preliminary. Due to the known uncertainties in dose calculation because of bowel gas movement, patients with pancreatic cancer should be treated only in prospective studies at experienced centers.

Current status of neoadjuvant radio (chemo) therapy in locally-advanced primarily non-resectable pancreatic cancer

The interpretation of the literature regarding the optimal therapy for locally-advanced pancreatic cancer is difficult for different reasons. First of all, very different treatment strategies exist, ranging from aggressive approaches with curative intent such as multimodal neoadjuvant treatment including regular surgical exploration to purely palliative systemic treatment approaches, with all conceivable steps in between. Further on, even if only series using very similar neoadjuvant approaches aiming at secondary resectability are assessed, they differ extensively in terms of patient selection. The distinction between resectable and irresectable lesions is flawed by a certain subjectivity, which clearly correlates with the experience of surgeon and center. Even if a lesion is deemed primarily irresectable, different sub-terms are in use. In most of the US literature, patients are sub-divided in borderline-resectable and unresectable depending on the extent of vessel involvement, while this differentiation is not commonly used in most parts of Europe and Asia. This results in the inclusion of very different advanced lesions in neoadjuvant approaches compromising reasonable comparisons. The primary aim of neoadjuvant approaches in patients with locally-advanced irresectable pancreatic cancer is the induction of tumor shrinkage and thereby the achievement of secondary resectability *per se* and the increase of the rate of microscopic negative (R0) resections. Secondary aims are response evaluation for further treatment stratification and improvement of quality of life by prevention of local symptoms in case of persistent irresectability. The impact of neoadjuvant

radio(chemo)therapy has been evaluated in numerous retrospective and prospective studies. These show a wide range of results and therefore seem less reliable when taken individually^[53]. However, Gillen *et al*^[2] described some fundamental findings in an impressive metaanalysis including 111 studies with 4400 patients. They included trials evaluating primarily resectable and primarily irresectable patients but analyzed them separately. In the group deemed primarily resectable they found a final resection rate of 74% after neoadjuvant therapy which is very similar to the rate reported for surgery alone. In the group primarily deemed irresectable, they observed a final resection rate of 33% after neoadjuvant (mainly radiochemo-) therapy. Radiological response assessment after neoadjuvant therapy described complete and partial remission in 4% and 29% with a 21% progression rate. These rates were not different between resectable and irresectable patients. However, the most important finding was a median survival of 21 mo and a 2-year overall survival rate of 50% in the group of patients, who reached secondary resectability after neoadjuvant treatment. This equals the result in the primarily resectable group (median survival 24 mo, 2-year overall survival 47%), while patients in whom resection was not achieved showed a significantly worse overall survival (median 10 mo), independent of their initial resectability status. Morganti *et al*^[53] performed another metaanalysis including 13 trials with 510 patients, which were deemed irresectable and had received neoadjuvant chemoradiation with at least 45 Gy. Interestingly, they reported similar results: final resection rate was 27% with 88% being complete (R0). Median survival after secondary resection (24 mo) was significantly improved in comparison to persistent irresectability (10 mo)^[53]. One of the largest single center analyses from Heidelberg again showed similar results^[54]. In 257 patients treated with neoadjuvant (mainly radiochemo-) therapy and surgical exploration, secondary gross total resection rate was 40% with a median survival of 25 mo after R0-resection^[54]. Postoperative morbidity and mortality does not seem to be increased after neoadjuvant chemoradiation compared to surgery alone^[55,56]. In summary, neoadjuvant radiochemotherapy for patients with primarily irresectable, locally-advanced pancreatic

cancer results in secondary resectability in a substantial portion (30%-40%) of the patients, accompanied by a significantly improved prognosis in this subgroup. The median survival time (median approximately 24 mo) is similar to patients with primary resection and adjuvant chemotherapy. Even if secondary resectability is not achieved, the results after neoadjuvant chemoradiation are found at the upper end of the range reported for chemotherapy alone in the current literature, including the advantage of improved quality of life due to durable prevention of local complications by tumor progression.

Current status of neoadjuvant radio (chemo) therapy in primarily resectable pancreatic cancer

The rationale for neoadjuvant treatment approaches in resectable pancreatic cancer is based on several findings. First of all, locoregional progression is at least a component of disease progression in 50%-75% as shown by pattern of recurrence analyses after resection alone^[57]. Even with the use of adjuvant chemotherapy, several studies reported local recurrence rates of 30%-60%. This suggests that eradication of locally persistent tumor cells by chemotherapy alone is not safely ensured^[6,58]. As locoregional recurrences often result in local complications, the aim of achieving adequate local control seems justified with regard to quality of life.

Neoadjuvant strategies have replaced or at least supplemented sole adjuvant approaches as the standard of care in many other resectable gastrointestinal tumors, for example rectal cancer or esophageal cancer^[5,59]. Unfortunately, no randomized data comparing neoadjuvant and adjuvant approaches in resectable pancreatic cancer have been published so far, although neoadjuvant approaches are investigated increasingly because of their potential benefits. Benefits include an improved local control rate for example due to an increased R0-resection rate, early initiation of at least a systemic therapy component to control potentially existing distant micrometastases, a simplified access to additional therapies and of course an optimal patient selection by exclusion of patients with early distant failure. The ongoing development will be illustrated exemplarily by the work of the MDACC group, which designed and performed a number of consecutive phase II trials over nearly 2 decades. They started with conventionally-fractionated radiation therapy combined with 5-FU^[60], went on with a shortened radiochemotherapy with additional IOERT^[61] and ended up with paclitaxel^[62] and finally gemcitabine-based preoperative chemoradiation^[63]. The last concept was evaluated in a phase II trial with 86 patients, who received a shortened radiation therapy (10 × 3 Gy in 2 wk) in combination with weekly gemcitabine 400 mg/m² over 7 wk. Resection was finally achieved in 74% of the patients. The median overall survival for the entire cohort was 23 mo with a 5-year survival rate of 27%. Resectable patients had a significantly improved median survival of 34 mo compared to 7 mo in irresectable patients. The same was true for the 5-year survival rate (36% vs 0%). The local control rate in resected patients was 89%^[63].

The authors concluded, that neoadjuvant chemoradiation allows a good selection of patients, who probably will not profit from major surgery. They recommended further investigation of neoadjuvant gemcitabine-based chemoradiation in resectable patients based on the very encouraging overall survival results^[63], especially because a parallel trial by the same group with additional induction chemotherapy showed no further benefit^[64]. The consistency regarding definition of resectability, surgical treatment and histological examination further suggests a good comparability of the results in the different MDACC trials^[65]. Gemcitabine-based radiochemotherapy resulted in improved response rates, improved R0-resection rates and longer median survival in comparison to combinations with 5-FU or paclitaxel^[65]. A pooled analysis of the MDACC studies including 240 patients treated with surgery after neoadjuvant therapy finally revealed a median disease-free survival of 15 mo and a median overall survival of 34 mo^[66]. The potential benefit of neoadjuvant radiation therapy for resectable pancreatic cancer is further supported by a SEER-analysis on more than 3800 patients, which described a significant improved median survival of 24 mo after neoadjuvant radiation therapy compared to 17 mo with adjuvant and 12 mo without radiation therapy^[67].

In summary, neoadjuvant radio- or radiochemotherapy offers several potential benefits compared to adjuvant strategies, although no randomized data are currently available to support this assumption. Nevertheless, neoadjuvant radio(chemo)-therapy has shown outstanding results in terms of response, local control and overall survival at least in phase II trials. These results clearly justify further investigation of neoadjuvant radiation therapy approaches. In this context, further shortening of neoadjuvant radiation therapy schemes might be beneficial, as currently investigated in several prospective trials evaluating modern photon or proton techniques^[28,50].

Future directions

As mentioned earlier, this article focuses on radiotherapeutic strategies including radiotherapy alone or combined with concurrent chemotherapy in the neoadjuvant setting. Within such approaches, chemotherapy is used mainly as a radiation sensitizer rather than as systemic treatment resulting in low doses and usually single drug treatment to keep combined toxicity acceptable. However, recently new chemotherapy agents and combinations like Gemcitabine/nab-Paclitaxel^[68] or FOLFIRINOX^[69] have been successfully introduced into the treatment of metastatic pancreatic cancer and resulted in improved response and overall survival. Therefore it seems reasonable to use these schemes also in the neoadjuvant setting either to target possible distant micrometastases as early as possible in patients with resectable disease or to induce tumor shrinkage in irresectable patients to achieve secondary resectability. Due to the increased toxicity profile of these potent combinations, concurrent application of radiation does not seem possible even with the most sophisticated radiation techniques. Therefore sequential

applications for example induction chemotherapy with FOLFIRINOX followed by chemoradiation with 5-FU or gemcitabine seem to be very promising and are currently under investigation (for example in the German CONKO 007 study), with some groups already showing very encouraging preliminary results^[70]. Therefore additional aims for future radiation research in pancreatic cancer should include the evaluation of optimal sequencing of systemic and radiotherapeutic approaches as well as the identification of biomarkers to predict the pattern of disease progression in the individual patient.

Biomarker for stratification

One of the main challenges in the treatment of pancreatic cancer remains the insufficient possibilities for an early prediction of disease progression. This compromises a reasonable stratification of patients in terms of treatment combinations. Established and new biomarkers could be helpful. This will be illustrated exemplarily in the following with CA 19-9 serving as example for an established and SMAD for a new marker. Several groups established an association between increased pretreatment CA 19-9 levels and an unfavourable outcome^[71], with very high values indicating an already disseminated disease. Kim *et al.*^[72] for example showed stage-dependent median CA 19-9 levels between 40 and 748 U/mL in stage IA-III compared to a median CA 19-9 level of 3239 U/mL in stage IV. However, two major disadvantages limit the value of pretreatment CA 19-9 levels for prediction of disease prognosis: 5%-10% of patients with pancreatic cancer show negative CA 19-9 levels due to a defect in the gene coding for Lewis enzyme^[73] and CA 19-9 levels can be heavily influenced by other factors, for example cholestasis. Therefore increasing interest has been paid to new markers like SMAD4. The SMAD family of proteins plays a role in TGF- β signaling, which is heavily involved in the regulation of cell proliferation, differentiation and apoptosis^[74]. SMAD4 has been recently suggested as the most important candidate in regard to pancreatic cancer because it has been linked not only with tumor development but also with the pattern of disease progression^[75]. In this context, the presence of intact SMAD4 seems to be associated with a rather locally-destructive growth, while loss of SMAD4 correlates with early distant metastasis^[76]. These findings were supported by a trial performed by Crane *et al.*^[77], which found that 73% of the patients with intact SMAD4 showed locoregional progression while 74% of the patients with inactive SMAD4 developed distant failure. In summary, although current knowledge about biomarkers seems premature in regard to treatment stratification, this might be an encouraging opportunity to allow an improved allocation of patients to locally-aggressive vs systemic treatment approaches to strengthen personalized medicine also for pancreatic cancer in the future.

SUMMARY

In the absence of randomized data, published studies

show consistently that neoadjuvant chemoradiation for locally-advanced, primarily non- or borderline resectable pancreas cancer results in secondary resectability in a substantial proportion of patients with consecutively markedly improved overall prognosis in this subgroup. Even if the goal of secondary resectability is not reached, radiation therapy may contribute to improved quality of life by the prevention of local complications. Neoadjuvant chemoradiation should therefore be considered as possible alternative in multidisciplinary pretreatment evaluations.

In resectable pancreatic cancer, outstanding results in terms of response, local control and overall survival have been observed with neoadjuvant radio- or radiochemotherapy in several phase I / II trials. These undoubtedly justify further evaluation of this strategy. In this context, further shortening of the radiation therapy series to allow a simplified integration into multimodal concepts is evaluated in ongoing trials.

Further investigation of neoadjuvant chemoradiation strategies should be performed preferentially in randomized trials in order to improve comparability of the current results with other treatment modalities. This should include the evaluation of optimal sequencing with newer and more potent systemic induction therapy approaches. Advances in patient selection based on new molecular markers might be of crucial interest in this context.

Finally modern external beam radiation techniques (IMRT, IGRT, SBRT), new radiation qualities (protons, heavy ions) or combinations with alternative boosting techniques (IOERT) widen the therapeutic window and contribute to the reduction of toxicity by improving normal tissue sparing and/or increasing efficacy by dose escalation or enhanced biological effectiveness. These techniques offer innovative treatment strategies, which should be further evaluated in prospective controlled trials.

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Ubiquitin proteasome system research in gastrointestinal cancer

Jia-Ling Zhong, Chang-Zhi Huang

Jia-Ling Zhong, Clinical Laboratory Department, Sichuan Academy of Medical Science and Sichuan Provincial People's Hospital, Chengdu 610072, Sichuan Province, China

Chang-Zhi Huang, Department of Etiology and Carcinogenesis and State Key Laboratory of Molecular Oncology, Cancer Institute and Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, China

Author contributions: Zhong JL and Huang CZ wrote and revised the manuscript.

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Correspondence to: Chang-Zhi Huang, Professor, Department of Etiology and Carcinogenesis and State Key Laboratory of Molecular Oncology, Cancer Institute and Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, No. 17 Panjiayuan Nanli, Chaoyang District, Beijing 100021, China. huangpumc@163.com
Telephone: +86-10-87787605
Fax: +86-10-87788426

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Abstract

The ubiquitin proteasome system (UPS) is important for the degradation of proteins in eukaryotic cells. It is involved in nearly every cellular process and plays an important role in maintaining body homeostasis. An increasing body of evidence has linked alterations in the UPS to gastrointestinal malignancies, including esophageal, gastric and colorectal cancers. Here, we summarize the current literature detailing the involvement of the UPS in gastrointestinal cancer, highlighting its role in tumor occurrence and development, providing information for therapeutic targets research and anti-gastrointestinal tumor drug design.

Key words: Ubiquitin proteasome system; Gastrointestinal cancer

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Core tip: The ubiquitin proteasome system (UPS) is involved in almost every cellular process, playing an important role in maintaining body homeostasis. Increasing evidence indicates that alterations in the UPS are correlated with gastrointestinal malignancies. Here, we review current information describing UPS members involved in gastrointestinal cancer, providing a resource for further study and clinical application.

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INTRODUCTION

The ubiquitin proteasome system (UPS) is important for the degradation of proteins in eukaryotic cells. Approximately 80%-90% of intracellular proteins involved in every cellular function are degraded through the UPS^[1]. Compared with the lysosomal system, the UPS is highly selective. It can regulate the proteins that it degrades *via* the ubiquitination-proteasome-deubiquitination mechanism to maintain homeostasis in the body. When regulatory proteins are stabilized by a decrease in degradation or are lost due to accelerated degradation, an imbalance is generated and diseases such as cancer occur.

Gastrointestinal cancer is a cancer of different organs of the digestive system, the most frequently occurring cancers of which are esophageal, gastric and colorectal. Despite improvements in diagnostic and therapeutic methods, gastrointestinal cancers remain a significant threat to patients^[2]. Recently, growing evidence has indicated that the UPS is linked to the development of gastrointestinal cancer. In this review, we discuss the members of the UPS thought to be involved in gastrointestinal cancer and highlight their roles in tumor occurrence and development.

The UPS is an important pathway for intracellular protein degradation

In cells, there are two main systems utilized for protein degradation: The autophagy-lysosome system and the UPS^[3,4]. The lysosomal pathway degrades extracellular proteins imported into the cell by endocytosis or pinocytosis, while the UPS controls the degradation of intracellular proteins^[5,6].

The UPS is composed of ubiquitin, ubiquitination enzymes, deubiquitination enzymes (DUBs), and proteasomes.

Ubiquitin is a highly conserved 76 amino acid protein, wild-expressed in eukaryotic cells^[7]. During the ubiquitination process, multiple ubiquitin proteins can be covalently attached to a target protein by ubiquitination enzymes^[8]. These ubiquitination enzymes include ubiquitin activating enzyme (E1), ubiquitin carrier protein (E2), and ubiquitin protein ligase (E3). Initially, E1 activates ubiquitin in an ATP-dependent manner, forming a thioester linkage between the carboxy-terminal glycine residue of ubiquitin and a cysteine in the active site of the E1 enzyme. Activated ubiquitin is then transferred from an E1 enzyme to a cysteine residue of an E2 enzyme. E3 then catalyzes the final step of the ubiquitination process by transferring ubiquitin to lysine residues of targeted proteins, forming a polyubiquitin chain that earmarks the targeted proteins^[8-10]. Humans possess two E1 enzymes (UBA1 and UBA6), several dozen E2 enzymes, and several hundred E3 enzymes^[11-13]. The specificity of the E3 enzymes determines the specific recognition of target proteins, providing selectivity in which proteins are targeted to the proteasome for degradation^[10,14,15].

Upon ubiquitination, targeted protein is degraded by

the 26S proteasome in an ATP-dependent manner^[16,17]. The 26S proteasome is a complex consisting of a proteolytic core particle (20S proteasome) that is capped at both ends by 19S regulatory particles (19S regulatory complex). The 20S proteasome is a barrel-shaped complex comprised of four stacked rings and contains multiple catalytic centers in the chamber. The 19S proteasome recognizes a polyubiquitinated protein, unfolds it, liberates it from the polyubiquitin chain, and translocates the protein into the proteasome chamber for degradation. The ubiquitin molecules are recycled, and the peptides generated are used for antigen presentation or are degraded into amino acids that are recycled for new protein synthesis^[9,10,17].

DUBs are a cluster of enzymes that oppose the action of the E3 ligases by cleaving the isopeptide bonds between lysine residues of targeted proteins and the C-terminal glycine of ubiquitin. They play important roles in maintaining the balance of the UPS. Analysis of the human genome has indicated the presence of ~ 100 functional DUBs^[18-20].

Tumorigenesis consists of several steps, including self-sufficiency in growth signals, insensitivity to growth inhibitor signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis^[21-24]. The UPS is an important regulator of protein degradation that is involved in every cellular function, including cell proliferation, apoptosis, migration and invasion. Thus, deregulation of this system may lead to tumorigenesis.

The UPS in esophageal cancer

Esophageal cancer (EC) is the eighth most common type of cancer, and ranks as the sixth leading cause of cancer-related mortality worldwide. The incidence of EC varies internationally, with the highest rates found in Eastern Asia and in Eastern and Southern Africa^[2,25].

Several E2 enzymes, including ubiquitin-conjugating enzyme H10 (UBCH10, also known as UBE2C), ubiquitin-conjugating enzyme E2L3 (UBE2L3, also known as UBCH7), E2-EPF ubiquitin carrier protein (UCP), and ubiquitin-conjugating enzyme E2D3 (UBE2D3) have been shown to be involved in the development of EC. UBCH10 is expressed in cancerous and dysplastic esophageal lesions, but not in normal tissue. Its expression is positively correlated with lymph nodes metastasis (LNM), TNM classification, and clinical stages, and negatively correlated with relapse-free survival period. Down-regulation of UBCH10 can inhibit cell proliferation and induce the sensibility to the treatment of MG-262^[26,27]. Knockdown of UBE2L3 expression can reduce the anoikis resistance of EC cells^[28]. Higher level of UCP has been linked to a greater tumor burden, poor response to neoadjuvant therapy, and worse overall survival for EC patients. Furthermore, UCP down-regulation can inhibit the proliferation, migration and invasion of EC cells, probably through the VHL/HIF-1 α -TGF- β 1 pathway^[29]. UBE2D3 expression is significantly lower in EC tissues and is correlated with histological grade, N stage, and

recurrence, suggesting that it acts as a tumor suppressor in EC. UBE2D3 may be involved the hTERT signal pathway, which can promote the development of invasive esophageal squamous cell cancer by interacting with the epidermal growth factor receptor and p53^[30,31].

Numerous E3 enzymes participate in the development of EC, some of which promotes tumor development. C-terminal Hsp-interacting protein (CHIP) exhibits a higher expression level in metastatic lymph nodes and is positively correlated with a poor survival rate in stage III EC patients^[32]. F-box protein 31 (FBXO31) is an ubiquitin ligase whose cytoplasmic expression is concordant with the nuclear expression of cyclin D1. In EC tissues, higher FBXO31 expression level is significantly correlated with depth of tumor invasion, clinical stage, and poorer prognosis^[33]. p53-associated cellular protein-testes derived (PACT) is highly up-regulated in EC. Experimental studies have revealed that knockdown of PACT significantly attenuates the p53-Hdm2 interaction, reduces p53 polyubiquitination, and enhances p53 accumulation, leading to both apoptosis and cell growth retardation^[34]. SMAD specific E3 ubiquitin protein ligase 2 (Smurf2) targets TGF pathway-restricted Smad2. In EC, high expression of Smurf2 is correlated with depth of invasion, LNM, and poor survival rate. High expression of Smurf2 can down-regulate Smad2, which in turn regulates the TGF signaling pathway^[35]. S-phase kinase-interacting protein 2 (Skp2) can interact with the S-phase kinase Cdk2/cyclinA and is involved in the ubiquitin-dependent degradation of p27. Skp2 expression is closely correlated with TNM stage. High expression of Skp2 is associated with poor overall survival in resectable EC. Further study reveals that knockdown of Skp2 inhibits cell migration and invasion and sensitizes cancer cells to anoikis, at least in part through the phosphoinositidyl 3-kinase-Akt pathway^[36,37]. Ubiquitin-like with PHD and ring finger domains 1 (UHRF1) is overexpressed in EC tissues. Its expression is correlated with the T-stage and N-stage as well as with differentiation. Down-regulation of UHRF1 can enhance the radio-sensitivity of TE-1 cells by altering cell cycle progression, increasing apoptosis and decreasing DNA damage repair capacity^[38].

Some E3 enzymes act as tumor suppressors in EC. F-box protein 4 (FBX4) is a ubiquitin ligase that directs the ubiquitylation of cyclin D1. Increased FBX4 function can enhance the normal activity of EC cells, and 14-3-3 ϵ is involved in regulating its function^[39]. F-box and leucine-rich repeat protein 19 (FBXL19) functions as an antagonist of Rac3 by regulating its stability, and it also regulates the TGF β 1-induced down-regulation of E-cadherin. Over-expression of FBXL19 attenuates TGF β 1-induced E-cadherin down-regulation and the elongation phenotype of EC cells^[40,41]. F-box and WD repeat domain containing 7 (FBXW7, E3 ubiquitin protein ligase) can induce the degradation of positive cell cycle regulators, such as Myc, cyclin E and Jun. In EC tissues, a decrease in FBXW7 copy number regulates FBXW7 mRNA expression, and reduced expression of FBXW7 is an independent prognostic factor in EC^[42].

Besides, there are still some E3 enzymes play controversial roles in EC. MDM2 (MDM2 proto-oncogene) is a key negative regulator of P53, but its expression state and clinicopathological parameters in esophageal squamous cell carcinoma are controversial. This may be due to a lack of sufficient case numbers in each study or the use of different methods to detect MDM2 expression. Meta-analysis suggests that MDM2 acts as a potent marker of early primary tumor stages but poses a high risk of regional LNM in EC. Notably, the MDM2 309GG genotype may be associated with an increased risk of EC among Asians^[43,44].

DBUs, including ubiquitin specific peptidase 7 (USP7), ubiquitin specific protease-9X (USP9X), Ubiquitin-specific protease 22 (USP22), ubiquitin carboxyl-terminal hydrolase 37 (UCH37), and ubiquitin carboxyl-terminal hydrolase1 (UCHL1) are correlated with EC development. USP7 can deubiquitylate p53 and protect it from proteasome-mediated degradation. EC cells can be protected against metformin-induced growth inhibition by siRNA against USP7^[45]. Up-regulation of USP9X in EC tissues plays an important role in the formation and progression of precancerous lesions, and its increased expression is significantly correlated with poorer survival rate in EC patients^[46]. High expression of the USP22 protein is significantly associated with tumor progression, relapse and poor prognosis^[47]. The expression of UCH37 is higher in EC tissues and is associated with outcome and recurrence. UCHL1 is silenced by promoter region hypermethylation in EC, and the restoration of its expression suppresses EC cell colony formation^[48,49].

The UPS in gastric cancer

Gastric cancer (GC) is the second most common cause of cancer-related death, and its incidence rates are highest in Eastern Asia^[2].

The E2 enzyme UBCH10 is known to be involved in GC. UBCH10 is expressed at higher levels in primary stomach tumors compared with corresponding normal tissues^[50].

The oncogenic E3 ubiquitin ligases involved in GC are discussed in detail below. Autocrine motility factor receptor (AMFR) expression is significantly increased in GC tissues and is associated with invasion depth and LNM. Its expression is correlated with poor overall survival and an increased risk of recurrence in GC cases. AMFR is thought to participate in the EMT pathway because its expression is negatively correlated with E-cadherin expression and is positively correlated with N-cadherin^[51]. Cullin 1 (CUL1) overexpression is significantly correlated with GC TNM stage, depth of invasion, LNM, worse overall survival rate, and 3-year survival rate in GC patients. Experimental studies have demonstrated that CUL1 knockdown inhibits cell growth by up-regulating p27 expression and decreases cell adhesion by suppressing the expression of Src family kinases and focal adhesion kinase^[52]. MDM2 protein level is significantly up-regulated in GC and is significantly correlated with clinicopathologic

characteristics and a shorter overall survival of GC patients. Similar to its role in EC, the MDM2 309GG genotype may be significantly associated with an increased risk of GC^[43,53]. Makorin ring finger protein 1 (MKRN1) can simultaneously induce p53 and p21 ubiquitination as well as proteasome-dependent degradation. In GC cells, MKRN1 could affect gastric tumorigenesis by repressing cellular senescence and tumor-suppressive effects through the down-regulation of p14ARF in either a p53-dependent or -independent manner^[54]. RING box protein-1 (RBX1) exhibits a higher expression level in GC tissues, and silencing it significantly inhibits the proliferation of GC cells *in vitro*^[55].

E3 ubiquitin ligases with tumor suppressor activity in GC are discussed in detail below. checkpoint with forkhead and ring finger domains (CHFR) is reported to promote the ubiquitination and degradation of oncogenic proteins such as Aurora A and polo-like kinase 1^[56]. It is frequently down-regulated in GC as a result of CHFR promoter methylation, suggesting that it acts as a tumor suppressor in GC. Methylation of the CHFR promoter is correlated with tumor differentiation. CHFR methylation is significantly higher in poorly differentiated GC samples^[57]. Moreover, CHFR promoter methylation is a sensitive marker of the effect of docetaxel in GC patients^[58]. CHIP expression is significantly lower in GC tissues. CHIP down-regulation is correlated with LNM and tumor differentiation. Further study has demonstrated that CHIP down-regulation results in increased angiogenesis and contributes to GC progression and a poor prognosis, probably through the NF- κ B signaling pathway^[59-61]. FBXO31 expression is dramatically decreased in GC tissue and is significantly associated with tumor size, infiltration, clinical grade and patient prognosis. *In vitro*, FBXO31 overexpression significantly decreases colony formation, induces a G1-phase arrest, and inhibits the expression of CyclinD1 in GC cells. *In vivo*, ectopic expression of FBXO31 dramatically inhibits xenograft tumor growth in nude mice^[62]. FBXW7 mRNA expression in GC samples is markedly decreased, and its deregulation is associated with the presence of LNM and GC stage III-IV, as well as poor prognosis. Reduced FBXW7 expression is associated with MYC overexpression and a more invasive phenotype in GC cells^[63]. Neural precursor cell expressed, developmentally down-regulated 4-like (NEDD4L) is strongly related to the invasion and metastasis of GC. Tumors lacking NEDD4L expression exhibit a greater extent of LNM, lymphatic invasion, and venous invasion, and present poor clinical outcomes for GC patients^[64]. RNF180 (Ring finger protein 180) acts as a tumor suppressor in GC, and the methylated CpG site count of the RNF180 DNA promoter is highly associated with patient survival^[65]. Zinc and ring finger 3 (ZNR3) is down-regulated in gastric adenocarcinoma tissues. There is also a correlation between the down-regulation of ZNR3 and poor tissue differentiation. Further study has revealed that ZNR3 inhibits GC cell growth and promotes cell apoptosis by affecting the Wnt/ β -

catenin/TCF signaling pathway^[66].

E3 ubiquitin ligases with controversial functions in GC are discussed in detail below. Cbl proto-oncogene B (CBLB) is highly expressed in GC tissue with EGFR, and their expression levels have been linked to the invasion and development of GC. However, some studies have revealed that CBLB represses IGF-I-induced EMT, likely by targeting IGF-IR for degradation and further inhibiting the Akt/ERK-miR-200c-ZEB2 axis in GC cells^[67,68]. Constitutive photomorphogenic 1 (COP1, also known as RWD2) has been shown to regulate c-Jun and p53. One study found that COP1 mRNA was significantly decreased in GC tissues, and knockdown of COP1 in GC cells promoted cell proliferation and the expression of MMP1, MMP7 and MMP10^[69]. However, another study showed that COP1 overexpression was associated with poor prognosis in primary GC^[70]. Neural precursor cell expressed, developmentally down-regulated 4 (NEDD4) is a regulator of PTEN and plays a complicated role in GC. One study revealed that overexpression of NEDD4 was tightly associated with TNM stage and a lower GC survival rate. And knockdown of NEDD4 dramatically inhibited GC cell migration and invasion^[71]. However, another study demonstrated that NEDD4 increased in intestinal metaplasia compared to normal gastric mucosa and decreased in gastric carcinoma compared to dysplasia^[72].

The DUBs involved in GC are discussed in detail below. UCHL1 is frequently methylated in primary GCs and has been found to be more frequently methylated in diffuse-type GCs than in intestinal-type GCs. Moreover, UCHL1 is involved in galangin-induced apoptosis in human GC cells^[49,73]. Ubiquitin-specific protease 10 (USP10) is expressed at lower levels in GC tissues and cells compared to their wild-type equivalents. A lack of USP10 expression results in a marked propensity toward gastric wall invasion, LNM, highly malignant biological behavior, and poor survival^[74].

The UPS in colorectal cancer

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females. The highest incidence rates are in Australia and New Zealand, Europe, and Northern America.

The E2 enzymes related to CRC are discussed in detail below. UBCH10 is highly expressed in CRC. The depletion of UBCH10 hinders tumorigenesis both *in vitro* and *in vivo*, probably by regulating the expression of cell cycle proteins such as cyclin A and cyclin B1. Furthermore, n-acetyl-leu-leu-norleucinal (ALLN) treatment is more effective in tumors with lower UBCH10 expression^[75]. Ubiquitin-conjugating enzyme E2Q family member 2 (UBE2Q2) expression is increased in 65.11% colorectal carcinoma tissues compared with their corresponding normal tissues^[76]. Ubiquitin-conjugating enzyme E2I (UBE2I) RNAi suppresses the 3D growth of KRAS mutant CRC cells *in vitro* and attenuates tumor growth *in vivo*^[77].

The oncogenic E3 ubiquitin ligases in CRC are discussed in detail below. F-box and leucine-rich repeat

protein 20 (FBXL20) is overexpressed in human colorectal adenocarcinoma. Moreover, the inhibition of FBXL20 expression can effectively suppress cell proliferation and promote apoptosis in CRC cells, while the overexpression of FBXL20 promotes the invasive ability of CRC cells, possibly by inducing the degradation of SET and E-cadherin through caspase activation^[78,79]. HECT, UBA and WWE domain containing 1 (HUWE1) is required for the growth of CRC cells in culture and in orthotopic xenograft models. HUWE1 shRNA suppresses the clonogenic growth of CRC cells, and small molecule inhibitors of HUWE1 can inhibit MYC-dependent transactivation in CRC cells but not in stem and normal colon epithelial cells^[80]. UHRF1 expression is up-regulated in approximately two-thirds of CRC specimens and is particularly expressed in right compared with left hemicolon cancer. High UHRF1 expression tends to be associated with the depth of invasion and with E2F-1 expression. Knockdown of UHRF1 suppresses cellular growth in colon cancer cell lines^[81-83]. Ubiquitin-like with PHD and ring finger domains 2 (UHRF2) is up-regulated at both the transcriptional and translational levels in tumor tissues. Overexpression of UHRF2 is highly linked to clinical stage, depth of invasion, nodal involvement, tumor histologic grade and the presence of metastases. Patients with UHRF2-positive tumors have a much lower disease-free survival and overall survival^[84]. Skp2-siRNA effectively inhibits proliferation, increases the level of apoptosis, and induces G0/G1 phase arrest of colon cancer cells, along with increasing p27 and p16 protein levels. Tumorigenicity experiments show that the inhibition of Skp2 significantly increases the survival. Skp2 is associated with a poor therapeutic response and adverse outcomes in rectal cancer patients treated with neoadjuvant chemoradiotherapy^[87].

E3 ubiquitin ligases exhibiting tumor suppressor activity in CRC are discussed in detail below. CHIP is down-regulated, predominantly in the late stages of CRC, and the CHIP promoter is hypermethylated in CRC specimens. Overexpression of CHIP results in impaired tumor growth in nude mice and decreased migration and invasion abilities of tumor cells. Further study reveals that CHIP negatively regulates NF- κ B signaling by promoting the ubiquitination and degradation of p65. The suppressive effect of CHIP leads to decreases in the expression of NF- κ B-targeted oncogenes, including Cyclin D1, c-Myc, MMP-2, VEGF and IL-8^[88]. Neural precursor cell expressed, developmentally down-regulated 4-like (NEDD4L) mRNA is significantly down-regulated in all CRCs. NEDD4L protein is significantly decreased in CRC compared to adjacent normal mucosa. Moreover, NEDD4L inhibits canonical Wnt signaling at or below the level of β -catenin *in vitro*^[89]. Neuregulin receptor degradation protein-1 (NRDP1) is significantly decreased in CRC tissues. Knockdown of NRDP1 enhances the proliferation of CRC cells, while the overexpression of NRDP1 inhibits the proliferation of CRC cells. Further analysis shows that NRDP1 may induce the degradation of its target ErbB3 to inhibit the activation of both the

ERK/MAPK and PI3K/Akt pathways in CRC cells, which seems to affect cell proliferation *via* the nuclear retention of a major cell-cycle inhibitor, p27. In addition, NRDP1 inhibits the expression of MMP7, which is required for cell invasion^[90,91]. Ring finger protein 43 (RNF43) can negatively regulate Wnt signaling, and its gene mutations in this gene has been found in over 18% of colorectal adenocarcinomas. Truncating mutations of RNF43 are more prevalent in microsatellite-unstable tumors and show mutual exclusivity with inactivating APC mutations in colorectal adenocarcinomas. These results indicate that RNF43 is one of the most commonly mutated genes in CRC^[92].

FBXW7 plays a controversial role in CRC. On one hand, FBXW7 mRNA expression is significantly lower in tumor tissues, an expression pattern correlated with poorer prognosis. *In vitro*, FBXW7-specific siRNA enhances the expression of c-MYC and cyclin E and promotes cell proliferation^[93]. Moreover, studies have found that the FBXW7 mutation is correlated with colorectal tumorigenesis^[94]. On the other hand, a large-scale study has revealed that there is no strong association between patient prognosis and FBXW7 mutation^[95].

The DUBs involved in CRC are discussed in detail below. OTU deubiquitinase, ubiquitin aldehyde binding 1 (OTUB1) is overexpressed in CRC tissues, and its expression level is associated with metastasis. A high OTUB1 expression level is also associated with poor survival, and OTUB1 serves as an independent prognostic factor in multivariate analysis. Further study has revealed that OTUB1 promotes the metastasis of CRC cell lines *in vitro* and *in vivo* by regulating EMT^[96]. Ubiquitin specific peptidase 11 (USP11) overexpression is frequently observed in CRC tissues and is correlated with poor survival. CRC cell lines expressing high levels of USP11 exhibit strong resistance to Smac mimetic-induced cIAP2 degradation. Furthermore, USP11 down-regulation sensitizes these cells to apoptosis induced by TRAIL and BV6, and suppresses tumor growth in a xenograft model^[97]. Ubiquitin-specific protease 22 (USP22) expression is significantly higher in primary CRCs than in the paired non-cancerous tissues at both the mRNA and protein levels. Higher USP22 expression is significantly associated with shorter periods of disease-specific survival and shorter disease-free survival. In addition, USP22 expression is significantly correlated with BMI-1, c-Myc and cyclin D2 and is a novel regulator of the SIRT1-STAT3 signaling pathway^[98-100]. Ubiquitin-specific protease 28 (USP28) deletion results in the fewer intestinal tumors of the murine model in CRC. And in established tumors, USP28 deletion reduces tumor size and dramatically increases lifespan^[101]. Ubiquitin-specific protease 33 (USP33) expression is down-regulated in CRC samples, and a reduced USP33 mRNA level is correlated with increased tumor grade, LNM and poor patient survival. USP33 acts as a tumor suppressor in CRC by mediating the inhibitory function of Slit-Robo signaling on CRC cell migration^[102]. USP44

Table 1 The roles of ubiquitin proteasome system members in gastrointestinal cancer

Enzyme	Esophageal cancer			Gastric cancer			Colorectal cancer		
	P ¹	S ²	C ³	P ¹	S ²	C ³	P ¹	S ²	C ³
E2 enzyme	UBCH10 UBE2L3 UCP	UBE2D3		UBCH10			UBCH10 UBE2Q2 UBE2I		
E3 enzyme	CHIP FBXO31 PACT Smurf2 Skp2 UHRF1	FBX4 FBXL19 FBXW7	MDM2	AMFR CUL1 MDM2 MKRN1 RBX1	CHFR CHIP FBXO31 FBXW7 NEDD4L RNF180 ZNRF3	CBLB COP1 NEDD4	FBXL20 HUWE1 UHRF1 UHRF2 Skp2	CHIP NEDD4L NRDP1 RNF43	FBXW7
DUBs	UCH37 USP9X USP22	UCHL1 USP7			UCHL1 USP10		OTUB1 USP11 USP22 USP28	USP33 USP44	UCHL1

¹Tumor promoter role; ²Tumor suppressor role; ³Controversial role. AMFR: Autocrine motility factor receptor; CBLB: Cbl proto-oncogene B; CHFR: Checkpoint with forkhead and ring finger domains; CHIP: C-terminal Hsp-interacting protein; COP1: Constitutive photomorphogenic 1; CUL1: Cullin 1; DUB: Deubiquitination enzymes; FBXO31: F-box protein 31; FBX4: F-box protein 4; FBXL: F-box and leucine-rich repeat protein; FBXW7: F-box and WD repeat domain containing 7; HUWE1: HECT, UBA and WWE domain containing 1; MDM2: MDM2 proto-oncogene; MKRN1: Makorin ring finger protein 1; NEDD4: Neural precursor cell expressed, developmentally down-regulated 4; NEDD4L: Neural precursor cell expressed, developmentally down-regulated 4-like; NRDP1: Neuregulin receptor degradation protein-1; OTUB1: OTU deubiquitinase, ubiquitin aldehyde binding 1; PACT: p53-associated cellular protein-testes derived; RBX1: RING box protein-1; RNF: Ring finger protein; Smurf2: SMAD specific E3 ubiquitin protein ligase 2; Skp2: S-phase kinase-interacting protein 2; UBCH10: Ubiquitin-conjugating enzyme H10; UBE2L3: Ubiquitin-conjugating enzyme E2L3; UBE2D3: Ubiquitin-conjugating enzyme E2D3; UCP: E2-EPF ubiquitin carrier protein; UHRF: Ubiquitin-like with PHD and ring finger domains; UCH37: Ubiquitin carboxyl-terminal hydrolase 37; USP: Ubiquitin specific protease; UCHL1: Ubiquitin carboxyl-terminal hydrolase1; UBE2Q2: Ubiquitin-conjugating enzyme E2Q family member 2; UBE2I: Ubiquitin-conjugating enzyme E2I; ZNRF3: Zinc and ring finger 3.

is hypermethylated in all CRC cell lines and in most colorectal adenomas, but rarely in normal mucosa samples^[103]. UCHL1 plays a controversial role in CRC. Some investigations have shown that UCHL1 is more frequently methylated in CRC tissues than in normal colorectal tissues, whereas other studies have indicated that high UCHL1 expression is related to colorectal tumor progression, invasion, LNM, and poor clinical outcome^[104].

CONCLUSION

The UPS plays an essential role in controlling every cellular process and in maintaining the homeostasis of the body. In this review, we discussed the members of the UPS known to be involved in gastrointestinal cancer. Among the UPS, the dysregulation of the enzymes E2, E3 and DUBs play the most prominent role in tumorigenesis and development. As shown in Table 1, some enzymes may be just involved in one type cancer, while others may be involved in two or three types. Moreover, a single enzyme may play different roles in different cancers, as is the case for CHIP and UCHL1. This suggests that these enzymes may exhibit tissue specificity or may function through different mechanisms in different situation. Further study is necessary to better understand the biological function of the UPS and for the development of new therapeutic targets and anti-tumor drugs.

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Case Control Study

Risk factors for the development of colorectal carcinoma: A case control study from South India

Santhana Krishnan Iswarya, Kariyarath Cheriya Premarajan, Sitanshu Sekhar Kar, Sathasivam Suresh Kumar, Vikram Kate

Santhana Krishnan Iswarya, Kariyarath Cheriya Premarajan, Sitanshu Sekhar Kar, Departments of Preventive and Social Medicine, Jawaharlal Institute of Postgraduate Medical Education and Research, Pondicherry 605006, India

Sathasivam Suresh Kumar, Vikram Kate, Department of Surgery, Jawaharlal Institute of Postgraduate Medical Education and Research, Pondicherry 605006, India

Author contributions: Iswarya SK, Premarajan KC and Kate V designed the research; Iswarya SK collected data; Iswarya SK, Kar SS and Kumar SS analyzed the data and prepared the manuscript; Premarajan KC, Kumar SS and Kate V corrected and revised the manuscript; and Kate V is the guarantor.

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Correspondence to: Vikram Kate, MS, FRCS (Eng.), FRCS (Ed.), FRCS (Glasg.), PhD, MAMS, FIMSA, MASCRS, FACS, FACG, MFSTEd, Professor of General and Gastrointestinal Surgery, Department of Surgery, Jawaharlal Institute of Postgraduate Medical Education and Research, Temple Road,

Dhanvantri Nagar, Pondicherry 605006, India. drvikramkate@gmail.com
Telephone: +91-413-2296741
Fax: +91-413-2272066

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Abstract

AIM: To study the association of colorectal carcinoma (CRC) with diet, smoking, alcohol, physical activity, body mass index, family history and diabetes.

METHODS: All consecutive patients with CRC confirmed by histopathology diagnosis were included. Age (± 5 years) and gender matched controls were selected among the patients admitted in surgery ward for various conditions without any co-existing malignancy. Food frequency questionnaire (FFQ) was developed and validated after pretesting by investigator trained in data collection techniques. Cases and controls were interviewed ensuring privacy, in similar interview setting, with same duration of time for both cases and controls without any leading question. Biological variables like family history of CRC in first degree relatives, history of diabetes mellitus; behavioral factors like tobacco use both smoking and smokeless form, alcohol consumption and physical activity were recorded. Dietary details were recorded using a FFQ consisting 29 food items with seven categories. Analysis was done using appropriate statistical methods.

RESULTS: Ninety-four histopathologically confirmed cases of CRC and equal number of age and gender

matched controls treated over a period of two years were studied. Age distribution, mean age, male to female ratio, education level and socioeconomic status were similar in cases and controls. Intake of food items was categorized into tertile due to skewed distribution of subjects as per recommended cut off for consumption of food item. On univariate analysis red meat [OR = 7.4 (2.935-18.732)], egg [OR = 5.1 (2.26-11.36)], fish, fried food and oil consumption were found to be risk factors for CRC. On multivariate analysis red meat consumption of more than 2-3 times a month (OR = 5.4; 95%CI: 1.55-19.05) and egg consumption of more than 2-3 times a week (OR = 3.67; 95%CI: 1.23-9.35) were found to be independent risk factors for the development of CRC.

CONCLUSION: Egg and red meat consumption found to be independent risk factors for CRC. Smoking, alcohol, physical activity and family history were not associated with increased risk.

Key words: Dietary factors; Smoking; Rectal cancer; Red meat; Colorectal malignancy

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Core tip: In this hospital based case control study, egg consumption of 2-3 times a week and red meat consumption of 2-3 times a month were found to be independent risk factors for the development of colorectal carcinoma. On the other hand smoking, alcohol, physical activity, diabetes and family history were not associated with an increased risk. There was no conclusive evidence to suggest that fruits and vegetable consumption has protective effect on colorectal carcinoma. Since red meat and egg had an increased risk, the community needs to be educated to reduce the consumption of red meat such as mutton and egg.

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INTRODUCTION

Colorectal cancer (CRC) is one amongst the leading cause of cancer related morbidity and mortality. CRC share 10% of the total cancers worldwide and accounts for 8% of all cancer related mortality; caused 608000 deaths worldwide^[1,2]. In India data from population based cancer registry at Bangalore, Chennai and Delhi showed significantly increased incidence of CRC from 1982-2006^[3].

Epidemiological studies have estimated that up to 70%-80% of CRCs could be ascribed to dietary,

environmental and lifestyle factors; suggesting majority of the risk factors are modifiable^[4]. It has been demonstrated that diet significantly influences the risk of developing CRC, and up to 70% reduction in the cancer burden can be achieved by changing the food habits^[5]. Many epidemiological studies across the globe have tried to evaluate the role of dietary and life style factors in the development of CRC, however a fair share of controversies exist among the observations^[6]. Majority of the studies that investigated the role of high vegetable and fruit diet failed to prove any significant reduction in the incidence of CRC.

For a long time, it was believed that low meat intake and high fiber vegetarian diet by Indian population is the reason for the low incidence of CRC in India. It was found that only two studies have been reported in literature from India regarding factors associated with CRC^[7,8]. Identifying the factors associated with decreased CRC incidence among Indian population may help in the prevention of CRC. Hence an attempt was made to study these factors through a case control study. The objective of the study was to find the association of CRC with life style variables (diet, smoking, alcohol, physical activity) and Biological Variables [body mass index (BMI), family history of CRC in 1st-degree relatives, history of diabetes mellitus].

MATERIALS AND METHODS

The study was conducted in Department of Preventive and Social Medicine in collaboration with department of Surgery in a tertiary care referral and research institute of India. This study was conducted from period of two years. This study was approved by the Institute Ethics Committee. The nature, methodology of the study was explained to the patient and informed consent was obtained. All the information collected was kept confidential and patient was given full freedom to withdraw from the study at any point during the study. All provisions of the Declaration of Helsinki were followed in this study.

All consecutive patients with confirmed histopathology diagnosis were included. Histopathology was done either pre-operatively or postoperatively. Diagnosis of CRC was confirmed by per-rectal sigmoidoscopic or endoscopic biopsy. In case where resection for colorectal malignancy was done as an emergency surgical procedure, the diagnosis was confirmed post operatively. CRC patients with co-existing malignancy were excluded. Age (\pm 5 years) and gender matched controls were selected among the patients admitted in Surgery ward for various conditions like inguinal hernia, varicose veins, necrotizing fasciitis and diabetic foot.

Patients with co-existing malignancies, familial adenomatous polyposis and patients admitted with any abdominal disorders were excluded from the study. Controls were selected within one week after selecting the case. When more than one control was eligible then

control was selected by simple random methods using lots. During initial phase of the study, food frequency questionnaire (FFQ) was developed and face validation was carried out by circulating among the faculty who were involved in the study. Pre-testing was done among 10 patients admitted in the surgery ward by investigator trained in data collection techniques. It helped to estimate the average time taken for questionnaire administration, examination and to check for comprehensibility of participants to the questions.

After pre-testing of questionnaire necessary modifications were carried out. After obtaining informed consent cases and controls were interviewed ensuring privacy, in similar interview setting, with same duration of time for both cases and controls without any leading question. Average time taken for each interview was around 45 min. Anthropometric measurements was taken at the end of the interview. Pre-tested questionnaire which elicited information on demographic parameters like name, age, gender; Social variables like education, occupation, income, presenting complaints; biological variables like family history of CRC in first degree relatives, history of diabetes mellitus; behavioral factors like tobacco use both smoking and smokeless form, alcohol consumption and physical activity.

The alcohol consumption among study participants was measured and classified as per the World Health Organization STEPwise approach to surveillance of non-communicable diseases. The STEPS questionnaires used for the study are available in the internet from: http://www.who.int/ncd_surveillance/en/steps_framework_dec03.pdf. The alcohol consumption pattern of drinkers (amount, type and frequency) was noted and converted in terms of average alcohol consumed in grams per day. These were further classified as abstainers (who never consumed alcohol in past 12 mo), grade 1 (< 39.9 g/d), grade 2 (40-59.9 g/d) and grade 3 (> 60 g/d). The physical activity was measured using international physical activity questionnaire-short version. Metabolic equivalent (MET) levels for walking, moderate and vigorous intensity activities were taken as 3.3, 4.0 and 8.0. The activities were measured separately (MET level × minutes of activity/day × days per week) and expressed as total MET min/wk. Based on the total scores, study participants were categorized in to low (< 600 MET min/wk), moderate (600-3000 MET min/wk) and high (> 3000 MET min/wk) level of physical activity.

Dietary details were recorded using a FFQ consisting 29 food items with seven categories (never or hardly ever, once a month, 2-3 times a month, once a week, 2-3 times a week, 4-6 times a week, once a day or more) for egg, chicken, mutton, beef, pork, fruits, vegetables, fried foods, type of oil, type of food, tea, coffee; anthropometric measurements including weight, height, hip circumference, waist circumference also were recorded.

Sample size was calculated using *n* Master software 2.0 for matched case control study, taking exposure in controls for non-vegetarian food as 58% and OR 3.38

Table 1 Socio demographic details of study population, *n* (%)

Variable	Cases	Controls
Age (yr)		
< 40	9 (9.6)	7 (7.4)
40-49	21 (22.3)	18 (19.1)
50-59	28 (29.8)	29 (30.9)
60-69	30 (31.9)	32 (34)
≥ 70	6 (6.4)	8 (8.5)
Educational status		
Never attended school	37 (39.4)	33 (35.1)
1-4	23 (24.5)	34 (36.2)
5-7	15 (16)	10 (10.6)
8-10	14 (14.9)	9 (9.6)
11-12	1 (1.1)	6 (6.4)
Graduation	4 (4.3)	2 (2.1)
Occupation		
Non worker	23 (24.5)	19 (20.2)
Skill I	44 (46.8)	59 (62.8)
Skill II	25 (26.6)	16 (17)
Skill III	2 (2.1)	0
PCI in indian rupees/mo		
Class I > 4400	1 (1.1)	0
Class II 2200-4399	1 (1.1)	2 (2.1)
Class III 1320-2199	5 (5.3)	6 (6.4)
Class IV 660-1319	34 (36.2)	38 (40.4)
Class V < 660	53 (56.4)	48 (51.1)

PCI: Per Capita Income (after adjusting for Consumer Price Index of 2011).

at 95%CI, 80% power the minimum sample size was 93^[9].

Analysis was done using SPSS version 20^[10]. Socio-demographic details and frequency of food intake were expressed in proportions. Univariate analysis for categorical variables (diet, smoking, alcohol, physical activity, BMI, history of diabetes, family history) were done using χ^2 test. Seven frequencies of food item intake were categorized into tertile. Tertile1 corresponds to lowest frequency of intake and tertile 3 corresponds to highest frequency of intake. OR was calculated for highest tertile of intake relative to lowest tertile by logistic regression. Factors having *P* value < 0.05 in univariate analysis were included as parameter for multivariate analysis using logistic regression. Results of multivariate analysis were given as OR with 95%CI. All *P* values were two tailed and significant when values were less than 0.05.

RESULTS

A total of 94 cases and controls were included in the study. The mean age group of cases and controls were 54.1 ± 11.5 years and 55 ± 11.8 years respectively. Age distribution of cases and controls were in the range of 17-78 years. There was almost equal distribution of males and females 48.9% and 51.1% respectively among the study subjects (Table 1). Around 39.4% cases and 35.1% of controls never attended school. In both cases and controls more than 50% of them belonged to class V socio economic status.

The distribution of subjects as per recommended cut off for consumption of food item was much skewed

Table 2 Frequency of food intake among cases and controls, *n* (%)

Food item		Never or hardly ever	Once a month	2-3 times/mo	Once a week	2-3 times/wk	4-6 times/wk	Once a day	Total
Egg	Case	7 (7.4)	17 (18.1)	6 (6.4)	28 (29.8)	21 (22.3)	7 (7.4)	8 (8.5)	94
	Control	8 (8.5)	36 (38.3)	10 (10.6)	27 (28.7)	8 (8.5)	-	5 (5.3)	94
Chicken	Case	13 (13.8)	31 (33)	9 (9.6)	36 (38.3)	5 (5.3)	-	-	94
	Control	12 (12.8)	45 (47.9)	14 (14.9)	19 (20.2)	4 (4.3)	-	-	94
Mutton	Case	23 (24.5)	40 (42.6)	4 (4.3)	25 (26.6)	1 (1.1)	1 (1.1)	-	94
	Control	44 (46.8)	42 (44.7)	3 (3.2)	4 (4.3)	1 (1.1)	-	-	94
Fish	Case	26 (27.7)	49 (52.1)	2 (2.1)	6 (6.4)	10 (10.6)	1 (1.1)	-	94
	Control	27 (28.7)	61 (64.9)	1 (1.1)	2 (2.1)	1 (1.1)	2 (2.1)	-	94
Beef	Case	68 (72.3)	1 (1.1)	1 (1.1)	18 (19.1)	6 (6.4)	-	-	94
	Control	81 (86.2)	6 (6.4)	-	7 (7.4)	-	-	-	94
Pork	Case	81 (86.2)	9 (9.6)	1 (1.1)	2 (2.1)	1 (1.1)	-	-	94
	Control	87 (92.6)	5 (5.3)	-	2 (2.1)	-	-	-	94
Fried foods	Case	3 (3.2)	32 (34.0)	6 (6.4)	35 (37.2)	18 (19.1)	-	-	94
	Control	5 (5.3)	45 (47.9)	14 (14.9)	28 (29.8)	2 (2.1)	-	-	94
Fruits	Case	32 (34.0)	37 (39.4)	7 (7.4)	6 (6.4)	5 (5.3)	3 (3.2)	4 (4.3)	94
	Control	36 (38.3)	23 (24.5)	14 (14.9)	13 (13.8)	3 (3.2)	-	5 (5.3)	94
Vegetables	Case	-	-	-	-	13 (13.8)	7 (7.4)	74 (78.7)	94
	Control	-	-	-	-	2 (2.1)	8 (8.5)	84 (89.4)	94
Coffee	Case	81 (86.2)	-	-	-	-	-	13 (13.8)	94
	Control	87 (92.6)	-	-	-	-	-	7 (7.4)	94
Tea	Case	20 (21.2)	-	-	-	-	-	74 (78.7)	94
	Control	11 (11.7)	-	-	-	-	-	83 (88.3)	94

Table 3 Colorectal carcinoma risk associated with individual dietary item

Food item		Adjusted OR (CI)	P value
Egg	Tertile 1 Never or hardly ever	1	
	Tertile 2 Once a month	1.6 (0.85-3.33)	0.133
	Tertile 3 > 2-3 times a week	5.1 (2.26-11.36)	0.001
Chicken	Tertile 1 Never or hardly ever	1	
	Tertile 2 Once a month	0.6 (0.25-1.51)	0.297
	Tertile 3 Once a week	1.6 (0.64-4.19)	0.297
Mutton	Tertile 1 Never or hardly ever	1	
	Tertile 2 Once a month	1.8 (0.93-3.45)	0.070
	Tertile 3 More than 2-3 times a month	7.4 (2.93-3.45)	0.001
Fish	Tertile 1 Never or hardly ever	1	
	Tertile 2 Once a month	0.8 (0.44-1.60)	0.588
	Tertile 3 More than 2-3 times a month	3.2 (1.13-9.53)	0.028
Beef	Tertile 1 -	-	-
	Tertile 2 Never or hardly ever	1	
	Tertile 3 More than once a month	2.3 (0.13-4.99)	0.237
Fruits	Tertile 1 Never or hardly ever	1	
	Tertile 2 Once a month	1.8 (0.89-3.64)	0.099
	Tertile 3 More than 2-3 times a month	0.8 (0.39-1.68)	0.540
Vegetables	Tertile 1 2-3 times a week	1	
	Tertile 2 Once a day	0.4 (0.19-1.00)	0.050
	Tertile 3 -	-	-
Fried foods	Tertile 1 Never or hardly ever	1	
	Tertile 2 Once a month	0.61 (0.21-1.74)	0.350
	Tertile 3 2-3 times a month	2.52 (1.35-4.70)	0.004

as shown in (Table 2). Among cases 22.3% consumed egg 2-3 times a week compared to only 8.5% among the controls. In cases about one-fourth 24.5% never or hardly ever consumed mutton compared to 46.8% in controls. Beef consumption was reported to be low

among both cases and controls, 72.3% of cases and 86.2% of controls never or hardly ever consumed beef. Similarly more than 80% of cases and controls never or hardly ever consumed pork. Majority of cases 78.7% and controls 89.4% consumed vegetables once a day.

As distribution of subjects as per recommended cut off for consumption of food item was much skewed, intake of food items was categorized into tertile. The frequency cut-off into tertile is not same for all the food items. For certain food items (beef, pork, vegetables, tea, coffee) ranking into tertile was not possible due to its skewed distribution. Univariate logistic regression analysis was done considering these tertile groups as shown in (Table 3). It was observed that consumption of egg for more than 2-3 times a week increases the risk of getting CRC by five times [OR = 5.1 (2.26-11.36)] compared to those who never or hardly consume egg. Mutton consumption of more than 2-3 times a month increases the risk of CRC by 7 times [OR = 7.4 (2.935-18.732)] compared to those never or hardly consumes mutton. Consuming fish and fried foods more than 2-3 times a month increases the risk for CRC. Coffee consumption was not significantly associated with CRC [OR = 1.95 (0.76-5.43)]. Similarly Tea consumption also did not show any significant association with CRC in the present study [OR = 0.49 (0.22-1.70)].

Compared to never smokers, subjects who smoked < 10 pack years, 10-20 pack years and > 20 pack years were not at increased risk for CRC. Alcohol consumption of < 39.9 g/d, 40-59.9 g/d and > 60 g/d was not associated with increased risk for CRC compared to non-users. High (3000 METs/wk) and moderate (600-3000 METs/wk) level of physical activity was not protective for CRC. BMI greater than 25 is not associated with CRC risk. History of diabetes was not significantly

Table 4 Association of variables with colorectal carcinoma, n (%)

Variable	Cases	Controls	OR (CI)
Type of oil			
Refined	29 (30.9)	22 (23.4)	1
Groundnut	15 (16)	42 (44.7)	0.271 (0.12-0.61)
Palm	50 (53.2)	30 (31.9)	1.264 (0.62-2.59)
Type of food			
Moderate spicy	73 (77.7)	82 (87.2)	1
Very spicy	21 (22.3)	12 (12.8)	1.97 (0.91-4.28)
Smoking status			
Non-smoker	74 (78.7)	75 (79.7)	1
< 10 pack years	3 (3.19)	5 (5.31)	0.60 (0.14-2.63)
10-20 pack years	5 (5.31)	10 (10.6)	0.50 (0.16-1.55)
> 20 pack years	12 (12.8)	4 (4.3)	3.04 (0.93-9.85)
Alcohol use			
Non users	68 (72.3)	74 (78.7)	1
Grade I (< 39.9 g/d)	19 (20.2)	10 (10.6)	2.06 (0.89- 4.75)
Grade II (40-59.9 g/d)	4 (4.2)	6 (6.3)	0.72 (0.19-2.68)
Grade III (> 60 g/d)	3 (3.2)	4 (4.2)	0.81 (0.17-3.78)
Physical activity (METs/wk)			
Low (< 600)	18 (19.1)	24 (25.5)	1
Moderate (600-3000)	51 (54.3)	44 (46.8)	1.54 (0.74-3.21)
High (> 3000)	25 (26.6)	26 (27.7)	1.28 (0.56-2.91)
BMI (kg/m ²)			
< 18.5 (underweight)	19 (20.2)	10 (10.6)	1
18.5-22.99 (normal)	47 (50)	57 (60.6)	0.43 (0.18-1.02)
23-24.99 (over weight)	14 (14.9)	17 (18.1)	0.43 (0.15-1.22)
≥ 25 (obese)	14 (14.9)	10 (10.6)	0.73 (0.24-2.24)
Diabetes mellitus			
No	73 (53.3)	67 (46.7)	1
yes	21 (41.2)	30 (58.8)	1.62 (0.85-3.12)

METs/wk: Metabolic equivalents minutes per week; BMI: Body mass index.

associated with CRC risk (Table 4). Multivariate logistic regression results (Table 5) for those factors found to be statistically significant in univariate analysis (mutton, egg, fish, fried foods and type of oil) showed egg and mutton as independent risk factor.

DISCUSSION

Though population based cancer registries showed a statistically significant increase in the incidence of CRC in India from 1982-2006, very few studies have been done in India to document the association of modifiable risk factors with CRC. The present study attempted to identify the modifiable risk factors so that appropriate preventive measures can be planned. Red meat consumption more than 2-3 times a month found to be an independent risk factor in multivariate regression analysis and increased the odds of developing CRC by 5.41 (1.55-19.05) times compared to those never or hardly consume. This was similar to study by Nayak *et al.*^[7] which reported beef consumption more than once a week has increased risk compared to those who do not consume beef [OR = 4.25 (2.02-8.94)]. A study from Uruguay^[9] reported a positive association between CRC and high intake of red meat with OR = 3.38 (2.37-6.20). Similarly Singh *et al.*^[11] reported red meat intake more than once a week increased the risk compared to non-

Table 5 Factors independently associated with colorectal carcinoma

Food item	Adjusted OR (CI)	P value
Mutton		
Tertile 1	Never or hardly ever	1
Tertile 2	Once a month	2.62 (0.08-6.33)
Tertile 3	> 2-3 times a month	5.41 (1.55-19.05)
Egg		
Tertile 1	Never or hardly ever	1
Tertile 2	Once a month	1.54 (0.63-3.70)
Tertile 3	> 2-3 times a week	3.67 (1.23-9.35)
Fried foods		
Tertile 1	Never or hardly ever	1
Tertile 2	Once a month	0.76 (0.22-2.54)
Tertile 3	> 2-3 times a month	2.03 (0.95-4.43)
Fish		
Tertile 1	Never or hardly ever	1
Tertile 2	Once a month	0.02 (0.08-0.58)
Tertile 3	> 2-3 times a month	0.39 (0.09-1.62)
Type of oil		
Refined	NA	1
Ground nut	NA	0.4 (0.15-1.00)
Palm	NA	1.6 (0.75-4.04)

consumers [RR = 1.90 (1.16-3.11)].

In the western studies red meat consumption included beef, pork and mutton. However, in present study population due to cultural practices and beliefs beef and pork consumption were minimal. Subjects who consumed egg more than 2-3 times a week had 3.6 (1.23-9.35) times higher risk compared to those who never or hardly ever consume egg. This was similar to the study^[12] which reported consumption of egg more than 2-3 times/wk is associated with increased risk of CRC compared to those who never or hardly consume egg [OR = 2.95 (1.75-5)]. In the present study fish consumption more than 2-3 times a month is associated with increased risk for CRC in univariate analysis. In contrast Nayak *et al.*^[7] from Kerala showed 20% decreased risk of CRC with consumption of fish with every meal [OR = 0.32 (0.13-0.98)]. European study reported fish consumption more than 80 g/d was inversely associated with CRC compared to those consuming < 10 g/d [OR = 0.69 (0.54-0.88)]^[13]. Discrepancy between present study finding and other studies could be due to difference in type of fish consumed, amount of fish consumed, method of cooking and method of preservation.

Fruits and vegetable consumption was not found to be protective for CRC, similar to the findings reported in studies from Western countries^[14-16]. Frequent intake of fried food a proxy variable for high fat intake was associated with CRC [OR = 2.52 (1.35-4.70)] in univariate analysis but it was not an independent risk factor. In contrast studies reported consuming deep fried foods more than once a month was not associated with increased risk^[17,18]. Coffee consumption was not significantly associated with CRC [OR = 1.95 (0.76-5.43)]. A meta-analysis by Je *et al.*^[19] in 2008 showed no significant association between coffee consumption and colorectal cancer [RR = 0.91 (0.81-1.02)], nevertheless; studies have also shown protective effect of coffee in the development of CRC. Kato *et al.*^[20] in Japan found daily coffee consumption

had protective effect on both colon and rectal carcinoma compared with the non drinkers with RR = 0.43 (0.25-0.73) and RR = 0.53 (0.27-1.03), respectively. Reasons for varying results across studies are due to difference in type of coffee, serving size, brewing method and also cutoffs for high and low exposure categories varies between studies. Tea consumption did not show any significant association with CRC in the present study [OR = 0.49 (0.22-1.70)]. Similar findings were found by Nayak *et al.*^[27] where highest quartile of tea consumption has not shown any risk difference compared to lowest quartile with OR = 1.03 (0.62-1.71). In 2005, Michels *et al.*^[14] from United States reported that tea consumption of more than 5 cups per day was not significantly associated with CRC [HR = 1.01 (0.83-1.22)].

Smoking and alcohol use was not associated with CRC in contrast to increased risk reported in few studies^[21-25]. As smoking and alcohol were considered as undesirable behavior in community people tend to under report the use due to social desirability bias^[26]. This could be the reason for no association in the present study. High level of physical activity was not associated with decreased risk for CRC compared to low level of physical activity as reported in other studies^[27,28]. BMI was not significantly associated with CRC; in contrast studies reported high BMI increased risk for CRC^[29,30]. This could be due to underlying limitation of hospital based case-control study, where cases are ill and admitted to the hospital in late stage of disease. By the time patients seek medical attention they would have lost considerable amount of weight. The weight recorded at the time of admission may not find the true association.

Selection of appropriate controls is crucial to establish the true association between exposure (diet, smoking, alcohol, physical activity) and outcome (CRC). Selection of controls remains a major concern when designing a case-control study due to the issues involved in the internal validity and cost. Scientifically there is scope for introducing bias (selection bias and information bias) while selecting hospital based controls^[31]. However there is several advantage of selecting hospital controls such as feasibility, cost, travel time and better recall among hospital controls. Validation studies conducted by Li *et al.*^[32], González *et al.*^[33], Inoue *et al.*^[34] showed that hospital based controls elicit similar information to community controls in assessment of dietary risk factors. Hospital controls are preferred in a hospital based case-control study in view of the issues of practicability. It also reduces the cost involved in the travel and decreases the time taken for face-to-face interviews at field. It has also been demonstrated that the capacity to recall and report the exposures are better in those who are actively seeking health care advise than the members randomly selected from the population^[35].

Since it measures long term, average and habitual dietary intake; FFQ as a mean of dietary assessment have been found appropriate in many nutritional and epidemiological studies^[36]. FFQ captures pattern of food consumption over a period of time ranging from months to years. Pandey *et al.*^[37] from India reported

FFQ had good correlation (0.8) with 5 d diet record and was reproducible. The quantity of food consumed is considered an important factor in estimating the dietary intake of an individual; however, the frequency rather than the serving size has been found to be a better contributor to the variance in the intake of most foods.

Primary limitation of the study was dietary items were not quantified. Though efforts were taken to minimize the recall bias, change in dietary pattern of cases after development of symptoms might have led to biased reporting of their diet.

In conclusion, this hospital based case control study showed egg consumption of 2-3 times a week and mutton consumption 2-3 times a month as independent risk factor. On other hand smoking, alcohol, physical activity, history of diabetes and family history were not associated with increased risk for CRC and no conclusive evidence to suggest fruits and vegetable consumption as protective factor. Cohort study is required to assess the risk associated with commonly consumed dietary items in a given population.

As it was found that persons consuming red meat (mutton) had an increased risk of developing CRC (OR = 5.4), the community needs to be educated to reduce the consumption of red meat such as mutton, so that they can minimize their risk for developing CRC. Similarly, egg consumption was found to increase the odds of developing CRC (OR = 3.6), people especially adults need to be advised to reduce the egg consumption.

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COMMENTS

Background

Epidemiological studies have shown that significant proportion of colorectal cancer (CRC) incidence could be ascribed to dietary, environmental and lifestyle factors; suggesting majority of the risk factors are modifiable. Regional variation in the dietary and social habit could play a vital role in the causation of CRC and may be responsible for the geographical variations in the occurrence of CRC.

Research frontiers

For a long time, it was believed that low meat intake and high fiber vegetarian diet by Indian population is the reason for the low incidence of CRC in India. Only two studies have been reported in literature from India regarding factors associated with CRC and more research studies are require to evaluate or to confirm the risk factors. Identifying the factors associated with decreased CRC incidence among Indian population may help in the primary prevention of CRC across the globe.

Innovations and breakthrough

This study found that red meat consumption of more than 2-3 times a month egg consumption of more than 2-3 times a week are independent risk factors for the development of CRC. Contrary to common belief the study showed no association between CRC and smoking, alcohol consumption, physical activity,

body mass index or diabetes. Consumption of coffee or tea were also not associated with CRC.

Applications

As it was found that persons consuming red meat (mutton) had an increased risk of developing CRC (OR = 5.4), the community needs to be educated to reduce the consumption of red meat such as mutton, so that they can minimize their risk for developing CRC. Similarly, egg consumption was found to increase the odds of developing CRC (OR = 3.6), people especially adults need to be advised to reduce the egg consumption.

Peer-review

This is a good case control study taken a very relevant and significant problem to be studied. The article evaluated important life style and dietary factors for the possible relationship with colorectal cancer in South Indian population. The study showed significant association between red meat and egg consumption and certainly gives better insight and understandings about the other risk factors including smoking, alcohol consumption, body mass index, diabetes and physical activity, *etc.*

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Retrospective Cohort Study

Correlation of *Helicobacter pylori* and interleukin-8 mRNA expression in high risk gastric cancer population prediction

Wilaiwan Chongruksut, Sirikan Limpakan (Yamada), Bandhuphat Chakrabandhu, Chidchanok Ruengorn, Sirisak Nanta

Wilaiwan Chongruksut, Sirikan Limpakan (Yamada), Bandhuphat Chakrabandhu, Department of Surgery, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand

Chidchanok Ruengorn, Faculty of Pharmacy, Chiang Mai University, Chiang Mai 50200, Thailand

Sirisak Nanta, Mae Sai District Hospital, Mae Sai, Chiang Rai 50200, Thailand

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Correspondence to: Sirikan Limpakan (Yamada), MD, PhD, FARCS(T), Associate Professor, Division Chief of Gastrointestinal Surgery and Endoscopy, Department of Surgery, Faculty of Medicine, Chiang Mai University, 239 Huai Kao Road, T. Suthep, A. Mueang, Chiang Mai 50200, Thailand. siyamada@yahoo.com
Telephone: +66-81-6716737

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Abstract

AIM: To evaluate (1) the association of the *Helicobacter*

pylori (*H. pylori*) test and interleukin-8 (*IL-8*) mRNA expression alone and the severity of gastric cancer (GC); (2) the association of both tests were added to patients' characteristics to identify Thai suspected patients of gastric cancer who would receive the most benefit; and (3) diagnostic value of levels of *IL-8* mRNA expression for gastric cancer.

METHODS: A cross-sectional analytical study was completed with 220 patients with 86 GC patients who underwent endoscopy with gastric surgery divided into non-metastasis and metastasis groups, and 134 patients with benign lesions who underwent endoscopic examination, at the Gastrointestinal Surgery and Endoscopy Unit, Chiang Mai University Hospital between 2006 and 2010. Of 220 patients, 86 cases of diagnosed gastric adenocarcinoma were in an advanced stage and 134 cases were non-cancer patients.

RESULTS: The *IL-8* mRNA expression showed predominant association with advanced GC when compared to *H. pylori* infection alone [OR (95%CI); 0.86 (0.49-1.53) vs 5.44 (3.08-9.62)] when including the patients' characteristics the highest of the area under the receiver operating characteristic curves (AuROC) of the model were males older than 40 years of age [AuROC (95%CI); 0.81 (0.75-0.86)]. However, preliminary testing for diagnostic indices of four cut-off points of *IL-8* mRNA expression to predict the severity of GC cases found an increasing suboptimal trend from the likelihood ratio of positive to differentiate the severity in the GC group. The *IL-8* mRNA expression showed a predominant association with GC when compared to *H. pylori* infection, especially in males older than 40 years of age who may benefit most from this test.

CONCLUSION: The future research of *IL-8* mRNA expression to predict severity in the gastric cancer group should be warranted.

Key words: *Helicobacter pylori*; Interleukin-8 mRNA

expression; Gastric cancer

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Core tip: The author reviewed updated basic research studies regarding linkages of inflammatory cytokine genetic expression level and gastric cancer (GC) risk prediction in the Thai population. The review focused on *interleukin-8 (IL-8)* mRNA expression and *Helicobacter pylori (H. pylori)* infection in which are found an increasing risk for GC and aggressive histologic types. We performed the epidemiologic data in-depth analysis, and make the various cut off points to discern which level of *IL-8* mRNA expression has remarkable predictive risk value in comparison with *H. pylori* infection to predict GC occurrence.

Chongruksut W, Limpakan (Yamada) S, Chakrabandhu B, Ruengorn C, Nanta S. Correlation of *Helicobacter pylori* and interleukin-8 mRNA expression in high risk gastric cancer population prediction. *World J Gastrointest Oncol* 2016; 8(2): 215-221 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v8/i2/215.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v8.i2.215>

INTRODUCTION

Gastric cancer (GC) is one of the most common cancers and continues to remain a major public health problem in the world, with a varying prevalence from 11% to 56% in different areas. The prevalence of GC in the United States was an estimated 74035 people^[1] and estimated 934000 cases, 56% of new cases from Eastern Asia, 41% from China, and 11% from Japan^[2]. In Thailand, the incidence rate of GC was 4.1:100000 for males and 2:100000 for females, especially in the northern part of Thailand which has a higher GC incidence rate with 6.6:100000 in males, and 4.5:100000 in females^[3].

GC is one of the most common causes of death from cancer worldwide, and most of the cases occur in developing countries^[4]. A gender difference of mortality of GC was reported; 14.3 per 100000 in men and 6.9 per 100000 in women worldwide^[5], as well as geographic countries; 20% mortality rate in Western countries vs up to 60% in Asia^[6].

Early diagnosis is crucial because of the possibility of early metastasis to other organs such as, liver, pancreas, omentum, esophagus, bile ducts, and lymph nodes^[7]. If GC was detected at an early stage, the five year survival was approximately 90%^[8]. Thus, in developing countries, early detection is most needed. The standard method or diagnosing GC is through the upper digestive endoscopy combined with biopsy and histopathological evaluation of the biopsy samples^[4]. This method has a high diagnostic accuracy of 95% to 99%^[9].

Helicobacter pylori (H. pylori) infection is widely regarded as the most important risk factor in the development of GC^[6] with from 0.5% to 2.0% developing

gastric adenocarcinoma^[10]. A meta-analysis of 34 cohort and case-control studied patients found that *H. pylori* carried a relative risk of GC of 3.02 (95%CI: 1.92-4.74) in high risk settings (China, Japan and Korea) and 2.56 (95%CI: 1.99-3.29) in low risk settings (Western Europe, Australia, and the United States)^[11]. Epidemiologic data indicates that GC occurs more frequently in populations with higher rates of *H. pylori* infection, and the World Health Organization has classified this bacterium as a Class 1 carcinogen for GC^[6]. *H. pylori* infection was important in the process of tissue remodeling, angiogenesis, tumor invasion and metastasis^[12] and induces a number of genes in host cells that are potential determinants of inflammation, angiogenesis, and metastasis including *interleukin-8 (IL-8)* gene expression^[13]. However, it remains unclear how *H. pylori* infection activates specific transcription factors and induces gene expression. Yamada *et al*^[14] indicated that the *H. pylori* infection in Thai GC patients was reported by combined histopathology and *H. pylori* IgG antibody test with 77.1% and 97.4% of sensitivity and specificity, respectively.

Moreover, *IL-8* mRNA expression is one of the factors that were possible influences which affect GC^[15], Yamada *et al*^[14,16] reported that GCs were detected in more than 80% of Thai patients with high levels of *IL-8* mRNA expression, while *H. pylori* infection and *IL-8* mRNA expression were relative risks for Thai GC, therefore *IL-8* mRNA expression may be a useful diagnostic and prognostic risk marker for GC. Similarly, Macri *et al*^[17] indicated that the level of serum *IL-8* mRNA expression may act as marker of GC. The high expression of *IL-8* mRNA expression was directly demonstrated with a poor prognostic histologic type in GC^[14,16].

Although, the association of *H. pylori* infection and *IL-8* mRNA expression and GC were demonstrated from several studies, most studies did not evaluate the association of these biomarkers and the GC severity, *i.e.*, no cancer, non-metastasis, and metastasis stage. There might be an increasing possibility of GC by gradient of *IL-8* mRNA expression. Moreover, some studies showed an association with independent factors such as advanced age, sex, and alcohol drinking. Therefore, this present study aimed to evaluate (1) the association of *H. pylori* test and *IL-8* mRNA expression alone and the severity of GC; (2) the association of both tests added to patients' characteristics to identify Thai suspected patient risk of GC who will receive the greatest benefit for follow up endoscopy; and (3) diagnostic value of four different levels of *IL-8* mRNA expression for GC cases.

MATERIALS AND METHODS

A cross-sectional analytical study was conducted in patients over 18 years of age. Eighty-six patients who underwent endoscopy were diagnosed with GC, and 134 patients who underwent endoscopic examination were diagnosed as non-GC, at the Gastrointestinal Surgery and Endoscopy Unit, Chiang Mai University Hospital between 2006 and 2010. All patients were comprehensively examined

by a gastrointestinal pathologist for *H. pylori* infection and combined histopathological diagnostic results. The outcomes of the study were divided into non-GC, and GC. In GC patients, those who were categorized in cancer Stages I, II, III and IV were in the GC group.

Tissue samples were taken by endoscopy with tissue *IL-8* mRNA expression conducted by real time relative quantitation polymerase chain reaction. Additionally, baseline characteristics; gender, age, alcohol drinking, smoking, stages of cancer, histological pathology were obtained by a physician and nurse, using a case record form. All enrolled patients were examined by endoscopy with a pathology result for *H. pylori* infection and received biopsy of tissues with *IL-8* mRNA expression. This study excluded all patients without results of pathology or tissue *IL-8* mRNA expression. The present study was approved by the Institutional Review Boards of the Faculty of Medicine, Chiang Mai University.

Statistical analysis

The demographic data were analyzed using χ^2 to test between groups, and the test for trend was used to test for proportion. An ordinal logistic regression, both univariable and multi-variable models, were performed to determine association of *H. pylori* and *IL-8* mRNA expression and severity of GC with or without patients' characteristics presented with crude and adjusted odds ratio with 95%CI. The area under the receiver operating characteristic curves (AuROC) was calculated and compared using a standard method. *IL-8* mRNA expression level was divided into four different cut-off points, and AuROC was compared to select the best cut-off point. Performance of each *IL-8* mRNA expression cut-off point was then evaluated for sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (LHR+), and negative likelihood ratio (LHR-) in only GC cases. A statistical significance level or alpha of 0.05 was selected for Type I error.

RESULTS

Of the 220 patients enrolled in this study, 86 cases were diagnosed with GC and underwent endoscopy with gastric surgery, and 134 non-cancer patients underwent endoscopic examination. Among those diagnosed with a non-GC, 45 cases were normal, 46 had benign lesions (polyps, erosion, mild superficial gastritis), and 42 cases had chronic active gastritis. When categorized by staging, 41 cases (47.67%), and 34 cases (39.53%) were in Stage III B, and Stage IV, respectively.

Two groups of patients were therefore assigned by order of severity to non-cancer, and advanced GC. Patients' characteristics found statistically significant when testing for trend were sex, age, and smoking status. The majority of GC patients were male, aged ≥ 40 years, and had a history of smoking (Table 1).

According to *H. pylori* active infection status and pathology, no statistical differences were found among

Table 1 Characteristics of gastric cancer and non-gastric cancer patients, *n* (%)

Characteristics	Gastric cancer <i>n</i> = 86	Non-gastric cancer <i>n</i> = 134	<i>P</i> value
Sex			< 0.001
Male	52 (60.47)	41 (30.60)	
Female	34 (39.53)	93 (69.40)	
Age			0.003
≥ 40	5 (5.81)	28 (20.90)	
< 40	81 (94.19)	106 (79.10)	
Mean \pm SD	56 \pm 11.29	48.5 \pm 11.21	
Alcohol drinking,	36 (41.87)	51 (38.06)	0.679
Smoking	24 (27.91)	12 (8.96)	< 0.001
Diseases			
Normal	0	45 (33.83)	
Benign lesion	0	46 (34.59)	
Chronic active gastritis	0	42 (31.58)	
Gastric cancer	86 (100)	0	
Stage			
I a	-	-	
I b	-	-	
II a	1 (1.16)	-	
II b	2 (2.23)	-	
III a	8 (9.30)	-	
III b	41 (47.67)	-	
IV	34 (39.53)	-	
Histological grade			
Poorly differentiated	25 (29.76)	-	
Signet ring cell	36 (42.82)	-	
Moderate differentiated	16 (19.05)	-	
Well differentiated	7 (8.33)	-	

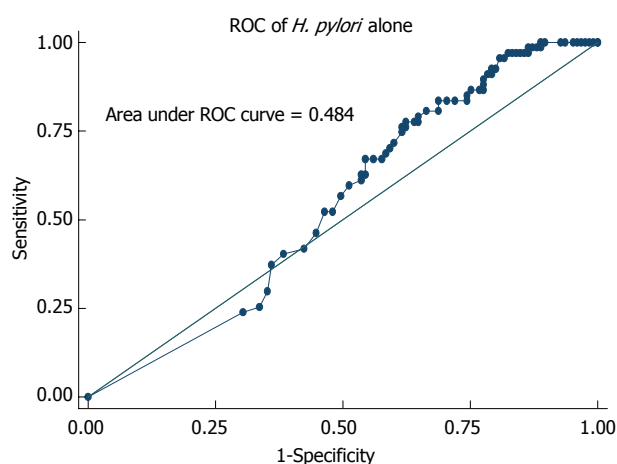
the groups. While, *IL-8* mRNA expression had the highest level in the metastatic GC group (median 325) and non-cancer group (median 19.72), respectively. An *IL-8* mRNA expression was transformed to log₁₀ and divided into five cut-off points. The higher the cut-off point, the higher proportion of the severity of GC was demonstrated (Table 2).

The value of *H. pylori*, and *IL-8* mRNA expression as biomarkers, alone or combined with patients' characteristics, were determined. The results showed the predominant performance of *IL-8* mRNA expression over *H. pylori* pathology and serum IgG results (Model 1 vs Model 2). *H. pylori* pathology results in accordance with significant demographic characteristics, *i.e.*, sex and age showed lower performance compared to *IL-8* mRNA expression alone (Model 3 vs Model 2). Adding *IL-8* mRNA expression in a model with sex and age, the AuROC of probability to predict severity occurrence of GC was increased (Model 4 vs Model 2). However, adding *H. pylori* pathology in the last model did not enhance a predictability of GC severity in the last model (Model 5 vs Model 4). Therefore, *IL-8* mRNA expression is useful to differentiate severity of GC especially when combined with sex and age (Table 3). The *IL-8* mRNA expression used was the best cut-off point of two to predict the severity of GC; AuROC of cut-off-point one through four was 0.64, 0.71, 0.60, and 0.53, respectively (data not shown).

We further analyzed the prediction ability of the model containing *IL-8* mRNA expression across age group and sex. The likelihood positive ratio of all models

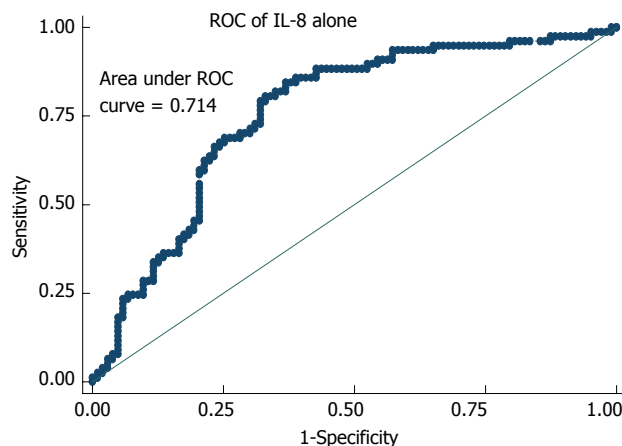
Table 2 Serum interleukin-8 mRNA expression and *Helicobacter pylori* infection status detection results in gastric cancer and non-gastric cancer patients, *n* (%)

Variables	Gastric cancer <i>n</i> = 86	Non-gastric cancer <i>n</i> = 134	<i>P</i> value
<i>H. pylori</i> pathology			0.267
Negative	32 (37.21)	60 (44.78)	
Positive	54 (62.79)	74 (55.22)	
<i>H. pylori</i> infection status			0.121
Negative	20 (24.4)	37 (36.0)	
Positive	62 (75.6)	96 (64.0)	
IL-8 raw RQ			< 0.001
< 100	33 (38.37)	105 (78.36)	
≥ 100	53 (61.63)	29 (21.64)	
Median (IRQ)	325 (2326.37)	19.72 (105.74)	
IL-8 Log 10			< 0.001
Min-0.99	5 (6.49)	36 (34.95)	
1.00-1.99	16.36 (22.08)	38 (36.90)	
2.00-2.99	28 (36.36)	13 (12.62)	
3.00-3.99	20 (25.97)	12 (11.65)	
≥ 4.00	7 (9.09)	4 (3.88)	

H. pylori: *Helicobacter pylori*.**Figure 1** Area under the receiver operating characteristic curve of *Helicobacter pylori* in prediction of gastric cancer. *H. pylori*: *Helicobacter pylori*; ROC: Receiver operating characteristic curves.

was statistically significant. The largest yield of the LHR+ was the model with all variables. Apparently, *IL-8* mRNA expression has the highest yield of the LHR+ in males who are older than 40 years old compared to the younger age or in the female group (LHR+ 14.54 vs 3.38) due to acceptable LHR+ (more than 5.0, theoretical suggested LHR+). Therefore, *IL-8* mRNA expression may be most useful in the Thai male with the age older than 40 years (Table 4).

Because the trend of prediction in the severity of GC of *IL-8* mRNA expression was observed, diagnostic indices were determined only in 86 GC patients who were categorized by metastatic status although it was still localized. Under four different cut-off points of *IL-8* mRNA expression, the sensitivity was highest in the *IL-8* mRNA expression Level one (96.8%) and continuously declined to 12.9% in the cut-off point of Level four. In the

**Figure 2** Area under the receiver operating characteristic curve of interleukin-8 mRNA Expression in prediction of gastric cancer. IL-8: Interleukin-8; ROC: Receiver operating characteristic curves.

opposite, specificity for GC metastasis increased from 8.7% in *IL-8* mRNA expression level one to 93.5% in Level four. The LHR+ increased from 1.06 to 1.98 of the Level one to Level four (Table 5). The AuROC of all of the cut-off points were not statistically significant (*P*-value of difference = 0.832) with less than a 60% range in all groups. There might have been a lack of ample sample size so the *IL-8* mRNA expression level could differentiate severity in the diagnosed GC group (Table 5).

The AuROC of *H. pylori* alone and AuROC of *IL-8* mRNA expression alone in prediction of GC occurrence is shown in Figures 1 and 2. Comparable AuROC of *IL-8* mRNA expression in an adjusted model by sex, age, and *H. pylori*, and additional AuROC of both *IL-8* mRNA expression and *H. pylori* infection in prediction of GC are shown in Figure 3.

DISCUSSION

In this study, performances of *H. pylori* infection and *IL-8* mRNA expression were determined as to whether there was an association with GC which was divided into two groups: Non-GC, and GC. The *IL-8* mRNA expression showed a predominant association with GC when compared to *H. pylori* infection, especially in males older than 40 years of age. In addition, there was a trend of the probability of GC with increasing levels of *IL-8* mRNA expression. Further, preliminary testing for diagnostic indices of four cut-off points of *IL-8* mRNA expression to predict severity of GC cases was performed. However, we found an increasing suboptimal trend from the likelihood ratio of positive (less than five times) which may be due to the small sample size to differentiate severity in GC groups.

GC has a high incidence rate in the northern part of Thailand. Epidemiological studies have shown that *H. pylori* is associated closely with the development of GC and it is widely regarded as the most important modifiable risk factor for GC^[18]. However, when categorizing GC

Table 3 Logistic models and the area of the receiver operating characteristic curves comparing *Helicobacter pylori* pathology results and interleukin-8 mRNA expression with/without demographic characteristics

Variable	Model 1 Crude OR (95%CI)	Model 2 Crude OR (95%CI)	Model 3 Adjusted OR (95%CI)	Model 4 Adjusted OR (95%CI)	Model 5 Adjusted OR (95%CI)
Sex	-	-	2.99 (1.72-5.20)	3.31 (1.84-5.94)	3.29 (1.83-5.93)
Age group	-	-	3.79 (1.38-10.41)	4.25 (1.47-12.34)	4.19 (1.44-12.20)
<i>H. pylori</i>	0.86 (0.49-1.53)	-	0.94 (0.52-1.71)	-	0.91 (0.48-1.70)
<i>IL-8</i> mRNA expression ≥ 2	-	5.44 (3.08-9.62)	-	6.05 (3.32-11.02)	6.07 (3.33-11.04)
AuROC	0.48 (0.42-0.54) ^a	0.71 (0.64-0.77) ^a	0.70 (0.63-0.76)	0.80 (0.75-0.86) ^b	0.81 (0.75-0.86) ^b

^aP value difference of AuROC = 0.532; ^bP value difference of AuROC < 0.001; *H. pylori*: *Helicobacter pylori*; AuROC: Area under the receiver operating characteristic curves.

Table 4 Likelihood ratio of positive of models including interleukin-8 mRNA expression, sex, and age group

Variable	LHR+ (95%CI)	P value
Interleukin-8 mRNA expression alone	2.90 (2.02-4.16)	< 0.001
Interleukin-8 mRNA expression + age > 40 or male	3.38 (2.26-5.04)	< 0.001
Interleukin-8 mRNA expression + age > 40 + male	14.54 (4.56-46.36)	< 0.001

Table 5 Diagnostic values of each interleukin-8 mRNA expression cut-off point, 95%CI and gastric cancer diagnosis only gastric cancer cases (*n* = 86)

IL-8 level cut-off point	Sensitivity	Specificity	PPV	NPV	LHR+	LHR-	AuROC
1	96.8 (92.8-100.0)	8.7 (2.4-15.0)	41.7 (30.6-52.7)	80.0 (71.1-88.9)	1.06 (0.95-1.18)	0.37 (0.04-3.16)	0.53 (0.48-0.58)
2	64.7 (54.6-74.8)	38.4 (28.2-48.7)	40.7 (30.4-51.1)	62.5 (52.3-72.7)	1.05 (0.76-1.46)	1.09 (0.62-1.93)	0.58 (0.41-0.62)
3	41.9 (30.9-53.0)	69.6 (59.3-79.8)	48.2 (37.0-59.3)	64.0 (53.3-74.7)	1.38 (0.75-2.52)	1.20 (0.84-1.71)	0.56 (0.45-0.67)
4	12.9 (5.4-20.4)	93.5 (88.0-99.0)	57.1 (46.1-68.2)	61.4 (50.6-72.3)	1.98 (0.48-8.23)	1.07 (0.92-1.25)	0.53 (0.46-0.60)

P value of all AuROC = 0.832. PPV: Positive predictive value; NPV: Negative predictive value; LHR+: Positive likelihood ratio; LHR-: Negative likelihood ratio.

by severity, *H. pylori* lost its association in our findings. Unlike, *IL-8* mRNA expression, the results from our study found the superiority of prediction of GC severity over *H. pylori* infection. *IL-8* mRNA expression is one of factors that possibly affects GC^[15]. Thai people in the advanced stage of GC showed that gastric mucosal tissue *IL-8* mRNA expression has a higher level value and percentage of poorer differentiated cell type more than in favorable histology or differentiated cell type^[14]. This finding was consistent with the results of Yamada *et al.*^[14,16] which showed that a high level of *IL-8* mRNA expression was detected more than 80% in Thai advanced GC patients, of cases and they demonstrated that gastric mucosal *IL-8* mRNA expression was a relative risk for Thai GC. Thus *IL-8* mRNA expression may also be a useful diagnostic risk marker for GC. It is possible to use *IL-8* mRNA expression as a good indicator for advanced GC or aggressive types of cancer treatment selection especially in poor prognostic cell type.

Moreover, this study demonstrated the AuROC of *IL-8* mRNA expression when comparing gender with age found that males more than 40 years of age predicted the severity of GC with LHR+ 14.5 times. This may explain recent indications that men have a higher incidence rate and may have a poorer prognosis than women^[19]. The increased incidence rate of males could be due to the difference in the lifestyles and habits from females; such as smoking and alcohol consumption^[20]. A previous study on sex differences in GC incidence based on the study of etiological hypothesis indicated that the predominance of GC in men was a global phenomenon, and was related to a 10 to 15 year delay in the appearance and onset of GC of intestinal subtype in women compared with men^[21,22]. Our data showed that *IL-8* mRNA expression may be a helpful tool to identify advanced risk of GC in Thai patients especially males with an age older than 40 years.

We further investigated diagnostic performances

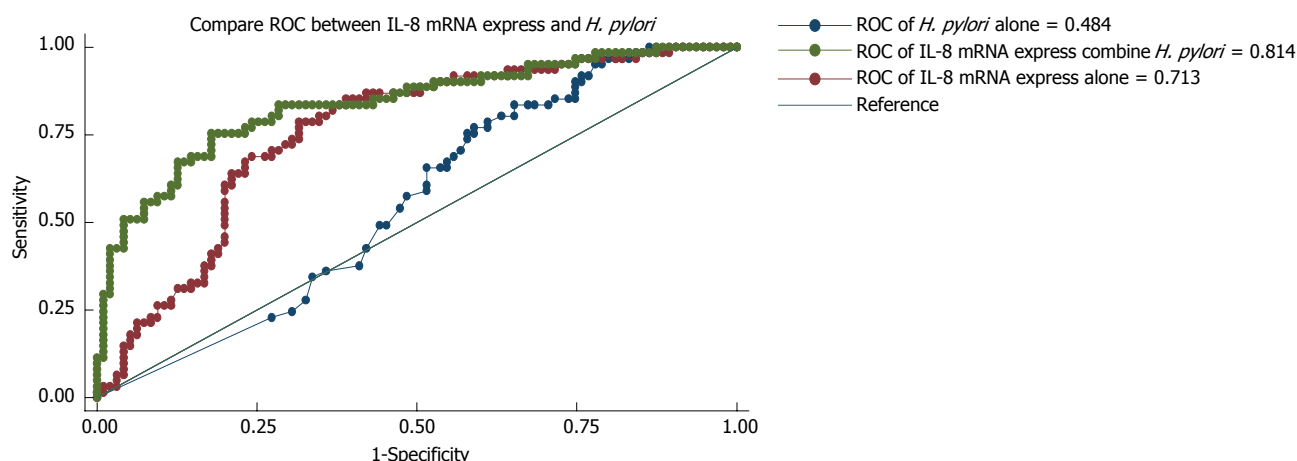


Figure 3 Comparable area under the receiver operating characteristic curve between interleukin-8 mRNA expression and *Helicobacter pylori* in prediction of gastric cancer. *H. pylori*: *Helicobacter pylori*; IL-8: Interleukin-8; ROC: Receiver operating characteristic curves.

of *IL-8* mRNA expression for advanced stages of GC. Although there was no statistical significance among the four cut-off points of *IL-8* mRNA expression of the AuROC, and the LHR+ values were lower than five times, there was a trend of increasing predictability of GC severity and prognosis. Future study is warranted to prove the predictive values of *IL-8* mRNA expression in GC patients with a larger clinical sample size. The limitation of this study was its retrospective nature and as a result some important available data could have been omitted due to a lack of medical records.

The *IL-8* mRNA expression showed predominant association with GC when compared to *H. pylori* infection, especially in males with age older than 40 years who may be benefit the most from this test. The preliminary testing for diagnostic indices of four cut-off points of *IL-8* mRNA expression showed a suboptimal trend to differentiate severity in the GC group.

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COMMENTS

Background

The association of *Helicobacter pylori* (*H. pylori*) infection and interleukin-8 (*IL-8*) mRNA expression and gastric cancer (GC) were demonstrated from several studies. However, there was a lack of evidence of the association of these biomarkers and GC severity.

Research frontiers

The association of *H. pylori* test and *IL-8* mRNA expression alone and the severity of GC; (1) the association of both tests added to patients' characteristics to identify Thai suspected patient risk of GC who will receive the greatest benefit for follow-up endoscopy; and (2) diagnostic value of four different levels of *IL-8* mRNA expression for GC cases.

Innovations and breakthroughs

The *IL-8* mRNA expression showed predominant association with GC when compared to *H. pylori* infection, especially in males with age older than 40 years who may be benefit the most from this test. The preliminary testing for diagnostic indices of four cut-off points of *IL-8* mRNA expression showed a suboptimal trend to differentiate severity in the GC group.

Peer-review

It might be interesting to analysis *IL-8* expression level in different stage and different histological grade, in order to demonstrate whether *IL-8* is more sensitive than *H. pylori* in different stage and grade GC.

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Evaluation of antibody-dependent cell-mediated cytotoxicity activity and cetuximab response in *KRAS* wild-type metastatic colorectal cancer patients

Cristiana Lo Nigro, Vincenzo Ricci, Daniela Vivenza, Martino Monteverde, Giuliana Strola, Francesco Lucio, Federica Tonissi, Emanuela Miraglio, Cristina Granetto, Mirella Fortunato, Marco Carlo Merlano

Cristiana Lo Nigro, Daniela Vivenza, Martino Monteverde, Federica Tonissi, Laboratory of Cancer Genetics and Translational Oncology, Oncology Department, S. Croce and Carle Teaching Hospital, 12100 Cuneo, Italy

Vincenzo Ricci, Emanuela Miraglio, Cristina Granetto, Marco Carlo Merlano, Medical Oncology, Oncology Department, S. Croce and Carle Teaching Hospital, 12100 Cuneo, Italy

Giuliana Strola, Laboratory Department, S. Croce and Carle Teaching Hospital, 12100 Cuneo, Italy

Francesco Lucio, Radiotherapy Department, S. Croce and Carle Teaching Hospital, 12100 Cuneo, Italy

Mirella Fortunato, Pathology Department, S. Croce and Carle Teaching Hospital, 12100 Cuneo, Italy

Author contributions: Lo Nigro C, Ricci V and Merlano MC designed the study; Ricci V, Miraglio E and Granetto C enrolled the patients and collected clinical data; Vivenza D, Monteverde M and Tonissi F performed ADCC assays and genotyping analyses; Strola G performed all the cytofluorimetric studies; Fortunato M selected patient's tumoral samples; Vivenza D and Lucio F performed the statistical analysis; Lo Nigro C, Ricci V and Merlano MC critically visioned the results and were equally involved in the writing of the paper with Vivenza D.

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Correspondence to: Cristiana Lo Nigro, PhD, Laboratory

of Cancer Genetics and Translational Oncology, Oncology Department, S. Croce and Carle Teaching Hospital, Via Carle 25, 12100 Cuneo, Italy. lonigro.c@ospedale.cuneo.it
Telephone: +39-0171-616338
Fax: +39-0171-616331

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Abstract

AIM: To investigate the prognostic role of invariant natural killer T (iNKT) cells and antibody-dependent cell-mediated cytotoxicity (ADCC) in wild type *KRAS* metastatic colorectal cancer (mCRC) patients treated with cetuximab.

METHODS: Forty-one *KRAS* wt mCRC patients, treated with cetuximab and irinotecan-based chemotherapy in II and III lines were analyzed. Genotyping of single nucleotide polymorphism (SNP)s in the *FCGR2A*, *FCGR3A* and in the 3' untranslated regions of *KRAS* and mutational analysis for *KRAS*, *BRAF* and *NRAS* genes was determined either by sequencing or allelic discrimination assays. Enriched NK cells were obtained from lymphoprep-peripheral blood mononuclear cell and iNKT cells were defined by co-expression of CD3, TCRV α 24, TCRV β 11. ADCC was evaluated as *ex vivo* NK-dependent activity, measuring lactate dehydrogenase release.

RESULTS: At basal, mCRC patients performing ADCC activity above the median level (71%) showed an improved overall survival (OS) compared to patients with ADCC

below (median 16 *vs* 8 mo; $P = 0.026$). We did not find any significant correlation of iNKT cells with OS ($P = 0.19$), albeit we observed a trend to a longer survival after 10 mo in patients with iNKT above median basal level (0.382 cells/microliter). Correlation of OS and progression-free survival (PFS) with interesting SNPs involved in ADCC ability revealed not to be significant. Patients carrying alleles both with A in FCGR2A and TT in FCGR3A presented a trend of longer PFS (median 9 *vs* 5 mo; $P = 0.064$). Chemotherapy impacted both iNKT cells and ADCC activity. Their prognostic values get lost when we analysed them after 2 and 4 mo of treatment.

CONCLUSION: Our results suggest a link between iNKT cells, basal ADCC activity, genotypes in FCGR2A and FCGR3A, and efficacy of cetuximab in *KRAS* wt mCRC patients.

Key words: Metastatic colorectal cancer; Single nucleotide polymorphism in Fc- γ receptors; Cetuximab; RAS family; Antibody-dependent cell-mediated cytotoxicity; Invariant natural killer T cells

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Core tip: A high number of invariant natural killer T (iNKT) cells and a high antibody-dependent cell-mediated cytotoxicity (ADCC) activity, evaluated before therapy, do correlate significantly with a longer overall survival in metastatic colorectal cancer patients treated with irinotecan-based chemotherapy and cetuximab in II and III lines. Chemotherapy impacted both iNKT cells and ADCC activity. The prognostic value of ADCC above the median basal level, get lost when we analysed those parameters after 2 and 4 mo of treatment. Correlation of overall survival and progression-free survival with interesting single nucleotide polymorphisms reported as involved in ADCC ability, either in the FCGR2A, FCGR3A or in the 3' untranslated regions of *KRAS* gene, revealed not to be significant.

Lo Nigro C, Ricci V, Vivenza D, Monteverde M, Strola G, Lucio F, Tonissi F, Miraglio E, Granetto C, Fortunato M, Merlano MC. Evaluation of antibody-dependent cell-mediated cytotoxicity activity and cetuximab response in *KRAS* wild-type metastatic colorectal cancer patients. *World J Gastrointest Oncol* 2016; 8(2): 222-230 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v8/i2/222.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v8.i2.222>

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer worldwide, accounting for 940000 million new cases annually and nearly 500000 deaths each year. Metastatic colorectal cancer (mCRC) previously untreated patients have demonstrated substantial improvements, with a median overall survival time now reaching more than

24 mo, by the development of systemic chemotherapy, including molecular-targeted therapy^[1].

The epidermal growth factor receptor (EGFR) signalling pathway is involved in cell differentiation, proliferation, migration, angiogenesis and apoptosis, all processes dysregulated in cancer cells.

Cetuximab is a chimeric immunoglobulin G1 (IgG1) monoclonal antibody (mAb) which binds EGFR with high affinity and inhibits ligand binding^[2].

KRAS activating mutations have been reported in 40% of mCRC showing a negative effect on response to anti-EGFR antibodies^[3,4]. Mutations in other downstream effectors of the *EGFR* signalling pathway, such as *BRAF*, *NRAS* and *PI3*kinase, might also impact the efficacy of monoclonal therapy. Thus, the absence of mutations in *RAS* appears to be a reliable marker for predicting the efficacy of cetuximab which was been restricted to mCRC patients with wild-type *RAS*^[5]. Several studies supported the biological activity of cetuximab in advanced CRC. Cetuximab enhances response rate and progression-free survival (PFS) in first-line therapy in combination with Folfiri and Folfox regimen of chemotherapy^[6,7]. However, some clinical studies have failed to show a significant correlation between EGFR expression and the response to cetuximab^[8]. The proposed working mechanism of cetuximab is thought to include antibody-dependent cell-mediated cytotoxicity (ADCC)^[9].

ADCC utilizes the response of innate immune cells to provide antitumor cytotoxicity triggered by the interaction of the Fc portion of the antibody with the Fc receptor on the immune cell. Immunotherapeutics that target natural killer (NK) cells, $\gamma\delta$ T cells, macrophages and dendritic cells can, by augmenting the function of the immune response, enhance the antitumor activity of the antibodies^[10].

Invariant CD1d-restricted natural killer T (NKT) cells are T lymphocytes characterized by an invariant T-cell antigen receptor-chain rearrangement that co-express NK cell markers^[11].

Molling *et al.*^[12] in 2007 demonstrated that a severe circulating invariant NKT (iNKT) cell deficiency was related to poor clinical outcome in head and neck squamous cell carcinoma patients, suggesting their critical contribution to antitumor immune responses. Furthermore, screening for iNKT cell levels may be useful for determining which patients can benefit from immunotherapeutic adjuvant therapies aimed at reconstitution of the circulating iNKT cell pool.

Whether ADCC is associated with EGFR expression and/or the mutational status of *RAS* and *BRAF* in CRC remains unclear. Seo *et al.*^[13] demonstrated that the ADCC activities were significantly associated with the cell surface expression levels of EGFR but not with the mutational status of *KRAS* and *BRAF*.

In this study we aimed to evaluate the prognostic and predictive value of cetuximab-mediated ADCC and circulating iNKT cells levels in mCRC and to analyse their correlation with *EGFR* level, mutational status of

Table 1 Characteristics of 41 patients in II and III line and tumours

	Number of patients	Rates	Median age (range) yr
Gender			
Male (M)	23	56%	67.5 (51-84)
Female (F)	18	44%	64.6 (49-83)
Primary tumour			
Right colon	7	17%	
Left colon	21	51%	
Rectal	13	32%	
Grade			
G1/G2	27	65.8%	
G3	13	31.7%	
NA	1	2.5%	
Metastasis			
Liver only	12	29.3%	
Liver plus other sites	14	34.1%	
Extra-hepatic sites	15	36.6%	
Response			
Responders			
CR	4	9.8%	
PR	12	29.3%	
SD	8	19.5%	
Non-responders			
PD	17	41.4%	
Line of treatment			
II	33	8%	
III	8	2%	

NA: Not available; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

KRAS, *NRAS*, *BRAF*, PFS and overall survival (OS) in a prospective cohort of mCRC patients treated with cetuximab-based therapy.

MATERIALS AND METHODS

Patients and clinical samples

A total of 41 mCRC patients were enrolled in this study from March 2008 to September 2014. Characteristics of the 41 patients are described in Table 1. An informed consent for tissue collection and use for scientific purpose was obtained from each patient enrolled in this study, approved by the local Ethical Committee and carried out in the respect to Helsinki Declaration. Inclusion criteria for mCRC patients were: Suitability for combination therapy including cetuximab with irinotecan-based chemotherapy in second and third lines and *KRAS* wild type (wt) status. Patients were evaluated for PFS, OS and response at the end of treatment with CT scan according to RECIST criteria^[2]. Median follow-up was 25 mo (range 10-70).

DNA extraction, genotyping and mutational analyses

Genotyping of rs1801274 (A > G) in the *FCGR2A*, rs396991 (T > G) in *FCGR3A* and rs61764370 in the 3' untranslated regions (3' UTR) of *KRAS* gene was done on genomic DNA isolated from whole peripheral blood samples using the EZ1 DNA Blood 200 Kit (Qiagen, Germany) according to the manufacturer's instructions. Analyses were determined using the appropriate

"allelic discrimination assay" from Life Technologies (Foster city, CA, United States): c_9077561_20 for rs1801274; c_25815666_10 for rs396991 and 1350086 for rs61764370 using the ABIPRISM 7000 Sequence Detection System (Applied Biosystems Foster City, CA, United States).

Mutational analyses for *KRAS* (codons 12-13-59-61-146), *BRAF* (codon 600) and *NRAS* (codons 12-13-59-61-117-146) genes were determined on patients' DNA extracted from Formalin Fixed Paraffin Embedded (FFPE) tumor tissues archived at diagnosis in the Pathology Department of our Institution, by a standard protocol that included proteinase K treatment (EuroClone, Pero, IT).

KRAS and *BRAF* gene analyses were performed by pyrosequencing using PyroMark ID System (Biotage, Uppsala, Sweden), while a Real-Time PCR (OncoSreen *NRAS*; Relab, Jesi, Italy) was employed for *NRAS* gene using the Rotor-Gene 6000 (Corbett Research, Pty Ltd; Sydney, Australia) according to the manufacturer's protocol.

Antibody-dependent cell-mediated cytotoxicity assay

Twelve milliliter peripheral blood samples were collected at start of therapy for all the 41 patients and ADCC and NK cells were evaluated at basal level. After 2 and 4 mo of treatment a second collection of blood was done in 30 and 23 patients respectively where ADCC and NK cells were longitudinally studied.

Enriched NK cells were obtained from lymphoprep-peripheral blood mononuclear cell pellets using the human NK Cell Isolation Kit (Miltenyi Biotec, Cologne, Germany). NK cells were defined as CD56⁺/CD3⁻; T cells as CD3⁺/CD56⁻ and invariant NKT (iNKT) cells by co-expression of CD3, TCR Vα24, TCR Vβ11.

ADCC was evaluated as *ex vivo* NK-dependent activity with a standard lactate dehydrogenase (LDH) assay (Cytotox 96[®] non radioactive cytotoxicity assay, Promega, Madison, WI) as set up in our Laboratory^[14].

Statistical analysis

Statistical analyses were performed using the GraphPad Prism 5 (San Diego, CA, United States) and SPSS version 13 (SPSS, Chicago, IL) programs. The association between ADCC median levels was analyzed using the Fisher's exact test or the Pearson's test when appropriate. OS analyses were based on the time from treatment start to death or last contact in which the survivors were censored. PFS analyses were based on the time from treatment start to first event; patients without an event were censored at their last follow-up. OS was calculated using the Kaplan-Meier method with log-rank test for statistical significance. A *P*-value < 0.05 was considered statistically significant.

RESULTS

Clinical and molecular characteristics of patients

Clinical characteristics of the 41 mCRC patients are

Table 2 *FCGR2A* (rs1801274; A > G), *FCGR3A* (rs366991; T > G) and single nucleotide polymorphism rs61764370 of *KRAS* 3' untranslated region genotypes with the correspondent aminoacid change in the 41 metastatic colorectal cancer patients included in this study

Gene	SNP	Genotype	Aminoacid change	Number of subjects (%)
<i>FCGR2A</i>	rs1801274	A/A	H131H	14 (34.1%)
		A/G	H131R	20 (48.8%)
		G/G	R131R	7 (17.1%)
<i>FCGR3A</i>	rs396991	T/T	F158F	14 (34.1%)
		T/G	F158V	21 (51.2%)
		G/G	V158V	6 (14.7%)
<i>KRAS</i> 3' UTR	rs61764370	T/T	---	32 (78%)
		T/G	---	9 (22%)
		G/G	---	0 (0%)

SNP: Single nucleotide polymorphism; UTR: Untranslated region.

detailed in Table 1. Genotyping analyses for single nucleotide polymorphisms are reported in Table 2. All patients were wt for *KRAS* gene. Determination of *NRAS* and *BRAF* mutations failed in 1 out of the 41 patients, due to poor quality of DNA obtained from tumoral tissue. In particular we identified 6 mutations in *NRAS* gene (1 mutation in G12x-G13x; 1 in G61R; 1 in Q61K, 1 in Q61L and 2 in A59x-Q61H codons) and 1 mutation in *BRAF* gene (V600E).

Survival analysis according to iNKT cells

iNKT cells evaluated before treatment were analysed to seek correlation with OS and PFS either as number of cells/microliter or as % of T cells, since a low level of circulating iNKT cells has been reported to predict poor clinical outcome in patients with head and neck squamous cell carcinoma^[12]. iNKT cells median value at basal determination, before treatment, was 0.382 cells/microliter. We did not find any significant correlation of iNKT cells with OS ($P = 0.19$), albeit we observed a trend to a longer survival after 10 mo in the population of patients ($n = 21$) with iNKT above median level (Figure 1).

Survival analysis according to ADCC activity

Median ADCC activity before treatment for all the 41 mCRC patients was 71% (range 10%-99%). Comparison between patients with ADCC above and below median value is reported in Table 3. There were no differences in the clinical characteristics between the two groups, although *EGFR* over-expression was more common in patients with ADCC activity above the median level ($P = 0.052$; Fisher's exact test). Correlation with OS and PFS was evaluated. Median OS was 12 mo (range 3-37) and PFS was 6 mo (range 3-37). Patients performing ADCC activity above the median level showed an improved OS compared to patients with ADCC activity below this value (median 16 vs 8 mo; $P = 0.026$; Long-rank Mantel-Cox Test) (Figure 2). On the contrary, there was no difference in PFS between patients with ADCC below or above the median level (data not shown). When we

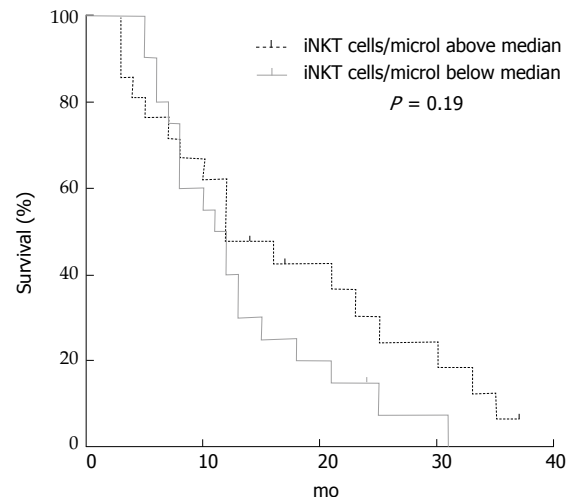


Figure 1 Overall survival in 41 metastatic colorectal cancer treated with cetuximab in II and III lines according to median basal level of invariant natural killer T cells (0.382 cells/microliter). iNKT: Invariant natural killer T.

stratified patients for both iNKT and ADCC activity at basal level, below and above the respective median level, we observed a better OS in patients having both values above the median level compared to all the other combinations (median 23 vs 10 mo; $P = 0.0075$; Long-rank Mantel-Cox Test) (Figure 3).

Survival analysis according to genotypes of *FCGR2A*, *FCGR3A* genes and in *KRAS* 3'UTR

Correlation in terms of OS and PFS with each genotype, either rs1801274 (A > G) in the *FCGR2A*, rs396991 (T > G) in *FCGR3A* or rs61764370 in the 3' UTR of *KRAS* gene reveal not to be significant (data not shown).

Patients carrying alleles both with A in *FCGR2A* (AA/AG genotypes) and TT in *FCGR3A* presented a longer PFS (median 9 vs 5 mo; $P = 0.064$; Long-rank Mantel-Cox Test) in comparison to all the other subgroups (Figure 4), although the difference was not significant.

Survival analysis according to mutational status in *RAS* family genes

Due to the limited number of patients we were not able to perform OS and PFS analyses according to all-*RAS* gene mutations. Nevertheless we observed that of ADCC activity, as median level, was not affected by the presence of a *NRAS* or a *BRAF* mutation (data not shown).

How the treatment influenced iNKT cells and ADCC activity

Both iNKT cells and ADCC activity were evaluated over time to seek for dynamic changes during treatment and to investigate the impact of therapy on patients' ability to perform ADCC and their clinical outcome. iNKT cells median number decreased from 0.382 cells/microliter at basal, before treatment, to 0.193 after 2 mo and to 0.165 after 4 mo of treatment. Likewise, ADCC activity was longitudinally evaluated up to 2 and 4 mo and its median level fell down from of 71% to 45% at two

Table 3 Comparison of characteristics of 41 patients on the basis of antibody-dependent cell-mediated cytotoxicity activity

	ADCC < n = 20 71%		ADCC > n = 21 71%		P
Gender					
Male (M)	11	55%	12	57%	0.9 ¹
Female (F)	9	45%	9	43%	
Primary tumour					
Right colon	5	25%	2	10%	0.28 ²
Left colon	8	40%	13	62%	
Rectal	7	35%	6	29%	
Grade					
G1/G2	13	65%	14	67%	0.87 ²
G3	7	35%	6	29%	
NA	0	0%	1	5%	
Metastasis					
Liver only and liver plus other sites	12	60%	14	67%	0.65 ¹
Extra-hepatic sites	8	40%	7	33%	
Response					
Responders					
CR	3	15%	1	5%	0.36 ²
PR	4	20%	8	38%	
SD	3	15%	5	24%	
Non-responders					
PD	10	50%	7	33%	
Line of treatment					
II	17	85%	17	81%	1 ²
III	3	15%	4	19%	
EGFR					
Neg; 1+; 2+	19	95%	14	67%	0.052 ²
3+	1	5%	5	24%	
NA	0	0%	2	10%	

¹Pearson's Test; ²Fisher's Exact Test; NA: Not available; Neg: Negative; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; ADCC: Antibody-dependent cell-mediated cytotoxicity; EGFR: Epidermal growth factor receptor.

withdrawal further analyses.

Survival analysis according to variation in ADCC activity during treatment

ADCC determination during treatment lost its prognostic value since there was no difference in OS between patients with ADCC activity above or below the median level after 2 and after 4 mo of treatment (data not shown). Variation of ADCC values was analyzed by stratifying patients on the basis of to the median values at basal level (71%, 41 patients), after 2 mo (45%, 30 patients) and after 4 mo (45%, 23 patients) of treatment. Combination of longitudinal values generated 4 groups of patients: the first included patients showing both ADCC activities above the median level [ADCCbas above/ II (or III) above, where II means on blood drawn after 2 mo and III after 4 mo], the second group a decrease from a basal above median to a II or III determination below median [ADCCbas above/ II (or III) below], the third group patients showing instead an increase from below at basal and above at II or III determination [ADCCbas below/ II (or III) above] and the fourth group patients with both ADCC activities below the median level [ADCCbas below/ II (or III) below].

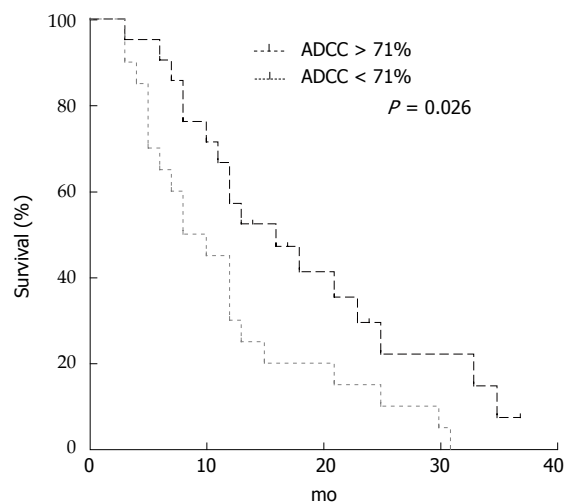


Figure 2 Overall survival in 41 metastatic colorectal cancer treated with cetuximab in II and III lines according to median basal level of antibody-dependent cell-mediated cytotoxicity activity (71%). ADCC: Antibody-dependent cell-mediated cytotoxicity.

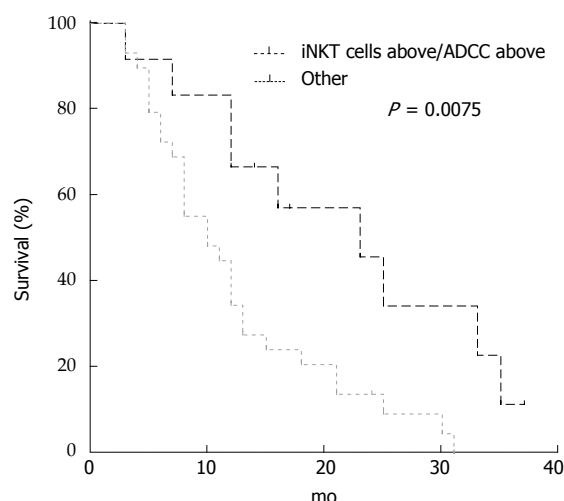


Figure 3 Overall survival in 41 metastatic colorectal cancer patients stratified for both invariant natural killer T and antibody-dependent cell-mediated cytotoxicity activity at basal level, below and above the respective median level. iNKT = 0.382 cells/microliter; ADCC = 71%. iNKT: Invariant natural killer T; ADCC: Antibody-dependent cell-mediated cytotoxicity.

We then analysed correlation with OS in the 4 groups. Patients performing ADCC activity above the median value both at basal level and either after 2 and/or 4 mo presented a trend in longer OS, albeit not significant (Figure 5).

When we focus on patients presenting ADCC values above the median levels in both determinations (basal and after 2 mo) we found that this 9 out of the 30 patients (30%) showed a higher OS compared to other patients (median 21 vs 13 mo, $P = 0.5$; Long-rank Mantel-Cox Test). After 4 mo, 8 patients out of 23 (35%) had both values above the median levels of ADCC activity, but their OS was not statistically different from that of the other patients (median 18.5 vs 15 mo; $P = 0.42$; Long-rank Mantel-Cox Test) (data not shown).

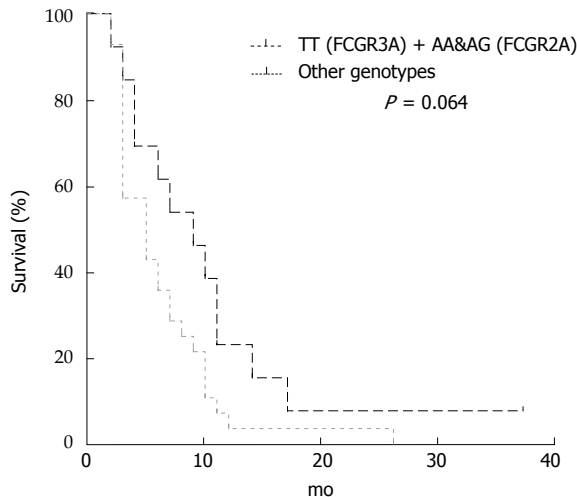


Figure 4 Progression free survival in compound heterozygote patients for single nucleotide polymorphisms rs1801274 in *FCGR2A* and rs396991 in *FCGR3A* genes in the 41 metastatic colorectal cancer patients.

DISCUSSION

Treatment of mCRC requires a multidisciplinary approach and multiple treatment options are nowadays available^[1]. Advances in the understanding of tumor biology have led to the development of *EGFR*-targeted therapies as mAbs. In fact, the *EGFR*-signalling pathway regulates important processes involved in cell differentiation, proliferation, migration, angiogenesis and apoptosis, all of which become deregulated in cancer cells. However, the mechanisms that mediate the therapeutic effect of these mAbs are still unclear.

Cetuximab is a chimeric monoclonal antibody that specifically targets *EGFR* with high affinity and prevents the ligand-mediated activation of the *EGFR*-dependent pathway. *KRAS* mutations occur in 35%-45% of mCRC and preclude responsiveness to *EGFR*-targeted therapy with cetuximab or panitumumab. Initial response rates of about 10% were seen with cetuximab monotherapy in patients with heavily pretreated mCRC. A phase II BOND study demonstrated the ability of cetuximab to circumvent irinotecan-based chemotherapy resistance^[2]. Less than 20% patients displaying wild-type *KRAS* tumors achieve objective response. In fact, it subsequently became clear that tumors without mutations in codon 12 or 13 of the *KRAS* gene responded in 13%-17% of cases, whereas only 0.1%-2% of the *KRAS* mutant tumors did^[15].

Alterations in other effectors downstream of the *EGFR* and deregulation of the *PIK3CA/PTEN* pathway have independently been found to give rise to resistance. Moreover, the *PIK3CA* gene is mutated in approximately 20% of CRCs. *BRAF* is the principal downstream effector of *KRAS* and its oncogenic V600E mutation is mutually exclusive with *KRAS* mutations in CRCs^[4,16].

It has recently become clear that IgG1 mAb, like cetuximab, may have mechanisms of action other than the selective blockade of tumoral membrane receptors. Among them, the Fc region of the mAb may also trigger

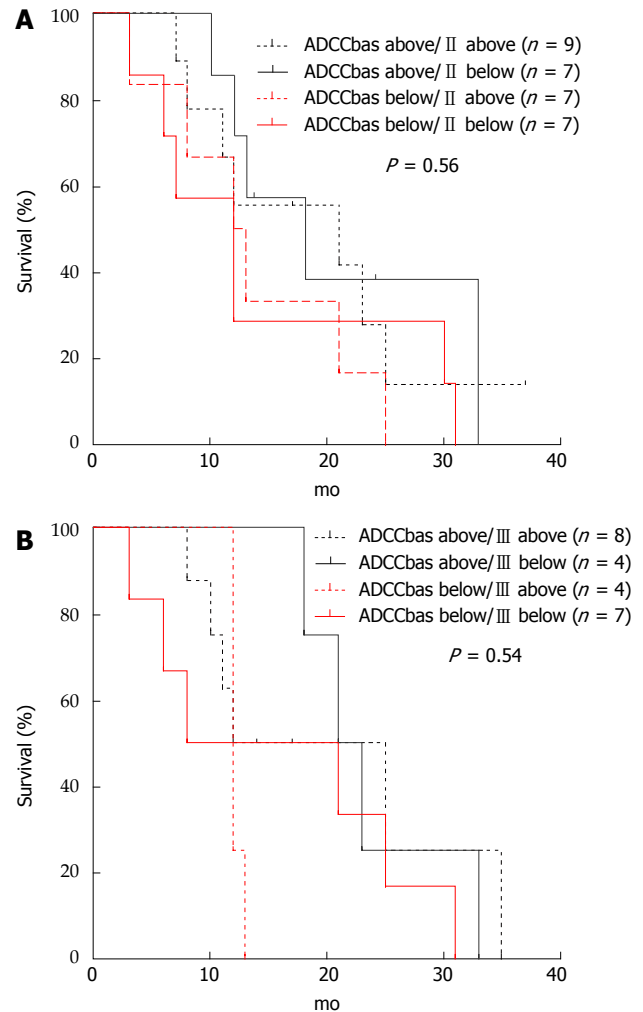


Figure 5 Overall survival in 30 metastatic colorectal cancer patients with a blood drawn after 2 mo (A) and in 23 patients with a blood drawn after 4 mo of treatment (B). Analysis was done in 4 groups of patients: The first included patients showing both ADCC activities above the median level [ADCCbas above/II (or III) above, where II means on blood drawn after 2 mo and III after 4 mo], the second group a decrease from a basal above median to a II or III determination below median [ADCCbas above/II (or III) below], the third group patients showing instead an increase from below at basal and above at II or III determination [ADCCbas below/II (or III) above] and the fourth group patients with both ADCC activities below the median level [ADCCbas below/II (or III) below]. ADCC: Antibody-dependent cell-mediated cytotoxicity.

ADCC, binding *via* Fv regions the target cell to any of the Fc- γ receptors, *i.e.*, CD16, CD32 and CD64, which are expressed, with different patterns, by cells of the innate immune system, namely monocytes, macrophages, granulocytes and NK. The contribution of the different cell types to the anti-tumor ADCC exerted *in vivo* by anti-*EGFR* mAbs is still debated. In general these cell are thought to play a relevant role controlling tumor growth and in preventing metastatic dissemination in humans^[17,18].

In particular, NK cells have been suggested to be the major mediators of the ADCC-dependent therapeutic effect of cetuximab^[19]. Moreover, invariant CD1d-restricted NKT cells has been reported to play an allegedly pivotal role in such responses *via* transactivation of immune effector cells. In particular, a severe circulating iNKT cell

deficiency was related to poor clinical outcome in head and neck squamous cell carcinoma patients^[12].

Thus, the number of iNKT and the level of ADCC activity exerted by NK cells from tumor patients in the presence of cetuximab might be useful prognostic or predictive parameters for response to treatment. With this in mind, we investigated 41 mCRC patients suitable for combination therapy including cetuximab with irinotecan-based chemotherapy in second and third lines and *KRAS* wild type.

Analyses were carried out at start of therapy for all the 41 patients and ADCC and iNKT cells were evaluated at basal level. After 2 and 4 mo of treatment additional determinations were done in 30 and 23 patients respectively where ADCC and NK cells were longitudinally studied.

Main aim of the project was to study *ex-vivo* the prognostic and predictive value of the number of iNKT cells and the level of cetuximab-mediated ADCC and to analyse their correlation with *EGFR* level, mutational status of *KRAS*, *NRAS*, *BRAF* and PFS and OS in our prospective cohort of mCRCs.

We did not find any significant correlation of iNKT cells at basal level with PFS nor with OS, albeit we observed a trend to a longer survival after 10 mo in the population of patients with iNKT above median level. Instead, patients performing, at basal determination, ADCC activity above the median level showed an improved OS compared to patients with ADCC activity below this value.

Moreover, if we combine iNKT number and ADCC basal level and we stratified patients for both determinations, as below or above the respective median level, we observed a better OS in patients having both values above the median level compared to all the other combinations. Of note, when we analysed the same parameters after 2 and 4 mo of treatment, levels of circulating T, NK, iNKT cells were significantly reduced. On the clinical side, we observed that cancer patients exhibited a lower capacity to perform ADCC as compared to the beginning of therapy; this observation has to be replaced in the global context of an immunosuppressed state of cancer patients and the immunosuppressive effect of chemotherapy and it is consistent with earlier reports^[20].

Intriguing, during treatment, neither low level of iNKT nor low ADCC activity did correlate to prognosis. In our study this could be in apparent contrast with what observed by us at the beginning of therapy and also with what reported by others, which is patients with a severe numeric iNKT cell deficiency have a strikingly poor clinical outcome in response to chemo and radiotherapy^[12].

On the other hand, we're analysing NK levels and their activity in peripheral blood; we did not have the picture of the functional properties of tumor-infiltrating T and NK cells in patients. A reduced number of NK and iNKT cells in periphery might be "the other side of the coin" and may reflect an increased activity in the tumor infiltrates^[21].

The impact of ADCC on the efficacy of cetuximab

might also be influenced by the occurrence of polymorphic forms of genes coding receptors for the antibody Fc region. The most relevant polymorphisms regulating Fc:FcR interactions are phenylalanine (F) or valine (V) expression at position 158 of the Fc fragment^[22]. In particular, differential response to therapeutic mAbs has been reported to correlate with specific polymorphisms in two of these genes: *FCGR2A* (H131R) and *FCGR3A* (V158F)^[23]. However, previous studies exploring the relation between the FCGR polymorphisms and cetuximab efficacy in mCRC have demonstrated conflicting and have been mostly low-powered studies with small sample sizes^[24].

More recently a variant allele in a let-7 microRNA complementary site within the 3'UTR of *KRAS* (rs61764370) has been correlated with clinical outcome in mCRC patients receiving cetuximab^[25].

In our cohort of mCRCs, correlation in terms of OS and PFS with each genotype, either rs1801274 (A > G) in the *FCGR2A*, rs396991 (T > G) in *FCGR3A* or rs61764370 in the 3' UTR of *KRAS* gene didn't reveal to be significant. Interestingly enough, patients carrying alleles both with A in *FCGR2A* (AA/AG genotypes) and TT in *FCGR3A* presented a longer PFS, although the difference was not significant, probably due to the low number of patients.

For the same reason, we were not able to perform OS and PFS analyses according to all-*RAS* gene mutations. It is well known, in fact, that activating *KRAS* mutations are negative predictors of the response to cetuximab therapy in patients with mCRC, since cetuximab is widely considered to be unable to block the signal initiated by oncogenic *KRAS*^[26,27].

Nevertheless we observed that of ADCC activity, as median level, was not affected by the presence of a *NRAS* or a *BRAF* mutation.

Seo *et al.*^[13] demonstrated cetuximab-mediated ADCC in human CRC cell lines and observed that ADCC activities for the tumor cells were higher in CRC patients with a high expression level of *EGFR*. Furthermore, the ADCC activity level was significantly associated with *EGFR*, but not with the *KRAS/BRAF* mutational status.

This has to be considered also in the light of the preclinical studies of nakadate and colleagues, who demonstrated that, in an ADCC assay, perforin-dependent target cell lysis was not affected by the *KRAS* mutation status. On the other hand, perforin-independent ADCC was observed only in CRC cells with wild-type *KRAS*, but not in cells with mutant *KRAS*. Their experiments also revealed that the Fas-Fas ligand (*FasL*) interaction was responsible for the induction of apoptosis and perforin-independent ADCC. Thus, their findings clearly suggested that ADCC is an important mode of action of cetuximab and that *KRAS* mutation impairs the therapeutic effect exerted by cetuximab-mediated ADCC. In our study, regrettably, the limited number of patients precluded any definitive confirmation of this in our clinical setting of mCRC patients^[27]. Therefore, all together, our results seem to suggest a link between iNKT cells, basal ADCC

activity, genotypes in *FCGR2A* and *FCGR3A*, and efficacy of cetuximab in *KRAS* wild-type mCRC patients.

The efficacy of monoclonal anti-EGFR antibodies, like cetuximab, has been proven in mCRC patients. It has been established clearly that response to anti-EGFR antibody treatment is only possible in selected patient groups. However, predictive factors for the efficacy of anti-EGFR therapy have still to be completely elucidated. A factor identified in multiple studies as essential for appropriate assessment of eligibility for cetuximab or panitumumab treatment is the absence of *KRAS* gene mutations. EGFR expression on the surface of cancer cells does not seem to have a decisive influence on the efficacy of the therapy. There are ongoing studies assessing the predictive value of the number of copies of the *EGFR* gene, mutations in the *NRAS*, *PI3KCA*, *P53* and *PTEN* genes, concentration of EGFR ligands and polymorphisms in the *EGF* and *EGFR*, and the *FCGR2A* and *FCGR3A*, genes. In our study, we observed that combining iNKT number and ADCC basal level allowed to identify a group of mCRC patients, having both determinations above the respective median level and a longer OS. This combination looks like the best prognosticator in our population of patients. However, it has not as of yet been examined in large randomized prospective studies and hence should still be better elucidated before using as a basis for mCRC patient eligibility for cetuximab treatment.

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COMMENTS

Background

The efficacy of monoclonal anti-epidermal growth factor receptor (EGFR) antibodies, like cetuximab, has been proven in metastatic colorectal cancer (mCRC) patients. It has been established clearly that response to anti-EGFR antibody treatment is only possible in selected patient groups. However, predictive factors for the efficacy of anti-EGFR therapy have still to be completely elucidated. A factor identified in multiple studies as essential for appropriate assessment of eligibility for cetuximab or panitumumab treatment is the absence of mutation in *RAS* genes. EGFR expression on the surface of cancer cells does not seem to have a decisive influence on the efficacy of the therapy. There are ongoing studies assessing the predictive value of the number of copies of the *EGFR* gene, mutations in the *NRAS*, *PI3KCA*, *P53* and *PTEN* genes, concentration of EGFR ligands and polymorphisms in the *EGF* and *EGFR*, and the *FCGR2A* and *FCGR3A* genes.

Research frontiers

In this study the authors aim to evaluate the prognostic and predictive value of cetuximab-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) and circulating invariant natural killer T (iNKT) cells levels in mCRC; the authors shall analyse their correlation with *EGFR* level, mutational status of *KRAS*, *NRAS*, *BRAF*, progression free survival and overall survival in a prospective cohort of mCRC patients treated with cetuximab-based therapy.

Innovations and breakthroughs

The prognostic value of circulating iNKT cell and ADCC basal level reported

here adds to previous notions and strengthens the hypothesis that human iNKT cells may also contribute to antitumor responses in cancer patients and to cetuximab efficacy. These results indicate also that the contribution of the indirect action of cetuximab may be relative high, compared with the direct anti-EGFR action, toward the clinical therapeutic effect.

Applications

In summary, the authors demonstrated here, in a prospective study, that a low level of circulating iNKT cells and a low ADCC activity before treatment in mCRC patients are significantly associated with poor survival. These data suggest that reconstitution of the iNKT cell pool (e.g., by adoptive transfer of *ex vivo* expanded autologous iNKT cells) provides a promising immunotherapeutic strategy for mCRC. Furthermore, screening for iNKT cells and ADCC levels in peripheral-blood samples might provide a noninvasive, straightforward prognostic parameter and may also be useful for determining which patients can benefit from cetuximab therapy.

Terminology

The antibody-dependent cell-mediated cytotoxicity is a mechanism of cell-mediated immune defense whereby an effector cell of the immune system actively lyses a target cell, whose membrane-surface antigens have been bound by specific antibodies. It is one of the mechanisms of the adaptive immune response through which antibodies can act to limit and contain tumors; classical antibody-dependent cell-mediated cytotoxicity is mediated by NK cells, which express CD16 which is an Fc receptor. This receptor recognizes, and binds to, the Fc portion of an antibody, such as the IgG1 anti-EGFR cetuximab; cetuximab is a recombinant chimeric mAb composed of the variable regions of a murine anti-EGFR antibody and of the constant regions of a human IgG1 kappa immunoglobulin. It is indicated for the treatment of squamous cell carcinoma of the head and neck and of *RAS* wild type mCRC.

Peer-review

This manuscript contributes to shed light to monoclonal therapy response in mCRC patients.

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Rectal neuroendocrine tumor with uncommon metastatic spread: A case report and review of literature

Nikolaos Tsoukalas, Michail Galanopoulos, Maria Tolia, Maria Kiakou, Georgios Nakos, Aristoula Papakostidi, Georgios Koumakis

Nikolaos Tsoukalas, Maria Kiakou, Department of Medical Oncology, 401 General Military Hospital, 11525 Athens, Greece

Nikolaos Tsoukalas, Maria Tolia, Aristoula Papakostidi, Georgios Koumakis, Second Department of Medical Oncology, "Agios Savvas" Anticancer Hospital, 11522 Athens, Greece

Michail Galanopoulos, Department of Gastroenterology, General Hospital of Athens "Evangelismos", 10676 Athens, Greece

Georgios Nakos, Department of Pathology, 401 General Military Hospital, 11525 Athens, Greece

Author contributions: Tsoukalas N and Galanopoulos M contributed equally to this work; all authors contributed to this work.

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Correspondence to: Nikolaos Tsoukalas, MD, MSc, PhD, Medical Oncologist, MSc in Bioinformatics, Consultant in Department of Medical Oncology, 401 General Military Hospital,

Kanelloupyloy 6, 11525 Athens, Greece. tsoukn@yahoo.gr
Telephone: +30-697-7366056

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Abstract

Neuroendocrine tumors of the gastrointestinal tract are rare neoplasms. Rectal neuroendocrine tumors consist approximately the 5%-14% of all neuroendocrine neoplasms in Europe. These tumors are diagnosed in relatively young patients, with a mean age at diagnosis of 56 years. Distant metastases from rectal neuroendocrine tumors are not very common. Herein we describe a case of a rectal neuroendocrine tumor which metastasized to the lung, mediastinum and orbit. This case underscores the importance of early identification and optimal management to improve patient's prognosis. Therefore, the clinical significance of this case is the necessity of physicians' awareness and education regarding neuroendocrine tumors' diagnosis and management.

Key words: Rectum; Uncommon metastatic spread; Neuroendocrine tumor; Rectal neuroendocrine tumor; Rectal neuroendocrine neoplasm

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Core tip: Rectal neuroendocrine tumors consist approximately 5%-14% of all neuroendocrine neoplasms in Europe. Distant metastases from rectal neuroendocrine tumors are not very common. Herein we describe a case

of a rectal neuroendocrine tumor with an uncommon natural history as well as a review of the literature. The present case underscores the importance of early identification and management of these tumors.

Tsoukalas N, Galanopoulos M, Tolia M, Kiakou M, Nakos G, Papakostidi A, Koumakis G. Rectal neuroendocrine tumor with uncommon metastatic spread: A case report and review of literature. *World J Gastrointest Oncol* 2016; 8(2): 231-234 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v8/i2/231.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v8.i2.231>

INTRODUCTION

The gastrointestinal tract has the largest component of neuroendocrine cells. In spite of this, neuroendocrine tumors of the colon and rectum are rare entities, with a reported incidence ranging from 0.3% to 3.9% of all colorectal malignancies^[1]. The introduction of more sensitive diagnostic tools (*e.g.*, immunohistochemical stains) and an overall increased awareness among physicians, have largely contributed to the rising incidence of neuroendocrine tumors^[2]. Here we describe an interesting case of a rare neuroendocrine neoplasm of the rectum with an uncommon natural history.

CASE REPORT

A 54-year-old man with free medical or family history came to our hospital reporting rectal bleeding in May 2005. Colonoscopy demonstrated a rectal polypoid mass, 15 mm in diameter, located 6 cm from the anus. Biopsies were taken and histopathology evaluation showed an adenocarcinoma which invaded submucosa. An extensive work up with computed tomography (CT) scans was negative for distant metastases but there was an infiltration of pericolic fat. After that, the patient underwent low anterior resection of the rectum and the mesorectum. The histopathological examination of the dissected specimen showed a grade 2 adenocarcinoma with infiltration of pericolic fat and regional lymph nodes (stage C1 Astler-Coller). Adjuvant chemotherapy with 6 cycles of FOLFOX4 was administered without radiotherapy.

Two years later, during the scheduled follow-up, the CT scans revealed a mass in the lower left lobe of the lung, which was surgically resected and the pathology showed a neuroendocrine tumor with well differentiation. The review of both histologic specimens (paraffin tube of rectum and lung specimens, Figures 1 and 2) showed that there were medium to large tumor cells, displaying a trabecular growth pattern with nuclear pleomorphism, hyperchromasia and prominent nucleoli. Tumor cells were often spreading individually infiltrating. No lymphovascular invasion was detectable. There were a few punctate foci of necrosis. The tumor cells invaded perirectal tissues and 2 regional lymph nodes were infiltrated. Pathologic staging was pT3N1M1 and the

clinical stage IV. Moreover, the immunohistochemistry analysis revealed positivity, in both specimens, for CK18, CK20, chromogranin, synaptophysin, CD56 and Ki-67, while CK7 and TTF1 were negative. Synaptophysin and chromogranin showed a diffuse positive staining of the tumor cells. These findings led to the conclusion that the primary tumor was that in the rectum and it was a neuroendocrine neoplasm well differentiated. In particular, Ki-67 was 8%-9% and the tumour was classified as well differentiated neuroendocrine tumor, intermediate grade (G2 NET). At that time patient refused to receive any further treatment.

One year later the planned follow-up showed a mass in the mediastinum. The octreoscan that followed showed increased uptake in the same anatomic region (Figure 3). Subsequently, the patient underwent radiotherapy (44 Gy) for the mass in the mediastinum. Moreover, the patient developed a mass in the left orbit, something that was discovered after a bilateral visual impairment and was treated with stereotactic radiosurgery (Cyber-Knife 18 Gy). Despite the medical advices patient refused to receive any systemic treatment. At the same period of time new lesions in left lung, mediastinum, adrenals and scalp were found. The patient was administered chemotherapy with the regiment Cisplatin 75 mg/m² d1 plus Etoposide 100 mg/m² d1-d3. Unfortunately, patient died after 4 cycles of chemotherapy due to uncontrolled systemic infection.

DISCUSSION

Rectal neuroendocrine neoplasms are usually small; polypoid lesions located in the mid-rectum, 5 to 10 cm from the anal verge and are submucosal in location, mainly discovered incidentally on routine surveillance endoscopies. If there are any symptoms, they include rectal bleeding, pain (as happened in our case) and change in bowel habits. However, 50% of patients are asymptomatic^[3].

They belong to a heterogeneous group of tumours, which all present a common phenotype with immunoreactivity for markers such as chromogranin A and synaptophysin^[4,5]. Neuron-specific enolase (NSE) and CD56 are frequently expressed in GEP-NETs, but are not specific. At present, immunohistochemistry for Ki-67 (MIB-1) is mandatory to grade the tumor according to the 2010 World Health Organization (WHO) classification and divides the tumors into NET G1, NET G2 and poorly differentiated neuroendocrine carcinoma (NEC G3)^[4].

Prognostic factors for metastases are tumor size, depth of invasion, and lymph node involvement of the rectal NETs. These factors may be assessed by transrectal ultrasound, if feasible, and pelvic MRI. One study revealed that metastases emerged in only 2% of tumors not bigger than 2 cm, which had not infiltrated the muscularis propria, compared to 48% of those infiltrating the muscularis layer^[6]. Although neuroendocrine tumours metastasize in 50%-75% of patients with the most common sites being lymph nodes, liver, and bones, metastases to the orbits, as happened in our case, have

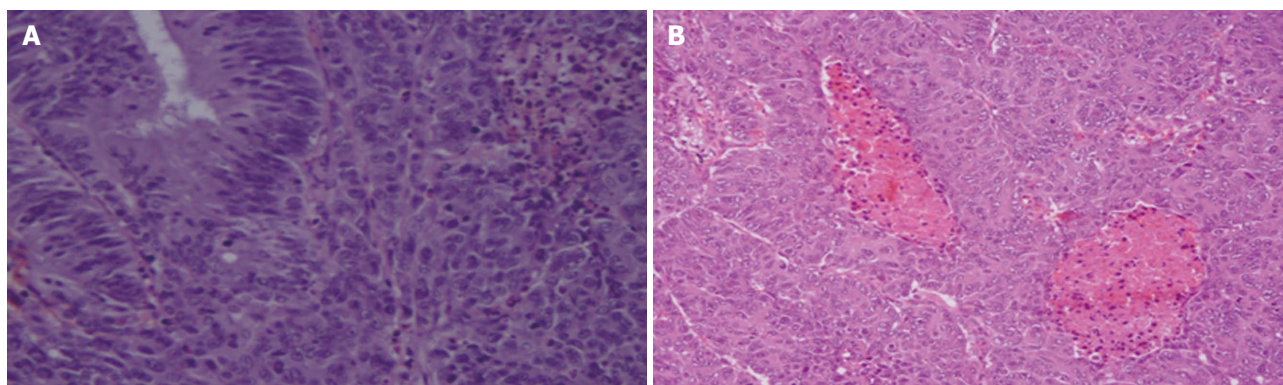


Figure 1 Biopsy of the rectal tumour (A) and lung tumour (B).

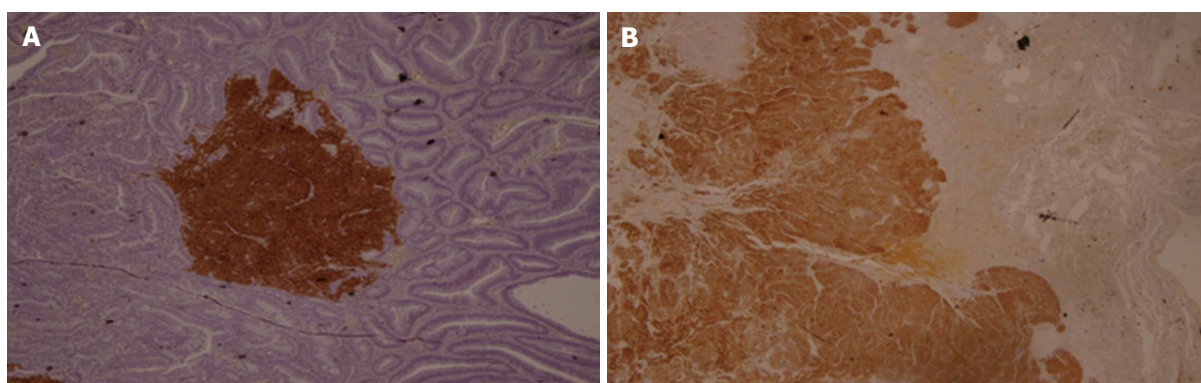


Figure 2 Immunohistochemical positivity for CD56 (rectum) (A) and CD56 (lung) (B).

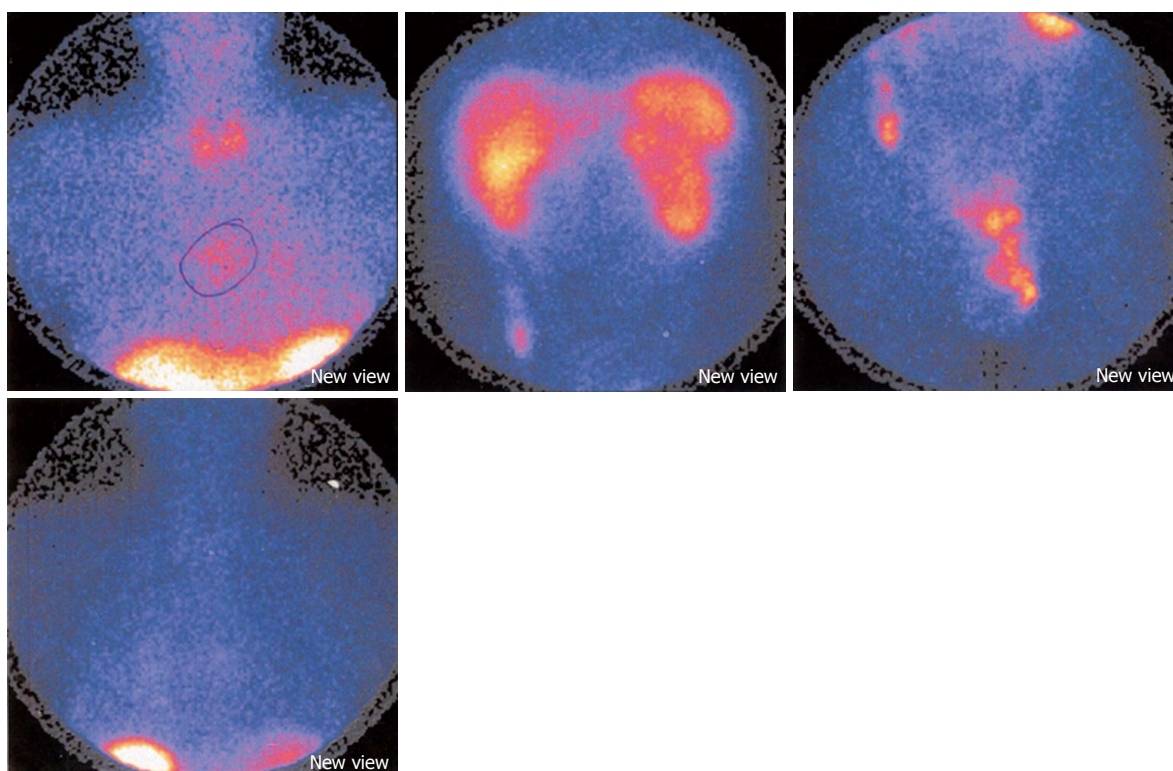


Figure 3 Octreoscan.

only rarely been reported (about 32 cases until 2006) and are believed to occur through hematogeneous

spread^[7]. Orbital neuroendocrine tumors tend to arise from the gastrointestinal tract, whereas bronchial neuroendocrine tumors show a propensity to uveal metastasis and typically present with a mass or diplopia while visual failure is unusual^[8], characteristics that verified in our case.

Obviously, metastatic disease at diagnosis will suggest a worse prognosis despite the available treatment options. In fact, surgery may have a palliative role to the complications associated with an advanced rectal tumour mass^[9]. Adjuvant therapy for well differentiated tumours after surgery is not considered, although an argument exists for applying chemotherapy in non-differentiated tumours with incomplete resection^[3]. Well differentiated neuroendocrine tumors is an uncommon indication for systemic chemotherapy^[10]. When used for progressive disease, streptozotocin combined with 5-fluorouracil with or without doxorubicin is most often applied even though the response rate is < 25%^[4]. The effectiveness of systemic chemo-regimens is optimal in poorly-differentiated tumours and the combination of cisplatin or carboplatin and etoposide have showed satisfactory results^[4]. Newer anti-angiogenesis or mTOR inhibitors may be used as well as peptide receptor radionuclide therapy peptide in patients with advanced or metastatic disease^[4,11]. Additionally, more chemotherapy regimens such as temozolomide and capecitabine are under clinical investigation for patients with advanced or metastatic neuroendocrine neoplasms^[12].

In conclusion, rectal neuroendocrine tumors are rare and cases with distant metastases are even rarer. This case underscores the necessity of physicians' awareness and education regarding neuroendocrine tumors' diagnosis and management.

COMMENTS

Case characteristics

A 54-year-old man with rectal bleeding.

Clinical diagnosis

A rectal polypoid mass, 15 mm in diameter.

Differential diagnosis

Rectal neuroendocrine tumor; Non-neoplastic polyp; Lung neuroendocrine tumor.

Laboratory diagnosis

A well differentiated rectal neuroendocrine tumor (G2 NET) with metastases to left lung, mediastinum and left orbit.

Imaging diagnosis

Computed tomography scans revealed masses in the lower left lobe of the lung, in the mediastinum and in the left orbit.

Pathological diagnosis

The histopathological examination showed a well differentiated rectal G2 NET.

Treatment

Chemotherapy with 6 cycles of FOLFOX4 at the beginning and then regimen Cisplatin 75 mg/m² d1 plus etoposide 100 mg/m² d1-d3.

Peer-review

This is an interesting case report describing a potentially malignant behavior of a primary neuroendocrine tumor of the rectum.

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TOPIC HIGHLIGHT

- 235 Complete mesocolic excision: Lessons from anatomy translating to better oncologic outcome
Zheng MH, Zhang S, Feng B

REVIEW

- 240 Endoscopic palliation of malignant biliary stricture
Salgado SM, Gaidhane M, Kahaleh M
- 248 Perioperative treatment options in resectable pancreatic cancer - how to improve long-term survival
Sinn M, Bahra M, Denecke T, Travis S, Pelzer U, Riess H
- 258 Primary prevention and treatment of venous thromboembolic events in patients with gastrointestinal cancers - Review
Riess H, Habbel P, Jühling A, Sinn M, Pelzer U
- 271 Gastric cancer development after the successful eradication of *Helicobacter pylori*
Uno K, Iijima K, Shimosegawa T

MINIREVIEWS

- 282 Emerging role of cystic fibrosis transmembrane conductance regulator - an epithelial chloride channel in gastrointestinal cancers
Hou Y, Guan X, Yang Z, Li C
- 289 Role of genetic detection in peritoneal washes with gastric carcinoma: The past, present and future
Chae HD
- 297 Is metastatic pancreatic cancer an untargetable malignancy?
Kourie HR, Gharios J, Elkarak F, Antoun J, Ghosn M

ORIGINAL ARTICLE

Basic Study

- 305 Expression of p-STAT3 and vascular endothelial growth factor in MNNG-induced precancerous lesions and gastric tumors in rats
Wang XY, Wang LL, Zheng X, Meng LN, Lyu B, Jin HF

Retrospective Study

- 314** Clinical and epidemiologic variations of esophageal cancer in Tanzania

Gabel JV, Chamberlain RM, Ngoma T, Mwaiselage J, Schmid KK, Kahesa C, Soliman AS

CASE REPORT

- 321** Myeloid sarcoma presenting as a colon polyp and harbinger of chronic myelogenous leukemia

Rogers R, Ettel M, Cho M, Chan A, Wu XJ, Neto AG

- 326** Rare case of entero-enteric intussusception caused by small bowel metastasis from a cardiac liposarcoma

Gomez G, Bilal M, Klepchick P, Clarke K

Contents

World Journal of Gastrointestinal Oncology
Volume 8 Number 3 March 15, 2016

ABOUT COVER

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Complete mesocolic excision: Lessons from anatomy translating to better oncologic outcome

Min-Hua Zheng, Sen Zhang, Bo Feng

Min-Hua Zheng, Sen Zhang, Bo Feng, Shanghai Minimally Invasive Surgery Center, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China

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Correspondence to: Bo Feng, MD, PhD, Shanghai Minimally Invasive Surgery Center, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, No. 197, Ruijin Er Road, Shanghai 200025, China. fengbo2022@163.com
Telephone: +86-21-63846590
Fax: +86-21-63846590

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Abstract

Since the introduction of complete mesocolic excision (CME) for colon cancer, the oncologic outcome of patients has been greatly improved, which has led to a longer survival

and a lower recurrence, just like the total mesorectum excision for rectal cancer. Despite the fact that the exact anatomy of the organ is one of the most vital things for surgeons to conduct surgery, no team has really studied the exact structure of the mesocolon and related attachments for CME, until the mesocolonic anatomy was first formally characterized in 2012. Therefore, this article mainly focuses on the anatomy development of the mesocolon and the achievement in this field. Meanwhile, we introduce the latest progress in laparoscopic surgery for colon cancer achieved by our team.

Key words: Colorectal cancer; "Page-turning" approach; Laparoscopic surgery; Complete mesocolic excision; Toldt's fascia

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Core tip: Despite that complete mesocolic excision (CME) has been conducted for many years, leading to a better outcome in colon cancer patients, there are limited studies on the structure of the mesocolon or related attachments, which is of great importance for surgeons to carry out surgery, until K. Culligan first formally characterized the mesocolonic anatomy, explaining the reason why CME would have a better oncologic outcome. Meanwhile, based on the exact anatomy of mesocolon, we introduce the latest progress in laparoscopic surgery for colon cancer achieved by our team, such as "page-turning" approach, and we also list the most important structure related to the CME.

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INTRODUCTION

In the past, patients with rectal cancer survived less than those with colon cancer. However, since Heald *et al.*^[1] first proposed total mesorectum excision (TME) for rectal cancer, the TME has standardized the surgical management of rectal cancer, which is based on the theory that mesorectum is composed of visceral and parietal planes covering rectum-supplying vessels and its lymphatic drainage like envelopes, leading to a lower recurrence rate as well as better 5-year cancer-related survival, which is even higher than that of colon cancer^[2-4] given that the surgery for colon cancer had not changed so much. In 2009, on the basis of the TME, Hohenberger *et al.*^[5] put forward the concept of complete mesocolic excision (CME). He stated that the mesocolon is covered by visceral and parietal planes by an envelope-style just like the mesorectum, and the "holy plane"^[6] extends to the mesocolon from the mesorectum. In his study, the patients who underwent CME had lower local recurrence and better survival, from 6.5% to 3.5% and 82.1% to 89.1% respectively. Since then, more and more studies have proven that superiority^[7,8].

Surgeons are meant to be ones who cure the disease through surgery with excellent knowledge about the organs, therefore, it is essential to stress the importance of accurately understanding mesocolic anatomy for surgeons to conduct CME. Although there have been studies on CME for years, no team really studied the exact structure of the mesocolon and related attachments, until K. Culligan *et al.*^[9] first formally characterized the mesocolonic anatomy in 2012. They made many undocumented and important discoveries, which could explain and confirm the feasibility and superiority of CME. Besides presenting the promising oncological outcome, this article mainly focuses on the anatomy of mesocolon and laparoscopic approach of CME to help peers comprehend CME better, and such improvement in anatomy-related understanding may explain why CME is a potential standardized procedure for colon cancer.

RESERACH

A systematic literature search was conducted using Pubmed and EMBASE with the terms of "complete mesocolic excision", "CME", "anatomy of CME", "laparoscopic CME" as well as "colon surgery". Many related studies were found and we summarized and presented the findings with our clinical experience.

The anatomy development of mesocolon

One of the earliest and most famous description of mesocolon was made by Sir Treves^[10] in 1885. He dissected and studied the anatomy of intestinal canal, peritoneum and mesentery with 100 cadavers and reported that mesocolon is discontinuous and fragmental. About half of the cadavers had neither an ascending nor a descending mesocolon, and 14 cadavers had them

both. The existence of mesocolon was always described as abnormal, even as "a cleft palate"^[11]. Indeed, before Sir Frederick Treves, Carl Toldt in 1879^[12], whose findings apparently differed from the above ones, studied the development of human mesentery, and noted the permanent existence of mesocolon in human being as well as a distinct fascial plane between the mesocolon and the underlying retroperitoneum. Known as Toldt's fascia, it was formed by the fusion of the visceral peritoneum of the mesocolon with the parietal peritoneum of the retroperitoneum. From today's point of view, Toldt's finding was almost true while Treves' not, but it did not work well at that time. Treves' discovery was so profound and foundational that it was spreaded and accepted extensively into surgery and teaching, even to recent days, it was still accepted widely as Rishabh Sehgal^[13,14] mentioned.

There has been no research really studying the exact structure of mesocolon for decades, until Culligan *et al.*^[9] first formally characterized the mesocolonic anatomy in detail. They chose 109 patients to undergo total abdominal colectomy, observed and recorded the anatomy of mesocolon, the related attachments and specimens, and finally noted many undocumented and meaningful findings: (1) mesocolon is continuous from the ileocaecal to rectosigmoid level; (2) Toldt's fascia is identified in the place where mesocolon is apposed to the retroperitoneum, such as ascending, descending mesocolon and non-mobile portion of mesosigmoid, while it does not show up in the transverse mesocolon and the mobile component of the mesosigmoid; and (3) the proximal rectum originates from the confluence of the mesorectum and mesosigmoid; and so on. What's more admirable, besides the important macroscopic discoveries above, Culligan's team first investigated the microscopic structures of mesocolon, Toldt's fascia and retroperitoneum before and after the colonic mobilization^[15,16]. They obtained samples from 24 cadavers, stained them with hematoxylin and eosin, Masson trichrome, and by immunohistochemistry to identify lymphatic vessels. Some samples were directly observed by scanning electron microscopy. Just like the macroscopic findings, they found consistent microscopic structures of mesocolon and associated fascia from the ileocecal to mesorectal level; in the place where mesocolon is apposed to the retroperitoneum, a connective tissue could be identified between them (*i.e.*, Toldt's fascia). Nowadays, we appreciate the excellent work done by Culligan's team. It was incredible and profound. For the first time, they described the exact anatomy of mesocolon, confirmed the continuity and surgical plane, and provided convincing proof for surgeons to conduct CME from the anatomical and histological aspects. Later, Gao *et al.*^[17] finished a similar study, also proving the continuity of mesocolon and the existence of visceral fascia. In his study he also thought the fascia to be able to block the tumor migration. Gao and Culligan used different terminologies to refer to the same thing, for example, Gao named it "visceral fascia" while Culligan called it "Toldt's fascia"^[18]. Such phenomenon is very normal, and there is also no

standardized nomenclature for CME, for instance, “right hemi-colectomy”, “enlarged right hemi-colectomy”, “Gerot’s fascia”, or “visceral fascia” and “parietal fascia”. Not being accurate and definite^[19,20] may confuse the new learners regarding explanations of the surgical procedures, which may have a bad impact on such improvement. Even though there is no “gold standard” for colon cancer, it is essential and necessary to have a unified terminology.

The laparoscopic approach of CME

Unexpectedly, despite the misconception in the textbook for centuries, surgeons have always seen mesocolon as a whole and conducted surgery based on the surgical plane between mesocolon and retroperitoneum^[19]. Back to 100 years ago, Jamieson (Jamieson and Dobson, 1909) suggested that surgery for colon cancer should resect the lesion, clean the regional lymph nodes to the vascular roots, and dissect lymph nodes of the interperitoneal colon, mesocolon and vascular roots, which was similar to the technique strategies of CME proposed by Hohenberger^[5]. Hohenberger demanded sharp separation of visceral and parietal fascia based on embryonic anatomy, ligation at the root of central supply vessels, and more radical lymph node dissection. The feasibility and promising outcome of CME have been confirmed in open surgery.

The improvement of laparoscopic techniques and further definition of equipment make it possible for surgeons to conduct colon cancer surgery. Taking the right-hemi colon cancer as an example, it is relatively more complicated and has more vessel variations. Feng *et al.*^[20] from our team first confirmed the feasibility and technical strategies of laparoscopic CME with medial access, following the dissection starting at the ileocolic vessel, proceeding along the superior mesenteric vein, and exposing the inborn surgical plane composed of Toldt’s and prerenal fascia to uncover the head of the pancreas and to mobilize the duodenum. The exposing range begins from the origin of transverse colon mesentery to the peritoneal reflection, and ligation at the origin of the central vessels to dissect the entire mesocolon was performed as a whole. Compared to the lateral access, the medial access complies more with the “no-touch” principle, since the lateral access starts with mobilizing the colon and then dissecting and ligating the central vessel. Not only is the pathological result comparable to the open surgery, but also the long-term outcome works well too^[2,4,21,22]. Based on the surgical experience through extensive surgeries, Feng *et al.*^[23] then exploited and distinguished two approaches for media access: Completely medial and hybrid medial approach (CMA and HMA). The major difference between them is the approach to dissect the inferior edge of the pancreas. The CMA uses a “bottom-to-top” fashion while the HMA uses a blending of “top-to-bottom and bottom-to-top” fashion. Compared to the HMA, the surgery time and ligation time of central vessel were significantly shorter, while the HMA induced more

vessel-related complications. Besides, our team recently found an improved surgical access based on CMA, which we named “page-turning” approach (CMAPA). It is conducted in a “bottom-to-top” and “inside-to-outside” direction, which adopted the strategy of “point-to-line” and “line-to-plane” [point: Taking the ileocolic vessels as a dissection trigger; line: Dissecting the vessels along the superior mesenteric vessel (SMV); plane: Extending the surgical plane by the “page-turning” approach, which was formed by Toldt’s fascia]. We suppose that this approach is technically feasible and complies more with the principle of tumor radical surgery.

However, CMA and HMA both emphasize the accurate recognition of the anatomical plane, especially the transverse retrocolic space which can extend to inter-mesenteric space and right retrocolic space to completely mobilize the right colon. Consequently, without a better understanding of the exact mesocolon, no surgeons can conduct perfect CME.

CONCLUSION

Just as the TME for rectal cancer, the CME follows the principle of embryology and anatomy by sharply dissecting the surgical plane between the visceral and parietal fascia to get an integrated mesocolon, ligation at the root of the central vessel and clearance of more lymph nodes, which can lead to a better survival. However, there is not an efficient evaluation system for CME except the grading system by West *et al.*^[24]: Muscularis propria plane (poor plane) if little mesocolon is excised with incision down to the muscularis propria; intramesocolic plane (moderate plane) if partial mesocolon is excised with an irregular shape but not down to the muscularis propria; mesocolic plane (good plane) if intact mesocolon is excised without defections on it and high ligation of the supply vessels. He further demonstrated a 15% increase of overall survival in the “mesocolic plane” group compared to “non-comparable” group. Even though the surgeons bear the concept in mind, there still have not been a global consensus about the accurate configuration of the mesocolon with related attachments. In addition, various words and terminologies are used in the CME, which makes people confused, especially the young. Besides, the reason why we describe CME as a better approach instead of a successful one, or why we think it has the potential to be the standard procedure for colon cancer, is that it has not been accepted universally, and there are still some debates. Some studies showed that extensive lymphadenectomy by CME failed to increase the survival^[25-28], although CME emphasizes the necessity to remove more lymph nodes, which can lead to a better survival. Besides, the operation time for CME is longer as the procedure is technically more challenging and complex, which may lead to more complications, such as bleeding, genitourinary dysfunction, or chyle leakage^[29,30]. What’s more, Culligan *et al.*^[18] first discovered abundant lymphatic vessels within Toldt’s fascia as well as mesocolon. This

means that it is possible that there are communications between them, which may cause tumor cell spread, and if that happens, there is little need to conduct CME. Further studies are needed to clarify this issue.

The majority of data we got were from single-center, retrospective studies, and not convincing enough for surgeons to accept CME as a standard procedure for colon cancer. So it is the time and it is essential for us to standardize the procedure of CME and conduct completely randomized and multi-center prospective studies to provide more proofs. At that time we will have great confidence to decide whether CME could be the standard procedure for colon cancer or not.

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Endoscopic palliation of malignant biliary stricture

Sanjay M Salgado, Monica Gaidhane, Michel Kahaleh

Sanjay M Salgado, Monica Gaidhane, Michel Kahaleh, Division of Gastroenterology and Hepatology, Weill Cornell Medical College, New York, NY 10021, United States

Author contributions: Salgado SM contributed to acquisition of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content; Gaidhane M contributed to acquisition of data, interpretation of data, critical revision of the manuscript for important intellectual content, study coordination; Kahaleh M contributed to study concept and design, acquisition of data, critical revision of the manuscript for important intellectual content, study supervision.

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Correspondence to: Michel Kahaleh, MD, AGAF, FASGE, Chief of Endoscopy, Division of Gastroenterology and Hepatology, Weill Cornell Medical College, 1305 York Avenue, 4th floor, New York, NY 10021, United States. mkahaleh@gmail.com
Telephone: +1-646-9624797
Fax: +1-646-9620110

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Abstract

Malignant biliary strictures often present late after

the window for curative resection has elapsed. In such patients, the goal of therapy is typically focused on palliation. While historically, palliative measures were performed surgically, the advent of endoscopic intervention offers minimally invasive options to provide relief of symptoms, improve quality of life, and in some cases, increase survival of these patients. Some of these therapies, such as endoscopic biliary decompression, have become mainstays of treatment for decades, whereas newer modalities, including radiofrequency ablation, and photodynamic therapy offer additional options for patients with incurable biliary malignancies.

Key words: Biliary strictures; Malignant; Endoscopic retrograde cholangiopancreatography; Photodynamic therapy; Endoscopy; Palliation; Endoscopic ultrasound; Radiofrequency ablation

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Core tip: Palliative therapies for malignant biliary strictures are crucial for a disease that so often presents with surgical ineligibility. In this paper, we highlight both the established and more novel endoscopic palliative approaches for these types of strictures. Perhaps the most established of these therapies is endoscopic biliary decompression *via* endoscopic retrograde cholangiopancreatography (ERCP), which is notably approached differently in extrahepatic and intrahepatic strictures. In cases where traditional ERCP fails or is not feasible, endoscopic ultrasound-guided biliary drainage has quickly become the second-line intervention. Finally, we end by discussing the literature behind more novel therapies, namely intraductal radiofrequency ablation and photodynamic therapy.

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INTRODUCTION

Biliary strictures should be considered in any patient presenting with clinical signs such as jaundice, pale stool, dark urine and pruritus. Once confirmed, a crucial step in the work-up includes differentiating between malignant and benign etiologies. Historically, this has been a challenge since as many of 15%-30% of such cases are eventually determined to be benign inflammatory processes after histologic assessment^[1,2]. Generally, the most common etiologies of malignant strictures include pancreatic adenocarcinoma (especially if located in the distal common bile duct) or cholangiocarcinoma (if in the mid- or proximal extrahepatic bile duct), although metastatic disease, ampullary neoplasia, gall bladder malignancy, hepatocellular carcinoma, and malignant periportal lymph nodes are all possibilities^[3].

Pancreatic adenocarcinoma and cholangiocarcinoma typically present late in the course of the disease, with the vast majority of patients ineligible for curative resection^[4], resulting in 5-year survival rates of under 5%^[5,6]. The role of endoscopy in these patients has expanded from a diagnostic tool to a therapeutic one, providing palliation that allows for improved quality of life at faster rates and lower cost than surgical methods^[6,7]. Endoscopic interventions for patients with malignant biliary strictures, includes endoscopic biliary drainage, intraductal radiofrequency ablation (RFA), and photodynamic therapy for cholangiocarcinoma.

A-ENDOSCOPIC BILIARY DECOMPRESSION

Extrahepatic biliary obstruction

While preoperative biliary drainage for malignant biliary strictures *via* endoscopic retrograde cholangiopancreatography (ERCP) remains controversial for surgically-eligible patients^[8], endoscopic stenting has become the gold standard for palliative biliary obstruction in non-surgical candidates. In this method, stents are placed at the site of obstruction *via* endoscope, allowing for minimally invasive relief of obstructive symptoms. Other, non-endoscopic options for palliative therapy include percutaneous transhepatic stenting of the biliary tree and surgical bypass. Early studies comparing endoscopic palliation to surgical bypass in patients with malignant strictures found that endoscopic intervention was superior to surgical bypass in terms of survival procedure-related mortality and complications^[9], survival (19 mo vs 16.5 mo^[10]), and total lifetime cost. Similarly, a randomized trial of 75 patients with malignant obstructive jaundice endoscopic palliation demonstrated that endoscopic stenting had significantly higher success in relieving jaundice while also boasting a lower 30-d mortality due to fewer liver-associated complications^[11].

A meta-analysis, which included 24 studies and over 2400 patients, found that, compared to surgical bypass, endoscopic intervention with plastic stents had similar

rates of technical and therapeutic success, as well as improvement in quality of life^[7].

Additionally, while endoscopic stenting with plastic stents had a lower risk of complications than surgical bypass, endoscopic intervention was, notably, also associated with a higher risk of recurrent biliary obstruction at 4 mo. Interestingly, given the recent increase in the use of metal (as opposed to plastic) stents in endoscopic palliation, assessing the comparative effectiveness of surgical vs endoscopic methods for palliative biliary obstruction becomes notably more difficult due to a paucity of available data. One small RCT ($n = 30$) that examined this comparison found no difference in complication rates, readmissions, or survival. However, those patients who received endoscopic therapy demonstrated better quality of life scores at roughly half the cost of surgical intervention^[12] suggesting a cost-effectiveness benefit to the utilization of endoscopic, as opposed to surgical, interventions. Nevertheless, ERCP has, over the past two decades, become the first line therapy for those ineligible for curative resection.

ERCP maintains biliary patency through stenting, options for which are continually evolving^[13]. Compared with plastic stents, self-expanding metal stents (SEMSs) have demonstrated significantly lower rates of migration and reocclusion^[7,14-17], an advantage mechanistically attributed to a wider stent diameter. However, the cost of SEMSs exceed that of plastic stents by order of magnitude^[18]. Therefore, the general consensus is that metal stents should be considered for patients with an estimated survival greater than 4-6 mo to maintain cost-effectiveness^[16,19,20].

SEMSs can be made of steel, nitinol or platinum and can be uncovered, partially covered, or fully covered. Covered stents have been manufactured with a coating designed to improve removability and prevent occlusion from tumor ingrowth or tissue hyperplasia^[21], however this theoretical advantage has not always been apparent from a clinical standpoint^[22,23] and until now the data of whether to use covered or uncovered metal stents in malignant disease is mixed among randomized controlled trials^[24-28] and even among metaanalyses^[29-31]. Underlying the inability powers those studies adequately. A recent study demonstrated longer patency of covered stent vs uncovered with the initial cost of the covered stent compensated by the benefit provide in patency.

Intrahepatic biliary obstruction

While biliary strictures commonly affect the extrahepatic biliary system, intrahepatic and hilar strictures tend to be less common, and can be asymptomatic in up to 30% of patients^[32,33]. Malignant obstruction of the biliary hilum have an exceptionally poor prognosis, with less than 10% of patients living longer than 5 years^[34]. The predominantly cause of malignant intrahepatic strictures is cholangiocarcinoma^[35], although squamous cell carcinoma, hepatocellular carcinoma, and metastatic disease are also potential etiologies^[36].

Hilar biliary strictures can be difficult diagnose. Typi-

cally presenting with ductal dilation with the absence of stones, the best available diagnostic tool is probably magnetic resonance imaging (MRI) or magnetic resonance cholangiopancreatography (MRCP), which not only can locate intrahepatic biliary strictures with 97% accuracy^[37], but also allows for the creation of a “road map” of the biliary tree to be used for planning endoscopic intervention (although notably, the specificity of MRCP may be more limited in the case of malignant strictures^[38]).

Therapeutic management of hilar strictures is predicated on surgical resectability. Tumors are deemed surgically unresectable in cases with (1) bilateral intrahepatic bile duct spread to secondary or segmental biliary radicals; (2) involvement of the main trunk of the portal vein; (3) bilobar involvement of hepatic arterial and/or portal venous branches; and (4) a combination of unilateral hepatic arterial involvement with cholangiographic evidence of extensive contralateral duct spread^[39].

A primary consideration in the management of hilar strictures is whether to unilaterally stent the obstructed duct or to, alternatively, place bilateral stents in both the left and right intrahepatic ducts. While unilateral stenting is less expensive than bilateral stenting^[40], a number of studies have suggested that bilateral stenting provides increased patency compared to unilateral stenting^[41,42] (although it is still debated^[43]). However, bilateral stenting is more challenging from a technical standpoint, requiring stent-within-stents or side-by-side deployment techniques^[44].

Although the use of plastic vs metal stents for intrahepatic strictures is still debated, there is growing evidence to suggest metal stents are preferable to plastic, with recent trials suggesting that SEMSs placement provides higher long-term patency, higher success rates, increased survival, and decreased costs^[14,41,45-47]. The use of metal stents is more crucial if the tumor is surgically resectable. Multiple studies examining the use of plastic stents in surgically resectable patients have noted high complication rates with no demonstrative mortality benefit^[48-50], whereas upon meta-analysis, metal stents have been found to reduce mortality up to 6%^[50], and are less likely to hinder future surgical intervention^[51].

Furthermore, the choice of using covered vs uncovered metal stents has important implications. Covered SEMS have the theoretic potential to obstruct the intrahepatic bile ducts in proximal biliary strictures, and although this limitation has been challenged in recent years^[52], most recommend placing uncovered metal stent in intrahepatic biliary obstruction. However, it warrants mention that compared to covered metal stents, uncovered stents have low migration rates, but are associated with a higher rate of stent dysfunction from tumor ingrowth and epithelial hyperplasia^[53]. For these reasons, uncovered SEMSs may be beneficial in cases of known malignant disease without eligibility for resection, but should be avoided if the diagnosis is uncertainly, or if there is any possibility of surgical resection, since covered SEMS better allows for future removal.

ENDOSCOPIC ULTRASOUND-GUIDED BILIARY DRAINAGE

In advanced disease, such as when tumor involves the second part of the duodenum, or in patients with alternated anatomy from bariatric surgeries or intestinal diversions^[54-56], endoscopic access to the biliary tree may be impaired, and ERCP not possible. Historically, upon failed ERCP, alternative therapies have included percutaneous transhepatic biliary drainage or surgical bypass. While in the past, arguments have been made in favor of these treatments^[57,58], surgical bypass is now limited to good surgical candidates, while external drainage *via* percutaneous intervention has been showed to have, negative impact on a patient's quality of life and long term failure^[59]. Furthermore, after a failed ERCP, attempting either of these interventions requires a separate intervention at a later date. Endoscopic ultrasound (EUS)-guided biliary drainage, has been offered for more than a decade in cases with (1) failed conventional ERCP; (2) altered anatomy; (3) tumor preventing access into the biliary tree; or (4) contraindication to percutaneous access (*i.e.*, ascites)^[56].

EUS-guided biliary drainage, a method first described in 1996^[60], is performed either through a transpapillary or transmural approach. The transpapillary approach consists of gaining access to bile ducts under EUS guidance, followed by placement of a guidewire across the obstruction. A conventional ERCP during a rendezvous can then be performed using the guidewire for access^[61]. Reported success rates of this procedure can vary greatly (70%-100%)^[62-65]. One of the largest study examining this procedure ($n = 58$) have reported favorable results, including success rates of over 98% with a complication rate of 6.9%^[66]. Furthermore, a recent meta-analysis demonstrated a success around 95% with adverse event of 15%^[67] related mainly to pneumoperitoneum, complication that has dramatically decreased since the use of CO₂.

In such cases where transpapillary drainage cannot be performed, transmural drainage may be taken, either through a transgastric-transhepatic (hepaticogastrostomy) approach for intrahepatic obstruction or a transenteric-transcholedochal (choledocoduodenostomy) for extrahepatic obstruction. The transgastric-transhepatic approach is typically performed through the lesser curvature of the stomach to allow for visualization and drainage of the left intrahepatic bile ducts, whereas the transenteric-transcholedochal^[68], is performed through the wall of the duodenum into the common bile duct^[56]. Similarly to traditional ERCP, plastic stents were originally placed for drainage, but SEMSs are being increasingly used^[69-71], due to their increased patency^[71,72].

The complications of EUS-guided biliary drainage include perforation, infection, and bleeding. Theoretically, EUS-guided draining may have a decreased rate of bleeding, as there is less manipulation of the papilla with this method^[73]. Furthermore, the manipulation of the wall integrity of the gastrointestinal tract could result in leakage

of bile, pneumoperitoneum, biloma, or bile peritonitis. However, the use of SEMSs should hypothetically minimize the risk of these complications by sealing the fistula created in these procedures^[74].

One single center randomized controlled trial of EUS-guided biliary drainage compared with percutaneous biliary drainage demonstrated similar levels of technical success and no difference in adverse effects or cost^[75]. Overall, EUS-guided biliary drainage in expert centers has become as a second-line therapy due to its minimal invasiveness and ability to be performed immediately after a failed ERCP.

C-INTRADUCTAL RFA

RFA is a technique in which monopolar or bipolar electrodes are inserted into tissue prior to applying an alternating electric current. The high resistance of the current in biological tissue results in the production of heat, which at sufficient levels, causes instant coagulative necrosis^[76]. RFA is used in a wide variety of palliative therapies for malignancies, including lung cancer^[77], renal cell carcinoma^[78], prostate cancer^[79], breast cancer^[80], osteoid osteomas^[81], and certain brain tumors^[82]. Among gastrointestinal tumors, RFA is an established treatment for inoperable liver neoplasm, and is increasingly being used as palliative treatment for malignancy-related biliary obstruction and advanced pancreatic tumors.

The biologic rationale of using RFA in the treatment of malignancies extends beyond its a priori destructive properties. Evidence suggests that malignant tumor cells necrosis at lower temperatures than normal cells^[83,84], indicating that cancerous cells will be disproportionately affected by thermal ablation. This effect is accentuated in areas of poor blood flow^[85], indicating that hypovascular tumors, in particular, would be ideal targets of hyperthermic treatment.

There are numerous advantages to RFA tumor therapy. Notably, it is less expensive and less invasive than surgery, carrying an eight-fold lower complication rate and a two-fold shorter hospital stay^[86,87]. For biliary obstruction, RFA is typically paired with SEMSs placement, with small trials demonstrating 100% patency at 30 d, and 85% patency at 90 d^[88], with significant improvement in stricture diameter of 3.7 mm^[89]. More impressively, in patients with malignancy-related biliary obstruction, a growing body of literature is suggesting that endoscopic RFA followed by stenting provides a significant survival benefit when compared to patients treated with stenting alone^[90-93], advocating for the use of combination endoscopic therapy. Interestingly, a 62 patients study assessing the treatment of biliary strictures related to various neoplastic etiologies found that pancreatic cancer, in particular, was a significant predictor of stricture improvement with RFA^[94], although the mechanism for this remains unknown.

D-PHOTODYNAMIC THERAPY

Photodynamic therapy (PDT) involves utilizing a specific

wavelength of light to activate a intravenously given photosensitizing agent and cause ablation by directly damaging tumor cells, interfering with microvasculature of the tumor bed, and potentiating an immune response^[95-99]. The photosensitizer-dependent wavelength light is typically delivered *via* optical fibers placed in the target tissue, with the penetrance dependent on the wavelength of light and the specific light source used^[100]. PDT causes tissue necrosis by a non-thermal cytotoxic effect, mediated by the light-induced transfer of oxygen from a photosensitizer to molecular oxygen, generating a reactive oxygen species. Unlike other ablative methods, PDT has the unique ability to trigger apoptosis in neoplastic tissue^[101] and is collagen-sparing, allowing for the maintenance of tissue architecture^[102].

There have been numerous studies examining PDT for cholangiocarcinoma, which have demonstrated that PDT provides a survival benefit^[103-106]. Further studies suggested that PDT in combination with endoscopic stenting was produced a mild survival benefit that was not matched by stenting alone^[107,108]. A randomized control trials comparing survival rates in patients treated with biliary stenting alone with those treated with combination biliary decompression and PDT was terminated early, due to the survival and quality of life benefit in the patient who received PDT^[109]. A meta-analysis of six studies found that, compared with biliary stenting, PDT was associated with improved biliary drainage, better quality of life, and longer survival with similar rates of biliary sepsis^[110]. Overall however, PDT for malignant biliary obstruction remain a strong therapy, although a large-scale randomized trial is in progress to further validate its benefits.

CONCLUSION

Palliative endoscopic therapies for malignant biliary strictures are crucial for a disease that so often presents with surgical ineligibility. Endoscopic options range from biliary decompression to more advanced therapies, such as RFA or photodynamic therapy. The potential advantages of full utilization these methods, especially in the setting of minimally invasive EUS-guided therapy, has redefined the management of patients with inoperable biliary malignancies.

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Perioperative treatment options in resectable pancreatic cancer - how to improve long-term survival

Marianne Sinn, Marcus Bahra, Timm Denecke, Sue Travis, Uwe Pelzer, Hanno Riess

Marianne Sinn, Sue Travis, Uwe Pelzer, Hanno Riess, Department of Medical Oncology and Haematology, Charité - Universitätsmedizin Berlin, 13353 Berlin, Germany

Marcus Bahra, Department of General, Visceral and Transplantation Surgery, Charité - Universitätsmedizin Berlin, 13353 Berlin, Germany

Timm Denecke, Institute of Radiology, Charité - Universitätsmedizin Berlin, 13353 Berlin, Germany

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Correspondence to: Dr. med. Marianne Sinn, MD, Department of Medical Oncology and Haematology, Charité - Universitätsmedizin Berlin, Augustenburger Platz 1, 13353 Berlin, Germany. marianne.sinn@charite.de
Telephone: +49-30-450553222
Fax: +49-30-450553959

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Abstract

Surgery remains the only chance of cure for pancreatic cancer, but only 15%-25% of patients present with resectable disease at the time of primary diagnosis. Important goals in clinical research must therefore be to allow early detection with suitable diagnostic procedures, to further broaden operation techniques and to determine the most effective perioperative treatment of either chemotherapy and/or radiation therapy. More extensive operations involving extended pancreatectomy, portal vein resection and pancreatic resection in resectable pancreatic cancer with limited liver metastasis, performed in specialized centers seem to be the surgical procedures with a possible impact on survival. After many years of stagnation in pharmacological clinical research on advanced pancreatic ductal adenocarcinomas (PDAC) - since the approval of gemcitabine in 1997 - more effective cytotoxic substances (nab-paclitaxel) and combinations (FOLFIRINOX) are now available for perioperative treatment. Additionally, therapies with a broader mechanism of action are emerging (stroma depletion, immunotherapy, anti-inflammation), raising hopes for more effective adjuvant and neoadjuvant treatment concepts, especially in the context of "borderline resectability". Only multidisciplinary approaches including radiology, surgery, medical and radiation oncology as the backbones of the treatment of potentially resectable PDAC may be able to further improve the rate of cure in the future.

Key words: Pancreatic cancer; Perioperative treatment;

Perioperative radiology; Chemotherapy

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Core tip: Pancreatic cancer remains one of the most challenging tumor entities and is predicted to become the second leading cause of cancer deaths. More effective chemotherapeutic concepts in combination with early and exact imaging and more extensive surgical approaches may improve the rate of cure.

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INTRODUCTION

Pancreatic ductal adenocarcinomas (PDAC) are predicted to become the second largest cause of cancer related death in the United States by 2030^[1]. This is mainly due to the lack of therapeutic options, making PDAC one of the few types of cancer with a still increasing mortality rate^[2]. Surgery remains the only chance of cure for this devastating disease, but only 15%-25% of patients present with resectable disease at the time of primary diagnosis^[3]. Important goals in clinical research must therefore be early detection with suitable diagnostic procedures, further broadening operation techniques, and determining whether chemotherapy or radiation therapy is the most effective perioperative treatment. Only multidisciplinary approaches including surgery, radiology, medical and radiation oncology and gastroenterology may be able to further improve the rate of cure in the future^[4].

This review of the treatment options of potentially resectable PDAC will focus on the 3 backbones - radiological assessment, surgical procedures and perioperative regimen (chemotherapy/radiochemotherapy) - and their potential impact on long-term survival. Current standards will be discussed as well as ongoing or recently completed clinical trials.

ROLE OF RADIOLOGY

Although explorative laparotomy is considered the gold standard for resectability assessment, radiological imaging plays a key role in planning surgical procedures. Until the recent refinement of national management guidelines for PDAC, the main concern on radiological assessment of patients with suspected PDAC was the determination of resectability^[5]. In view of the high mortality rate, it is necessary to offer curative resection, as this is the only chance for long-term survival. On the

other hand, this chance is rather small, with only about 20% 5-year survival rate in case of curative resection, and the procedure is not free of risk for mortality, which depends on the medical centers' experience and ranges from 3.8% to 16.3%^[6,7]. Furthermore, morbidity after resection can limit the use of adjuvant chemotherapy. Thus, the preoperative imaging must be used to determine whether a patient has a good chance of curative resection and should have explorative laparotomy or should rather undergo conservative treatment with chemotherapy without further delay. This decision is always accompanied by the double risk of either denying a patient a potentially lifesaving resection or performing unnecessary surgery in patients with unresectable tumors.

The achievement of an R0-resection is one of the most significant parameters for survival^[6]. Therefore, the first goal of radiological assessment is to confirm the probability of R0-resection without the necessity of arterial reconstruction^[8]. Both computed tomography (CT) and magnetic resonance imaging (MRI) have been shown to be effective to this end. The assessment accuracy of local resectability by CT was as high as 93% in a representative study conducted in a high volume center^[9]. The criteria have been extensively reviewed elsewhere and are summarized in current guidelines^[10-12]. Basically, arterial encasement of the celiac trunk, the common hepatic artery, the proper hepatic artery or the superior mesenteric artery is deemed unresectable, as survival remains poor even after technically successful tumor removal and arterial reconstruction (Figure 1)^[8]. Similarly, survival is impaired for those patients requiring resection of the mesentericoportal venous axis; however, long-term survival can be observed in some of these cases^[8]. Therefore, venous involvement of the tumor is not a criterion for unresectability if reconstruction of the mesentericoportal flow by vessel resection and reanastomosis, patch plastic or graft interposition is technically feasible (Figure 1). CT can predict clear infiltration by encasement of arteries and clear non-infiltration by non-contact to adjacent arteries (surrounding preserved fat plain) with acceptably high accuracy; however, once tumor contact to the vessels is present, the ability to decide whether the tumor can be surgically removed from the artery without resection and reconstruction of the artery decreases^[13,14]. In such cases, the accuracy of prediction of local resectability by radiological imaging is limited and repeatedly results in unexpected abortion of surgical exploration. Or - vice versa - could cause preclusion of a patient from resection if no exploratory attempt was undertaken because the disease was found to be too advanced on imaging. Even though these cases are becoming rare thanks to improvements in imaging in terms of spatial resolution and speed of image acquisition, with better vessel enhancement and tumor delineation as well as minimization of image artifacts^[9], it would be desirable to offer the remaining patients inside this grey zone of locally advanced tumors an approach to safely decide

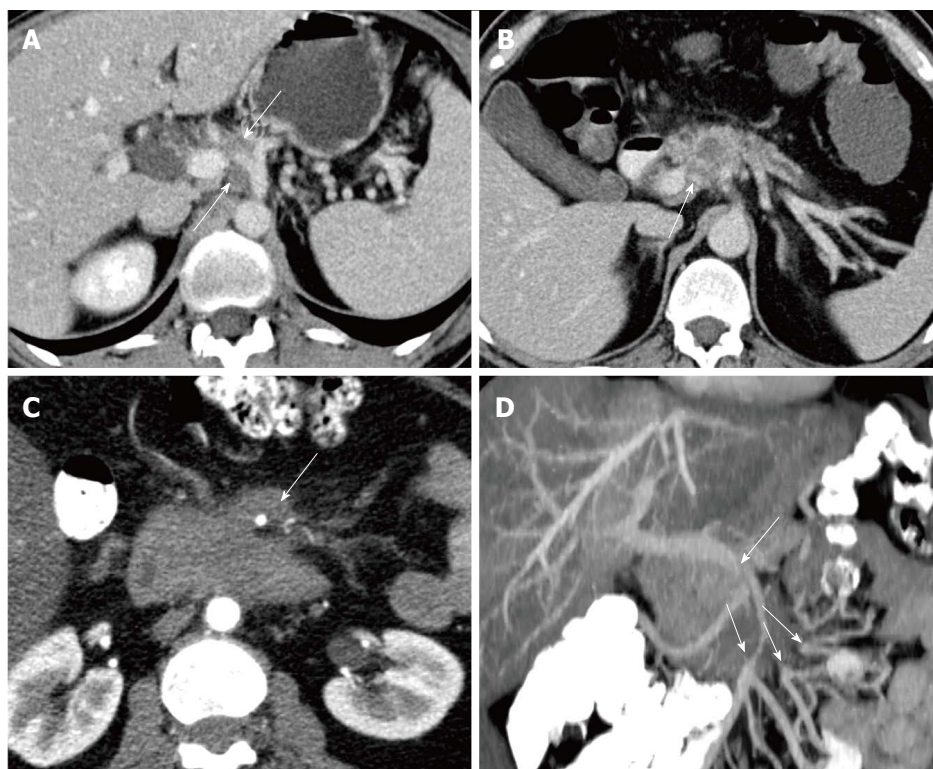


Figure 1 Examples of resectability assessment in untreated pancreatic ductal adenocarcinoma by means of computed tomography. A: Unresectability due to encasement of the common hepatic artery reaching to the celiac trunc (arrows); B: Borderline resectability with infiltration of the portal vein and one-sided contact to the common hepatic artery without extension to the celiac trunc. Neoadjuvant treatment and/or pancreatic left resection with *en-bloc* resection of the celiac trunc (after embolization) can be considered; C: Unresectability due to encasement of the superior mesenteric artery by more than 180°; D: Infiltration of the superior mesenteric vein and venous confluence with stenosis and multiple separated mesenteric venous branches unsuitable for surgical reconstruction.

whether it is worth undergoing the R0-resection or continuing with conservative treatment.

Preoperative treatment could serve as “problem solver” in such cases, termed “borderline resectable” cancers. They are characterized by intense tumor contact to the arterial mesenteric (up to 180° encasement) and celiac axes (up to short encasement of the common hepatic artery without extension to the celiac trunk) or infiltration of the mesentericoportal venous axis (if technically manageable) (Figure 1)^[5]. This concept could help increase the rate of R0-resection in this group *via* tumor shrinkage and aid in selection of patients for resection by filtering out those with aggressive tumors nonresponsive to chemotherapy. Observational cohort studies of this concept have been promising, but as yet no prospective controlled studies have been performed. Arguments against this approach could be that shrinkage of initially radiologically invisible micrometastases (*e.g.*, to the peritoneum and liver), will lead to false negative M0-assessments intraoperatively despite residual viable tumor cells in these remote lesions. In order to appropriately select patients for this new approach, the actually most accurate imaging strategy for detection of small metastases, MRI of the liver with diffusion weighted imaging (DWI) and hepatocyte specific contrast agents^[15-17] would be justified.

Finding the optimal imaging strategy is also desirable for the local assessment of tumor extent. On the other hand, introduction of a third type of assessment complicates the work of the radiologists and should not be used to avoid definite statements by calling tumors borderline resectable in case of any doubt. In consequence, diagnostic imaging should consist of state of the art imaging

under optimal conditions such as hardware and contrast agents used, as well as imaging protocols including dynamic high resolution contrast imaging in order to optimize delineability of the peripancreatic vasculature. One of the most important factors to be considered is how recent the imaging studies are, as it is known that regardless of the imaging quality, images older than 4 wk do not reflect the extent of tumors found on surgery^[18], making repeated imaging necessary before a decision can be made.

Another problem of preoperative therapy is the necessity of histological confirmation and grading of the tumor. It therefore becomes even more important to characterize the tumor by means of radiological imaging and to exclude other tumors such as neuroendocrine tumors, sarcomas, lymphomas, as well as non-malignant diseases (*e.g.*, mass forming pancreatitis, autoimmune related pancreatitis). This aids decision making regarding pretherapeutic biopsy strategies.

The reassessment of tumors after neoadjuvant treatment poses new challenges for diagnostic imaging. After initial reports showing results almost as good as in patients without preoperative therapy, more recent studies have increasingly shown that local tumor extent tends to be overestimated by imaging procedures after chemotherapy, and even more so after radiation^[19-21]. The reliability of a preoperative radiological statement is diminished and can only be compensated for by thorough surgical exploration. This again - knowing of the “point of no return” of the resection of a pancreatic head carcinoma, beyond which R0-resection may still be found to be impossible in some cases - can be a drawback of the preoperative concept, potentially resulting in R2-

resected patients. In this context new parameters are needed to identify response and shrinkage of viable tumors. DWI, perfusion measurement by MRI and CT (including dual energy CT), and positron emission tomography are currently under investigation to fill this gap with results pending. Alternative surgical strategies are also included, such as the very promising distal pancreatectomy with en-bloc resection of the celiac trunk for cases where pancreatic tail carcinoma has reached the celiac axis. This requires very precise planning based on radiologic imaging and a preoperative radiological intervention in order to train collateralization (see "surgical procedures" below)^[22,23].

In conclusion, with the advent of new management options in preoperative therapy of pancreatic cancer, radiological imaging - for planning of biopsy and surgery as well as pre-surgical reassessment and preconditioning - has moved to the frontline of treatment decision making and become the basis of measures to achieve the treatment goal: Prolonged survival of the patients.

SURGICAL PROCEDURES

Much progress has been made in the field of pancreatic resection in recent years and importantly, as the quality of surgery is considered a key factor in long-term survival, consensus has been reached on a number of surgical principles. An analysis of our own patient cohorts ($n = 428$) suggests that postoperative complications deteriorate long-term outcome after pancreatic resection in pancreatic cancer patients. We found that severe postoperative complications had a strong negative impact on long-term survival. This effect was significant and even comparable to that of the most relevant tumor characteristics such as lymph node involvement, grading or resection margin^[24]. An analysis by Birkmeyer *et al.*^[7] showed absolute differences in 5-year survival probabilities rates between low-volume hospitals (LVH) and high-volume hospitals (HVH): The absolute difference in 5-year survival in patients with pancreatic cancer between LVH and HVH was 5%. These findings underline the importance of centralization of pancreatic surgery for pancreatic cancer; in other words, patients should be transferred to specialized centers to further improve the results of surgical procedures^[7].

Perioperative medical management

Several studies have suggested that blood transfusion is associated with impaired long-term survival. A recent systematic review including 23 studies with 4339 patients confirmed this assumption. Patients receiving perioperative blood transfusion had a significantly lower 5-year survival rates after pancreatic resection in 13 of 19 studies in univariate and multivariate analyses^[25].

Extended pancreatectomy

The goal of any pancreatic resection for pancreatic adenocarcinoma should be the complete removal of

the tumor (R0-situation). To this end, more radical or "extended" surgical techniques have been established in the last decades, whereat "extended" in this context means a more aggressive marginal clearance^[26-28].

This may include total duodenopancreatectomy, resection of the portal-mesenteric axis, and extended lymph node dissection. Extended pancreatectomy is defined as standard pancreatoduodenectomy and includes the resection of the head of the pancreas and uncinate process, duodenum and first segment of the jejunum, common bile duct and gallbladder, lymphadenectomy, sometimes pylorus and/or antrum of the stomach and sometimes elements of the transverse mesocolon. Relevant vascular structures and any of the following organs involved in continuity are excluded: More than the antrum or distal half of the stomach; colon and/or mesocolon with relevant vascular structures of the transverse mesocolon (ileocolic, right, or middle colic vessels); the small intestine beyond the first segment of the jejunum; portal vein, superior mesenteric, and/or inferior mesenteric vein; hepatic artery, celiac trunk, and/or superior mesenteric artery; the inferior vena cava, right adrenal gland, right kidney and/or its vasculature; and last but not least the resection of liver and diaphragmatic crura.

The standard distal pancreatectomy is defined as the resection of the body and/or tail of the pancreas; the spleen with the splenic vessels combined with a standard lymphadenectomy; the resection of the fascia of Gerota if necessary; and potentially also elements of the transverse mesocolon apart from relevant vessels.

In contrast, an extended distal pancreatectomy refers to the standard distal pancreatectomy plus any type of gastric resection, colon and/or relevant vascular structures of the transverse mesocolon, small intestine; portal vein, superior mesenteric, and/or inferior mesenteric artery; inferior vena cava, left adrenal gland, left kidney, liver and diaphragmatic crura.

The extended resection approach may have problematic consequences: Operating time and blood loss; in addition the length of stay in the intensive care unit and hospital may be increased after extended surgery. Furthermore, surgical morbidity is increased after extended pancreatectomy. Perioperative mortality seems to be similar to that of standard pancreatectomies. Data regarding survival suggested no difference between patients after standard resection compared to those who underwent extended resection^[29]. However, recent long term follow-up findings suggest an inferior prognosis after extended pancreatic resection compared to those who underwent standard resection (11.7% 5-year survival vs 21% 5-year survival)^[30].

Extended lymph node dissection

The resection of the regional lymph nodes around the duodenum and the pancreas plus the lymph nodes on the right side of the hepatoduodenal ligament, the right side of the superior mesenteric artery and the

anterior and posterior pancreatoduodenal lymph nodes is defined as standard lymphadenectomy. Lymph node dissection beyond this area is considered extended. However, a prospective comparison of the surgical results of extended lymph node dissection and standard lymph node resection did not show any significant difference in survival between the two strategies^[31]. Extended lymph node dissection is therefore not considered to improve long-term survival.

Portal vein resection

A recent publication showed that resection of the portal vein was significantly associated with the four following factors: Larger and poorly differentiated tumors, higher numbers of positive lymph nodes and positive resection margins^[32]. Debate is currently ongoing as to whether routine portal vein resection during pancreatoduodenectomy for PDAC is reasonable. In this context, a French retrospective analysis has been published showing that patients (without microscopic venous tumor involvement of the portal vein) who underwent pancreatic resection combined with portal vein resection had a significantly longer overall survival than patients in a matched control group after pancreatic head resection without venous resection^[33]. These data may indicate that a “*per se*” portal vein resection results in a more radical local tumor elimination and may improve long-term survival.

Infiltration of the celiac trunk

As a particular situation in the context of locally advanced PDAC, tumor manifestation in the pancreatic body with involvement of the celiac trunk and/or the common hepatic artery needs mentioning. Normally such a situation is considered unresectable^[34]. However, a radical distal pancreatectomy with splenectomy and *en-bloc* resection of the celiac trunk without reconstruction of the celiac axis is a potential curative approach for these cases^[35]. The weak point of this concept is the interruption of the direct arterial blood supply to the liver, bile ducts and stomach. This may lead to acute liver failure, bile duct necrosis or ischemic necrosis of the stomach. A multidisciplinary approach is therefore needed to avoid such severe complications. Preoperative digital subtraction angiography is necessary to provide information about the perfusion of the crucial vessels^[23]. In cases where there is a pre-existing stenosis of the celiac trunk, spontaneous collateral blood flow from the superior mesenteric artery directly to the gastroduodenal artery *via* pancreaticoduodenal arcades and therefore to the hepatic artery can be seen. In such a situation, the tumor can be resected immediately including the celiac trunk. If there is no pre-existent celiac trunk stenosis, embolization of the celiac trunk is needed to enhance collateral blood flow before the operation. Important requirements for this multi-step approach are the existence of sufficient collateral arteries and no tumor invasion of the superior mesenteric artery. Sperti *et al*^[22] reported on similar survival rates to regular R0-resections after this sophisticated intervention.

Is there a role for pancreatic resection in resectable pancreatic cancer with limited liver metastasis?

A recent retrospective analysis conducted by our institution showed that even patients with advanced pancreatic cancer (locally advanced or locally resectable with hepatic oligometastasis) seem to benefit from pancreatoduodenectomy followed by gemcitabine-based chemotherapy. Forty-five patients who had undergone palliative intended pancreatic resection followed by chemotherapy were therefore matched with 45 patients after upfront gemcitabine-based palliative chemotherapy. A subgroup of patients with locally resectable tumor and limited liver metastasis (R0/M1) showed a significant improvement in overall survival compared to patients who had not had surgery (14.4 mo vs 7.2 mo), suggesting a potential role for pancreatic resections in patients with limited liver metastasis if the primary tumor is completely removable^[36].

ADJUVANT AND NEOADJUVANT CONCEPTS

Current standards

In the ongoing discussion about the role of perioperative treatment options in PDAC, ideal timing (neoadjuvant/adjuvant) and the ideal modality (chemotherapy, radio-chemotherapy) remain controversial^[11,37]. The most convincing contemporary data based on a high level of evidence are available for the effectiveness of adjuvant chemotherapy alone, based firstly on the results of the CONKO-001 trial. This randomized multi-center phase III trial included 368 patients and compared 6 cycles of adjuvant gemcitabine (Gemcitabine 1000 mg/m² d1, 8, 15, q29) to observation only^[38]. Median disease-free survival was significantly improved (13.4 mo for the gemcitabine group compared to 6.7 mo for the observation only group; $P < 0.001$) and led to a significant but numerically small advantage in overall survival (22.4 mo gemcitabine vs 20.2 mo observation only; $P = 0.01$). Indeed, the most important and clinically relevant result of the CONKO-001 remains that 5-year survival (an established surrogate marker for long-term survival) and rate of cure were doubled by adjuvant gemcitabine: 20.7% in the Gem group compared to 10.4% in the observation group. These data are based on long-term follow-up over more than 11 years^[39] and were confirmed to a large extent by the ESPAC-3 trial. This large randomized, multicenter phase III trial included 1088 patients and compared 6 cycles of gemcitabine to 6 cycles of 5-FU (425 mg/m² d1-5 bolus *i.v.*, q d29) and folinic acid (20 mg/m² d1-5 *i.v.*, q29). Median DFS (Gem 14.3 mo vs 14.1 mo; $P = 0.53$) and OS (Gem 23.2 mo vs 5-FU 23.0 mo; $P = 0.32$) were similar in both treatment groups (and to the CONKO-001 gemcitabine group), but more relevant toxicities were observed in the 5-FU group^[40]. In conclusion, both chemotherapeutic regimens are considered standard care in the adjuvant

Table 1 Standard adjuvant therapy

Gemcitabine 1000 mg/m ² <i>i.v.</i> 30 min	d1, 8, 15, q29
	6 cycles
5-fluorouracil 425 mg/m ² <i>i.v.</i> Bolus	d1-5, q29
Folinic acid 20 mg/m ² <i>i.v.</i> Bolus	6 cycles

setting^[41], but the better toxicity profile of gemcitabine should be taken into consideration^[34]. More recently, the Japanese JASPAC-01 trial compared gemcitabine to the 5-FU prodrug S1 (80/100/120 mg/d based on body surface area, p.o., d1-28, q6w, for 4 courses). S1 is known for its specific effectiveness in Asians and is widely used, especially in the treatment of gastric cancer^[42]. 385 patients were included in this randomized phase III trial which showed impressive results, with a significantly improved 2-year survival of 70% in the S1 group compared to 53% in the gemcitabine group (HR 0.56, $P < 0.001$). The data were presented at the annual ASCO-GI meeting in 2013^[43], but full publication and a confirmatory trial, especially in non-Asian patients, must be awaited (Table 1).

Therapeutic concepts combining chemotherapy with targeted therapy have been so far unsuccessful at further improving survival in resectable PDAC - a well-known phenomenon for unresectable PDAC as well^[44]. CONKO-005, designed as a follow-up randomized phase III trial of CONKO-001, investigated the additional effect of the EGFR-tyrosine-kinase inhibitor Erlotinib (100 mg daily p.o. d1-28, q29) in combination with gemcitabine (standard dosage) for 6 cycles in 436 patients after R0 resection. No improvement could be demonstrated for the primary endpoint DFS, but a trend was described for improved long-term survival in the erlotinib + gemcitabine group^[45]. Longer follow-up and ongoing molecular analyses will clarify whether it is possible to identify a subgroup which could benefit from erlotinib in the future. The parallel trial CONKO-006 for R1 resected patients investigated the concept of prolonged additive chemotherapy in this high risk patient cohort, as well as the role of the multikinase inhibitor sorafenib. All patients ($n = 122$) received 12 cycles of adjuvant gemcitabine and were randomized for gemcitabine alone or in combination with sorafenib (400 mg bid, p.o., d1-28, q29). No improvement in DFS or OS could be demonstrated for the overall study population by prolonging postoperative chemotherapy or using sorafenib^[46].

Several trials which investigated the role of adjuvant radiochemotherapy in PDAC also failed to show a clear survival advantage in the use of intensified treatment modalities. The CAPRI trial^[47] may be presented as a disappointing endpoint of this concept. In this phase III trial, 132 patients were randomized to receive either 5-FU as standard of care or an aggressive postoperative regimen of 5-FU (200 mg/m² daily), Cisplatin (30 mg/m² weekly) and Interferon alpha-2b (3 Mio IU 3 × weekly) in combination with radiation therapy (50.4

Table 2 Completed adjuvant phase III trials

Ref.	Treatment group	Median OS (mo)	3-yr-survival	5-yr-survival
CONKO-001 ^[38,39]	Gem	22.8	37%	21%
	Observation	20.2	20%	9%
ESPAC-3 ^[40]	Gem	23.6		
	5-FU/ folinsäure	23.0		
JASPAC-01 ^[43]	S1		2 yr 70%	
	Gem		2 yr 53%	
RTOG 97-04 (pancreatic head) ^[57]	RCT + Gem	20.5	31%	24%
	RCT + 5-FU/ FS	16.9	22%	11%
CONKO-005 ^[45]	Gem + Erlotinib	24.6	36%	28%
	Gem	26.5	33%	19%

5-FU: 5-fluorouracil; OS: Overall survival.

Gray), followed by 2 cycles of continuous 5-FU. No difference was found for the primary endpoint OS (26.5 mo vs 28.5 mo, $P = 0.99$) or DFS (15.2 mo vs 11.5 mo); instead a massive increase of grade 3/4 toxicities (85% vs 16%) was seen.

An overview of the completed and clinically relevant adjuvant phase III trials is given in Table 2.

Ongoing adjuvant trials

Several adjuvant phase III studies with different concepts are ongoing, such as the combination of two or more cytostatic substances, or chemotherapy in combination with immunotherapy or radiochemotherapy (Table 3).

The ESPAC-4 trial is investigating the role of a more intense chemotherapeutic regimen combining gemcitabine (standard dosage) with capecitabine (d1-14, q22) for 6 cycles in a randomized phase III trial. Recruitment for the pancreatic cohort (732 patients) has been completed and initial results can be expected soon.

More recently, the chemotherapeutic regimens which are more effective for the palliative situation, namely FOLFIRINOX^[48] and nab-paclitaxel in combination with gemcitabine^[49], are also available for adjuvant treatment. The actively recruiting APACT (adjuvant therapy for pancreatic cancer trial) will provide information about whether the combination therapy of nab-paclitaxel (125 mg/m² d1, 8, 15, q29) and gemcitabine is feasible in resected PDAC patients ($n = 800$) and also whether it is more effective than gemcitabine alone. The French ACCORD/PRODIGE study group will investigate a similar concept in a randomized phase III trial of 480 patients comparing 6 cycles of modified FOLFIRINOX (Irinotecan 150 mg/m², oxaliplatin 85 mg/m², folinic acid 400 mg/m², 5-FU 2400 mg/m² per 46 h d1, q 15) to gemcitabine monotherapy. As both trials are based on the current most effective and evidence-based chemotherapies for advanced PDAC with a clear advantage in comparison to gemcitabine monotherapy, they have the potential to further improve current standard therapy. The question remains as to whether these more aggressive concepts

Table 3 Ongoing adjuvant trials

Trial	Registration No.	Treatment groups	Patients planned
ESPAC-4	ISRCTN96397434	Gem + capecitabine	732
APACT	NCT01964430	Gem Gem + nab-paclitaxel	800
ACCORD24	NCT01526135	FOLFIRINOX Gem	490
ROG-0848	NCT01013649	Gem + RCT (capecitabine or 5-FU)	950
Algenpantucel Immunotherapy	NCT01072981	Gem ± RCT + Algenpantucel-I vaccine Gem ± RCT	722

5-FU: 5-fluorouracil; RCT: Randomized controlled trial.

will be feasible in PDAC patients with a partially reduced performance state and after extended abdominal surgery. Accrual of the trials and (long-term) follow up data must be awaited.

With regard to radiochemotherapy, the RTOG-0848 trial is planned to investigate an additional effect of postoperative radiochemotherapy to standard chemotherapy. All patients are to receive 6 cycles of gemcitabine as standard of care and if without signs of disease recurrence will then be randomized to either receive additive radiochemotherapy or not 950 patients are planned. The protocol was amended and the initial randomization to gemcitabine ± erlotinib was terminated.

Immunotherapy is one of the most fascinating recent innovations in the treatment of oncological disease, but most clinical research to date has had disappointing results in PDAC, which is considered to be non-immunogenic^[50]. Seven hundred and twenty-two patients have been recruited for a phase III study of chemotherapy and chemoradiotherapy with or without Algenpantucel-L (HyperAcute®-Pancreas), a new immunotherapeutic concept. Algenpantucel is a vaccine derived from 2 pancreatic cell lines which showed promising results in a single-arm phase II trial^[51]. Results of the phase III trial are pending.

Neoadjuvant concepts

Chemotherapies with response rates (in terms of tumor shrinkage) of about 30% - compared to formerly < 10% for gemcitabine monotherapy - have become available^[52,53] in the treatment of PDAC for the first time due to the introduction of FOLFIRINOX and gemcitabine/nab-paclitaxel. This is of special relevance for the further development of neoadjuvant and perioperative treatment strategies, as pancreatic cancer must be considered a systemic disease. Many patients experience early recurrence postoperatively, which in the majority of cases may in fact be metastatic spread which was undetected at the time of primary diagnosis. Analogue to the ongoing discussion on the role of induction chemotherapy in locally advanced unresectable PDAC^[11], the question as

to whether patients with resectable PDAC and disease progression under neoadjuvant therapies would really profit from primary resection must be posed (Table 4).

Two trials are ongoing to investigate these questions in clearly defined study protocols: The German NEONAX is investigating the role of perioperative gemcitabine/nab-paclitaxel and perioperative modified FOLFIRINOX is being investigated in the United States in a phase II trial by the University of Yale.

Treatment strategies for borderline resectable PDAC

In addition to the aim of improving the rate of cure in terms of hindering recurrence of disease after curatively intended surgery, a further aim must be to render more patients resectable after intensified induction treatment. In this context, the term "borderline resectable" PDAC is relevant and relatively recent but different definitions are currently in use^[4] (Table 4).

Katz *et al.*^[54] presented data at the Annual ASCO meeting in 2015 of a small phase II trial which may be the starting point for a new era in borderline resectable PDAC. Twenty-two patients with ECOG 0 and 1 were included and prospectively analyzed. Participants were centrally reviewed to assess borderline criteria [tumor-vessel interface (TVI) with superior mesenteric/portal vein (SMV) $\geq 180^\circ$, TVI with superior mesenteric artery (SMA) $< 180^\circ$, TVI with any degree of hepatic artery]. All patients were intended to be treated with neoadjuvant modified FOLFIRINOX (oxaliplatin 85 mg/m², irinotecan 180 mg/m², leucovorin 400 mg/m² on day 1 followed by 5-FU 2400 mg/m² × 48 h for 4 cycles) and CRT (50.4 Gray in 28 fractions) with capecitabine (825 mg/m² BID) prior to pancreatectomy and postoperative gemcitabine (1000 mg/m² d1, 8, 15 for 2 cycles). Of the 15 (68%) patients who underwent pancreatectomy, 14 (93%) of the operations were R0, suggesting efficacy for this therapeutic concept.

The German NEOLAP trial includes borderline resectable and non-resectable PDAC and focuses in particular on the fact that the effectiveness of neoadjuvant treatment may not be reflected by a radiologically measurable response^[21]. After an induction treatment with gemcitabine + nab-paclitaxel for 2 cycles, participants will be randomized for 2 further cycles of gemcitabine + nab-paclitaxel or 2 cycles of FOLFIRINOX. In the case of stable disease an obligatory explorative laparotomy/laparoscopy will be performed to answer the question of a possible discrepancy between radiological assessment and intraoperative evaluation of resectability.

CONCLUSION

Although the progress in the last decades in pancreatic surgery has been clinically relevant, as demonstrated by a clinically relevant decrease of mortality in specialized centers, more extensive operations will be needed to further improve long-term survival. Extended pancreatectomy, portal vein resection and pancreatic resection in resectable pancreatic cancer with limited liver metastasis seem to be

Table 4 Ongoing neoadjuvant/borderline resectable trials

Trial	Registration No.	Treatment groups	Primary endpoint	Patients planned
NEONAX	NCT02047513	2 cycles Gem + nab-paclitaxel neoadjuvant 4 cycles Gem + nab-paclitaxel adjuvant 6 cycles Gem + nab-paclitaxel adjuvant	DFS	166
Perioperative mFOLFIRINOX	NCT02047474	3 cycles FOLFIRINOX Neoadjuvant + adjuvant	DFS	46
NEOLAP	NCT02125136	2 cycles Gem + nab-paclitaxel + 2 × FOLFIRINOX + adjuvant 3 × Gem + nab-paclitaxel 4 cycles Gem + nab-paclitaxel + adjuvant 3 × Gem + nab-paclitaxel	Conversion rate	168 (including locally advanced PDAC)

DFS: Disease-free survival; PDAC: Pancreatic ductal adenocarcinomas.

the surgical procedures with the highest impact in this field.

In this context, the concept of borderline resectability appears very promising. Although this concept has already been adopted to some extent in some national guidelines, it is still evolving. Criteria for imaging are defined quite strictly and pragmatically, as they do not only reflect technical resectability but must be considered prognostic parameters indicating more advanced extent of tumors. Furthermore, variability of the vasculature of the individual patient is not reflected by the criteria, leaving the individual decision dependent on the experience of the treating medical institution. This includes alternative surgical strategies such as the promising distal pancreatectomy with *en-bloc* resection of the celiac trunk in case of pancreatic tail carcinoma reaching the celiac axis, which requires very precise planning based on radiologic imaging and a preoperative radiological intervention in order to train collateralization. The timing of imaging studies is another important factor, as it is known that regardless of imaging quality, images older than 4 wk rarely represent the tumor spread found upon surgery. In such cases imaging has to be repeated before the decision is made.

Adjuvant chemotherapy for PDAC can double the rate of cure, as demonstrated by the clinical trials outlined in this review. Although this is comparable to results for other cancers, further improvements are urgently needed, as the starting point is catastrophically unfavorable, with rates of recurrence of disease of about 90% without perioperative treatment. Since the approval of gemcitabine in 1997 more effective cytotoxic substances (nab-paclitaxel) and combinations (FOLFIRINOX) are now available for advanced PDAC, raising hopes for more effective adjuvant and neoadjuvant treatment concepts for potentially resectable tumours. The fact that therapies with a broader mechanism of action will become available for research projects in the near future is equally important. In addition to the omnipresent immunotherapy, stromal depletion, e.g., with hyaluronidase^[55] and anti-inflammatory concepts^[56], appear most promising.

The only hope for improving long-term survival in this still challenging disease is a multidisciplinary approach, ideally with the close collaboration of radiologists, surgeons and oncologists in specialized tumor centers.

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Primary prevention and treatment of venous thromboembolic events in patients with gastrointestinal cancers - Review

Hanno Riess, Piet Habbel, Anja Jühling, Marianne Sinn, Uwe Pelzer

Hanno Riess, Piet Habbel, Anja Jühling, Marianne Sinn, Uwe Pelzer, Department of Hematology, Oncology and Tumor Immunology, Charité University Medicine Berlin, 10117 Berlin, Germany

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Correspondence to: Dr. Hanno Riess, Professor, Department of Hematology, Oncology and Tumor Immunology, Charité University Medicine Berlin, Charitéplatz 1, 10117 Berlin, Germany. hanno.riess@charite.de
Telephone: +49-30-450513002
Fax: +49-30-450513952

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Abstract

Venous thromboembolism event (VTE) is a common and morbid complication in cancer patients. Patients with gastrointestinal cancers often suffer from symptomatic or incidental splanchnic vein thrombosis, impaired liver function and/or thrombocytopenia. These characteristics require a thorough risk/benefit evaluation for individual patients. Considering the risk factors for the development of VTE and bleeding events in addition to recent study results may be helpful for correct initiation of primary pharmacological prevention and treatment of cancer-associated thrombosis (CAT), preferably with low molecular weight heparins (LMWH). Whereas thromboprophylaxis is most often recommended in hospitalized surgical and non-surgical patients with malignancy, there is less agreement as to its duration. With regard to ambulatory cancer patients, the lack of robust data results in low grade recommendations against routine use of anticoagulant drugs. Anticoagulation with LMWH for the first months is the evidence-based treatment for acute CAT, but duration of secondary prevention and the drug of choice are unclear. Based on published guidelines and literature, this review will focus on prevention and treatment strategies of VTE in patients with gastrointestinal cancers.

Key words: Thromboembolism; Gastrointestinal cancer; Prophylaxis; Treatment; Anticoagulation

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Core tip: The risk for venous thrombosis and pulmonary

embolism is clearly elevated in patients with gastrointestinal cancers. This risk is highest for patients with pancreatic, gastric or colorectal cancer and those receiving anti-cancer therapies. Available guidelines usually refer to thromboembolism in cancer patients without differentiating between types of cancer. Those patients with gastrointestinal cancers are more likely to present with additional problems such as hepatopathy-associated low platelet counts and/or prolonged prothrombin times. Furthermore, symptomatic or incidental thromboembolism of the visceral veins may occur more often. Identifying the risk factors for the development of venous thromboembolism and bleeding events may be helpful for correct initiation of primary pharmacological prevention and treatment of cancer-associated thromboembolism. Based on published guidelines and literature, this review will focus on prevention and treatment strategies of venous thromboembolism in patients with gastrointestinal cancers.

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INTRODUCTION

Venous thromboembolism (VTE) commonly presents as deep vein thrombosis (DVT) or pulmonary embolism (PE), occasionally as thrombosis of the hepatic, portal, or splanchnic veins. Patients with cancer are at increased risk for VTE^[1-4]. In addition to prothrombogenic cancer-related factors, the risk for symptomatic VTE is modulated by patient-specific and treatment-related factors (Table 1). Among the different subtypes of malignancy, some gastrointestinal cancers have a clinically relevant VTE incidence of more than 5% in the first year after cancer diagnosis (Figure 1), such as pancreatic (16%-22%), gastric (12%-17%) and colorectal (8%-12%) cancers^[2,5-8]. Symptomatic VTE is not only associated with substantial morbidity and complications in clinical management, but has been shown to have a detrimental effect on cancer survival^[9-11]. Acute and follow-up complications such as intestinal necrosis and esophageal bleeding due to portal hypertension may be life threatening if intra-abdominal veins are involved^[12-15].

Furthermore, anticoagulation with a vitamin K antagonist (VKA), such as warfarin, is complicated by drug interactions and variable drug absorption due to the changing nutritional status of cancer patients^[16]. In fact, therapeutic anticoagulation with VKA does not prevent VTE recurrences as effectively as in non-cancer patients, and major bleeding complications are also more than twice as common^[17]. Acute anticoagulation and secondary prevention with low molecular weight heparin (LMWH) has been established as an effective treatment

of VTE in cancer patients^[18], but daily subcutaneous application may result in local complications such as hematoma or infections, thus negatively influencing quality of life.

Most research into the primary prevention and treatment of VTE included both cancer and non-cancer patients. Recommendations for patients with malignancies are therefore based on the sparse available evidence and the guidelines differ only marginally (Table 2)^[19-27]. Most clinical studies focusing on VTE in cancer patients do not differentiate between different kinds of cancer, resulting in a lack of specific data concerning VTE in gastrointestinal cancers.

PERIOPERATIVE PROPHYLAXIS OF VTE IN CANCER PATIENTS UNDERGOING SURGERY

The risk for VTE is higher in cancer patients undergoing surgery without prophylaxis in comparison to non-cancer surgical patients^[27,28]. Administration of LMWH or fondaparinux (FPX) in these patients significantly reduces the rate of VTE^[29,30]. Dosages of LMWH in the range of 3000-5000 anti-FXa units per day are more effective than and as safe as lower doses. Patients undergoing major abdominal or pelvic surgery for malignancy remain at risk for VTE for up to five weeks after surgery. Thus, a prolonged pharmacologic thromboprophylaxis (LMWH or FPX once daily) should be considered, unless contraindicated because of high bleeding risk or active bleeding^[31,32]. Prophylactic regimens begin 12-24 h pre- or 6-24 h postoperatively^[29-32]. Based on these studies^[31-33], and according to the different guidelines (Table 2), antithrombotic prophylaxis is recommended for a minimum of 7 d and up to 35 d postoperatively. FPX (2.5 mg/d) was found to be as effective and safe in preventing VTE after abdominal surgery as the LMWH dalteparin (5000 antiFXa units/d). A post-hoc analysis of 1407 (out of 2048) patients with cancer demonstrated a significant (39%) risk reduction of VTE^[29]. Accordingly, the European (ESMO) guideline^[20] recommends extended prophylaxis for all patients undergoing elective cancer surgery. In the American guidelines (ASCO, NCCN)^[19,34], extended prophylaxis is only recommended in the presence of high thromboembolic risk factors such as residual or advanced cancer, aged 60 or older, obesity, previous history of VTE, duration of surgery longer than 2 h or prolonged postoperative immobilization. In patients with contraindications to pharmacological anticoagulant prevention strategies (e.g., increased risk of hemorrhage), the use of intermittent pneumatic compression devices or compression stockings is advised^[19-27].

The recommendations for patients undergoing minimally invasive or laparoscopic surgery are even less evidence-based. In a recently published randomized study evaluating postoperative antithrombotic prophylaxis with LMWH for one vs four weeks in patients undergoing

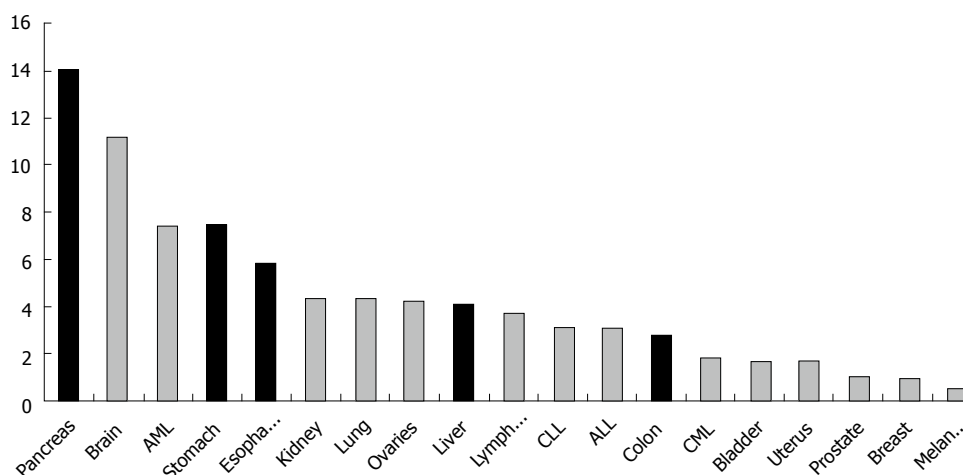


Figure 1 Venous thromboembolic event-risk according to different cancer entities (modified from Wun *et al.*^[8] 2009). VTE-Incidence in the first year after cancer diagnosis (all stages) California Cancer Registry 1993-1999 (Patient Hospital Discharge Dataset). VTE: Venous thromboembolism event.

Table 1 Some venous thromboembolic event risk factors in cancer patients

Cancer-related	Patient-related	Treatment-related
Reduced mobility		
Primary cancer (e.g., pancreatic cancer > colo-rectal cancer)	Age	Operation
Stage (IV > III)	History of VTE	Chemotherapy
Histology (e.g., adeno- > squamous cell-carcinoma)	Infection/fever	Central line/port-catheter
Grade (3 > 2)		Parenteral nutrition
Thrombocytosis		Radiation therapy
Leukocytosis		
Acute phase (elevated CRP)		
Elevated D-dimer		

CRP: C-reactive protein; VTE: Venous thromboembolic event.

laparoscopic surgery for colorectal cancer, extended antithrombotic prophylaxis was safe and reduced the 3-mo risk of VTE by more than 90%, and that of proximal DVT by 50%, as compared to the one week regimen^[35]. These data are consistent with the suggestion to follow the same recommendations regardless of whether a cancer patient is to undergo an open or laparoscopic surgical intervention^[36,37].

Prevention of portal vein thrombosis

Splanchnic vein thromboses (SVT), including portal vein thrombosis (PVT), mesenteric vein thrombosis, splenic vein thrombosis and the Budd Chiari syndrome are frequent events in patients with hepato-biliary-pancreatic cancers, with cancer-associated PVT responsible for 21% of all cases^[14]. The risk of VTE and SVT is increased in patients with cirrhosis^[38,39], and PVT is a relevant complication of hepato-biliary-pancreatic surgery^[40], reported to occur in 9% of patients after liver resections^[41]. In this context, thromboprophylaxis with LMWH was demonstrated to be effective and safe in a retrospective comparative cohort study of 201 patients undergoing liver resections for liver cancers, with a reduction in PVT from 10% to 2%^[42]. These data suggest that prophylactic anticoagulation with

LMWH - as recommended in patients undergoing major abdominal cancer surgery - is also effective in cancer patients undergoing liver resection.

PROPHYLAXIS OF VTE IN HOSPITALIZED MEDICAL PATIENTS WITH CANCER

Hospitalized patients with active cancer - a term not uniformly defined - were included in all published randomized clinical trials investigating the role of unfractionated heparin (UFH), LMWH or FPX for the prevention of VTE. Treatment of hospitalized patients for 6-14 d with low-dose enoxaparin (20 mg/d s.c.) was ineffective^[43], whereas higher prophylactic doses of LMWH (enoxaparin 40 mg/d, or dalteparin 5000 anti FXa units/d)^[43,44] or FPX (2.5 mg/d)^[45] demonstrated superiority compared to placebo in the prevention of VTE with minimal or no increase in major bleeding events. Subgroup analyses failed to identify a subgroup which did not benefit from pharmacological thromboprophylaxis^[46]. This was also true for a small subgroup (5%-15% of the study population) of cancer patients.

Subgroup analysis of 274 patients with active cancer from the CERTIFY study comparing UFH (3 × 5000 IU/d) with LMWH (certoparin 3000 anti FXa units/d) demonstrated a similar VTE risk (5.3% vs 4.1%) and similar rates of any (4.0 vs 3.9) or major (0.7% vs 0.5%) bleeding compared to the 2965 patients without cancer^[47,48].

Based on three studies, extended prophylaxis with the anticoagulant drugs LMWH^[49] or non-vitamin K oral anticoagulant drugs (NOACs)^[50,51] cannot be recommended in medical patients. In the MAGELLAN trial^[51], cancer patients ($n = 592$; 7.3%) had higher rates of VTE when prophylaxis with rivaroxaban was extended from 10 to 35 d.

Discussion is ongoing as to whether all cancer patients hospitalized for reasons such as infectious complications or complex chemotherapy regimens should generally receive medical thromboprophylaxis unless contraindicated by active bleeding or high bleeding risk^[52-55]. According to the

Table 2 Comprehensive outline of some guidelines focusing on the prevention and treatment of cancer associated venous thromboembolism

	Primary prophylaxis/prevention of VTE in cancer patients			Treatment of cancer-associated VTE	
	Surgical patients	In-patients (non surgical)	Out-patients	Acute/initial	Long-term/secondary prevention
ASCO Guidelines 2013 ^[15]	UFH, LMWH (Dalteparin, Enoxaparin), Fondaparinux A combined regimen of pharmacologic and mechanical prophylaxis may improve efficacy, especially in the highest risk patients. Patients undergoing major cancer surgery should receive prophylaxis starting before surgery and continuing for at least 7 to 10 d and it should be considered an extension up to 4 wk in patients undergoing abdominal and pelvic surgery	UFH, LMWH (Dalteparin, Enoxaparin), Fondaparinux	Not recommended routinely ¹ LMWH may be considered	UFH, LMWH (Dalteparin, Enoxaparin, Tinzaparin), Fondaparinux VKA not recommended	LMWH (Dalteparin, Enoxaparin, Tinzaparin) VKA (INR 2-3) acceptable if LMWH is not available Use of NOACs is not recommended Treatment of splanchnic or visceral vein thrombi diagnosed incidentally should be considered on a case-by-case basis, considering potential benefits and risks of anticoagulation
ESMO Guidelines 2011 ^[16]	LMWH (Dalteparin, Enoxaparin), UFH Fondaparinux not recommended Cancer patients undergoing elective major abdominal or pelvic surgery should receive in hospital and post-discharge prophylaxis with s.c. LMWH for up to 1 mo after surgery	LMWH (Dalteparin, Enoxaparin), Fondaparinux, UFH	Not recommended routinely May be considered in high risk patients	LMWH (Enoxaparin, Dalteparin), UFH	Treatment for a total of 6 mo. Initial dose of LMWH 100% for 1 mo, thereafter 5 mo with 75%-80% of the initial dose of LMWH
International Consensus Groupe 2013 ^[17]	LMWH (Dalteparin, Enoxaparin, Nadroparin, Tinzaparin), Fondaparinux, UFH For 10 ± 2 d or 25-31 d (28 d) extended use (Bemiparin sodium 3500 IU per day for 28 d)	LMWH (Dalteparin, Enoxaparin, Nadroparin, Tinzaparin), Fondaparinux, UFH	Not recommended routinely To be considered/recommended: Patients with locally advanced or metastatic lung or pancreatic cancer treated with chemotherapy and having a low bleeding risk	LMWH (Dalteparin, Enoxaparin), UFH	LMWH (Dalteparin, Enoxaparin) for 3 to 6 mo
British Committee for Standards in Haematology 2015 ^[18]	Patients undergoing abdominal and pelvic surgery for cancer should be considered for extended thromboprophylaxis	Patients with active or recent cancer should receive thromboprophylaxis throughout their admission unless contraindicated Patients without a history of venous thromboembolism receiving adjuvant hormonal therapies for cancer should not routinely receive thromboprophylaxis	Patients should be assessed for thrombosis risk and although most do not routinely require thromboprophylaxis, it should be considered for high risk patients	Initial treatment should be with LMWH for six months Cancer patients with incidental pulmonary embolus or deep vein thrombosis should be therapeutically anticoagulated as for symptomatic disease	Warfarin and other oral anticoagulants are acceptable alternatives if LMWH is impractical and anticoagulation is indicated Anticoagulation should be continued, taking pt status and wishes and bleeding risk into consideration. There is a rationale but little direct evidence for preferring to continue to use LMWH

Australian Governments National Health and Medical Research Council 2009 ^[19]	LMWH, continue for at least 7 to 10 d following major general surgery Consider using extended thromboprophylaxis with LMWH for up to 28 d after major abdominal or pelvic surgery for cancer, especially in patients who are obese, slow to mobilise or have a past history of VTE	LMWH, UFH	-	-	
MAYO CLINIC VTE Prevention and Management Guidelines 2014 ^[20]	UFH, LMWH (Enoxaparin, Dalteparin), Fondaparinux Cancer patients undergoing pelvic or abdominal surgery should receive 4 wk of LMWH	UFH, LMWH (Enoxaparin, Dalteparin), Fondaparinux	UFH, LMWH (Enoxaparin, Dalteparin), Fondaparinux VKA (INR2-3)	Anticoagulants are continued until there is no evidence of active malignant disease defined as any evidence of cancer on cross-sectional imaging or any cancer-related treatment (surgery, radiation, or chemotherapy) within the past 6 mo	
ASH Guidelines 2013 ^[21]	UFH, LMWH, Fondaparinux in all patients undergoing major surgical intervention for malignant disease Prolonged prophylaxis for up to 4 wk may be considered in patients undergoing major abdominal or pelvic surgery for cancer with high-risk features such as residual malignant disease, obesity, and prior history of VTE	UFH, LMWH, Fondaparinux	Routine VTE prophylaxis in ambulatory patients receiving chemotherapy is not recommended	LMWH	LMWH Continue treatment with LMWH is preferred for at least the initial 6 mo of treatment
German Guidelines ^[22,23]	LMWH, Fondaparinux, (UFH) Patients undergoing abdominal and pelvic surgery for cancer are recommended to get extended thromboprophylaxis (28 to 35 d)	LMWH, Fondaparinux, (UFH)	LMWH, Patients should be assessed for thrombosis risk and thromboprophylaxis should be considered for high risk patients	LMWH, Fondaparinux, UFH	LMWH for 3 to 6 mo If cancer persists extended secondary prophylaxis (with LMWH, VKA, or NOAC) is useful (till death)

¹For highly selected high-risk patients (*e.g.*, such as those with pancreatic cancer) preventive anticoagulation may be considered to be applied routinely. VTE: Venous thromboembolic event; LMWH: Low molecular weight heparins; UFH: Unfractionated heparin; VKA: Vitamin K antagonist; INR: International normalized ratios; NOAC: New oral anticoagulants.

available guidelines (Table 2), routine thromboprophylaxis should at least be considered. Italian investigators assessing the risk of VTE in hospitalized medical patients confirmed cancer to be a major predisposing factor for VTE (PADUA prediction score), without differentiating between cancers^[56]. However, the existence of active cancer on its own does not classify as a high VTE risk unless additional risk factors are present (Table 1).

VTE PROPHYLAXIS IN OUTPATIENTS WITH CANCER

Prevention of catheter-related VTE

Patients who have been recently diagnosed with cancer are at high risk for VTE^[57,58]. Nowadays these patients usually receive non-surgical therapies such as

radiation, chemotherapy or radiochemotherapy in an outpatient setting. Long-term central venous catheters (*e.g.*, port or picc line catheters) are used to facilitate drug administration in many of these patients. There is an increased risk of DVT in the subclavian or jugularis interna veins after insertion of a central line, with an estimated incidence of asymptomatic catheter-related DVT of about 20%, although less than 5% develop clinical symptoms, which very rarely result in symptomatic PE or relevant post-thrombotic sequelae^[59-64]. Multicenter randomized double blind placebo-controlled studies to assess the efficacy and safety of LMWH or VKA demonstrated a significant reduction in asymptomatic catheter-related thrombi, but failed to show significant benefit with regard to clinically symptomatic DVT^[59]. International guidelines therefore recommend against routine prophylaxis for this

Table 3 Primary prevention of cancer-associated venous thromboembolic event in gastrointestinal cancers

Study	Cancer	n	VTE placebo	VTE LMWH	VTE U-LMWH	RR
PROTECHT ¹	Gastrointestinal ²	148/272	2.7%	1.5%		-44%
Agnelli <i>et al</i> ^[68] , 2009	Pancreas	17/36	5.9%	8.3%		40%
SAVE-ONCO	Colo-rectal	461/464	2.0%		1.1%	-45%
Agnelli <i>et al</i> ^[69] , 2012	Pancreas	128/126	10.9%		2.4%	-78%
	Stomach	207/204	1.9%		0.5%	-75%
FRAGEM	Pancreas	60/63	23%	3.4%		-85%
Maraveyas <i>et al</i> ^[70] , 2012						
CONKO 004	Pancreas	152/160	9.9%	1.3%		-87%
Pelzer <i>et al</i> ^[71] , 2015						

¹Randomization 1:2; ²Gastric (n = 40/58), colon (n = 79/156) and rectal (n = 29/58) cancers. LMWH: Low molecular weight heparin; U-LMWH: Ultra-LMWH; RR: Relative risk; VTE: Venous thromboembolic event.

indication.

Antithrombotic prophylaxis in cancer outpatients receiving chemotherapy

The risk of VTE in cancer patients is especially high during the first weeks or months of specific therapy^[4,62,65]. Several clinical trials have evaluated the role of primary prevention in outpatients with selected or unselected malignancies. An early study on patients receiving chemotherapy for advanced breast cancer found low dose warfarin to be safe and efficacious, with 85% relative reduction in VTE compared to placebo^[66]. Two other randomized trials in patients with breast or lung cancers using prophylactic dosages of LMWH failed to show a benefit^[67]. The PROTECHT study, however, showed a statistically significant relative risk reduction of 50% for symptomatic VTE in cancer outpatients receiving chemotherapy for different types of malignancy (including gastrointestinal cancers) in favor of prophylactic dosages of the LMWH nadroparin (Table 3)^[68]. Moreover, despite a relatively low event rate of less than 3% in the placebo group, prophylactic dosages of semuloparin, an ultralow molecular weight heparin (not further developed), demonstrated a highly significant reduction (65%) of VTE in patients with metastatic or locally advanced solid cancers (1590 of 3212 with pancreatic, gastric or colorectal cancers) receiving chemotherapy^[69] (Table 3), without an increase in the incidence of clinically relevant or major bleeding complications. Whereas these trials investigated the effect of prophylactic dosages of antithrombotic drugs, two randomized open-label studies in patients with advanced pancreatic cancer undergoing chemotherapy investigated primary prophylaxis with higher doses of LMWH (Table 3). The phase II FRAGEM-trial^[70] examined the therapeutic dose of dalteparin (200 anti FXa units/kg per day), and the phase III CONKO-004 trial^[71] used half the therapeutic dosage of the LMWH enoxaparin (1 mg/kg per day). Despite differences in study design and primary endpoint of effectiveness, both trials found this intensity of anticoagulation to be safe and reported a more than 80% relative risk reduction of thromboembolic events compared to observation (Table 3).

Nevertheless, available international guidelines do

not recommend routine prophylaxis in all ambulatory cancer patients receiving anti-cancer chemotherapy (Table 2). ACCP and ASCO guidelines^[19,62] suggest considering antithrombotic prophylaxis in outpatients receiving chemotherapy with unspecified solid tumors and additional risk factors, whereas others recommend considering primary prevention, especially in patients with lung or pancreatic cancer^[21] in accordance with a recent meta-analysis^[72].

A number of attempts have been made to classify individual cancer outpatients receiving chemotherapy more precisely according to VTE risk. In this context, the scoring system developed by Khorana *et al*^[73] (Table 4), which associates pancreatic and stomach cancer with a very high VTE risk, demonstrated potential for the identification of cancer outpatients at risk for VTE prior to the start of chemotherapy with simple and commonly available criteria. A prospective randomized trial investigating the benefit of primary antithrombotic prophylaxis in high risk patients according to this score is ongoing. Until results from this study become available, the initiation of pharmacological prophylaxis in high risk patients is suggested by the standardization subcommittee of the ISTH^[74]. Supplementing the Khorana score with lab parameters such as D-dimer and soluble P-selectine^[75] or chemotherapy components such as gemcitabine and cis- or carboplatin^[76] may further categorize patients according to their VTE risk.

With the exception of patients suffering from pancreatic cancer, for whom primary prophylaxis with LMWH is highly warranted unless contraindicated by increased bleeding risk, insufficient evidence is available regarding other gastrointestinal cancers to recommend routine primary prevention with anticoagulant drugs. Until further evidence has accumulated, the Khorana score is helpful for classification of patients with gastrointestinal cancer according to their VTE risk and as an aid in decision-making regarding initiation of pharmacologic prophylaxis. While prophylactic dosages of LMWH may be effective in most gastrointestinal cancers, half-therapeutic dosages of LMWH should be considered for patients with advanced pancreatic cancer in the first three months of chemotherapy.

Table 4 Assessment scores for prediction of the venous thromboembolic event-risk in cancer out-patients receiving chemotherapy, according to Khorana *et al.*^[73], Pabinger *et al.*^[75] and Verso *et al.*^[76]

Khorana score criteria ^[73]	Score
Primary cancer	
With very high risk (pancreas, stomach) (high grade glioma ¹)	2
With high risk (lung, lymphoma, gynecologic, bladder, testicular)	1
Platelet count prior to chemotherapy > 350000/ μ L	1
Hb < 10 g/dL or ESA-application	1
Leukocyte count prior to chemotherapy > 11000/ μ L	1
Body mass index > 35 kg/m ²	1
High risk	> 3
Vienna prediction score (additional parameters to Khorana score) ^[75]	
D-dimer > 1.44 μ g/mL	1
Soluble P-selectin > 153.1 μ g/mL	1
High risk	> 4
Protecht prediction score (additional parameters to Khorana score) ^[76]	
Cisplatin or carboplatin	1
Gemcitabine	1
High risk	> 3

¹High grade glioma are considered very high risk site of cancer in the Vienna prediction score only. ESA: Erythropoiesis stimulating agents.

VTE risk and new drugs

The introduction of new drugs which target angiogenesis, tumor stroma or immunity in gastrointestinal cancer medicine led to the realization that individual VTE risk may be strongly influenced by the drugs. For example, the introduction of bevacizumab for the treatment of advanced colorectal cancer not only resulted in a clinically relevant increase in bleeding, but in thromboembolic complications as well^[75,77,78].

Moreover, drug-associated VTE may become an important factor limiting further cancer therapy development. At the ASCO meeting in May 2015, Hingorani *et al.*^[79] presented a phase II trial in pancreatic cancer patients treated with gemcitabine, nab-paclitaxel and recombinant pegylated hyaluronidase - an experimental drug - which had to be stopped due to high numbers of VTEs. A prophylactic dose of LMWH (40 mg enoxaparin/d) was not effective, but the use of half therapeutic doses of enoxaparin (1 mg enoxaparin/kg per day) allowed the continuation of the study.

TREATMENT OF ACUTE VTE IN CANCER PATIENTS

The treatment of patients with active cancer and acute VTE with the standard treatment for non-cancer VTE patients (LMWH or FPX plus concurrent introduction of anticoagulation with VKA) resulted in a two- to three-fold increase of both re-thrombosis and bleeding^[17,18]. Several randomized controlled trials compared prolonged application of LMWH to oral anticoagulation with VKA in patients with cancer-associated thrombosis (CAT) and demonstrated superior efficacy of LMWH with no increase

in bleeding complications (Table 5), results confirmed by meta-analysis^[18,19,80,81]. Based on these results, guidelines recommend a standard treatment of three to six months of weight-adjusted LMWH regardless of whether the patient suffers from gastrointestinal or another type of cancer (Table 2). The underlying malignancy, probably representing the most important risk factor for VTE, continues beyond six months after the initial VTE in most of these patients. Therefore, anticoagulation beyond this period may be warranted and is recommended in most guidelines (Table 2). Nevertheless, evidence for this recommendation is clearly weaker, as prospective randomized trials investigating type of anticoagulation, dosage of anticoagulant drugs or duration of prolonged anticoagulation have not been reported. Available data suggest that the pro-thrombotic risk correlates with disease progression due to increasing tumor load, decreasing performance status, progressive mobility reduction and increased use of palliative chemotherapy^[82-84]. On the other hand, an increased bleeding risk, associated with progression of cancer, prolonged anticoagulation and prolonged chemotherapy needs to be considered in order to balance the benefit of preventing recurrent VTE against the risk of bleeding^[83,84]. Furthermore, the impact of prolonged anticoagulation on the quality of life of cancer patients needs to be considered.

Although clinical guidelines (Table 2) currently recommend considering indefinite anticoagulation in patients with advanced malignancy, they do not recommend a specific anticoagulant drug due to the limited evidence available.

Treatment of acute PVT

Acute thrombosis of splanchnic veins - PVT in particular - is a frequent complication in patients with cancers of the hepato-biliary-pancreatic system^[23,85]. PVT results in portal hypertension and impairment of liver perfusion. Spread of thrombosis to splenic or mesenteric veins can result in fatal complications such as splenic or intestinal infarction. Anticoagulation is the therapy of choice in non-cirrhotic, non-cancer patients, with successful recanalization in about half of acute PVT cases^[86,87]. Anticoagulation is considered in patients with cirrhosis and PVT with a small increase in bleeding complications^[86], and can safely be combined with placement of transjugular intrahepatic portosystemic shunts^[87]. Unfortunately cancer patients, e.g., those with gastrointestinal cancers, were excluded from these trials^[86,87].

Treatment of cancer-associated PVT is therefore based on evidence from non-cancer patients with or without cirrhosis. Despite the fact that prophylactic and therapeutic anticoagulation seems to be effective and safe for cirrhotic patients^[86-88], an individual risk/benefit evaluation which takes prognosis, anticancer treatment options and acute anticoagulation-associated bleeding risk, as well as future complications with the associated consequences for surgical interventions and the risk of variceal hemorrhage into consideration is challenging.

Table 5 The CLOT and CATCH studies^[80,81]: Study-population characteristics and study outcomes

	CLOT	CATCH	VKA	Dalteparin	P	VKA	Tinzaparin	P
Study-population characteristics								
n	676	900						
Women	52%	59%						
Median age (yr)	63	59						
ECOG 0-1	63%	77%						
Metastasized cancer	67%	55%						
Brest cancer	17.6%	9%						
Colo-rectal cancer	17.8%	13%						
Lung cancer	14.8%	12%						
Gynecological cancer	11.2%	23%						
Pancreatic cancer	4.8%							
Urogenital cancers	14.2%							
Brain cancers	5.5%							
Hematological cancers	10%	10%						
Study outcomes								
VTE			15.8%	8.0%	0.002	10.0%	6.9%	0.07
DVT			11.0%	4.2%		5.3	2.7	0.04
Fatal PE			2.1%	1.7%		3.8%	3.8%	
Non-fatal PE			2.7%	2.7%		0.7%	0.4%	
Major bleeding			4%	6%	0.25	2.7%	2.9%	
CRNM-bleeding						16%	11%	0.03
Any bleeding			19%	14%	0.09			
6-mo mortality			41%	39%		41%	40%	
INR < 2			30%			26%		
INR 2-3			46%			47%		

VKA: Vitamin K antagonist; VTE: Venous thromboembolic event; DVT: Deep vein thrombosis; PE: Pulmonary embolism; INR: International normalized ratios.

Treatment of CAT in particular situations

Thrombocytopenia is a well-known effect of chemotherapy in cancer patients and higher degrees of thrombocytopenia increase the risk of hemorrhage. Anticoagulation should therefore be administered to thrombocytopenic patients after thorough evaluation of the risks and benefits. The risk of recurrent VTE is high in the month following the initial diagnosis on beginning cancer therapy^[1,4]. Full therapeutic anticoagulation with LMWH is considered appropriate in these situations when the platelet count is above 50000/ μ L, and a reduction of anticoagulation intensity to 75% or even 50% may be reasonable later in the course of treatment. When platelets are below 50000/ μ L but above 20000/ μ L, a half-therapeutic to prophylactic dose should be considered^[89].

It should be recognized that increased prothrombin times and elevated international normalized ratios (INR) as a result of hepatic dysfunction only reflect reduced synthesis of coagulation factors. In fact coagulation inhibitors are also decreased, resulting in a well-balanced but more labile equilibrium with normal thrombin generation capacity^[90,91]. For this reason patients with reduced hepatic capability are not protected against VTE, despite an increased INR, but patients with cancer and hepatic dysfunction have an increased VTE risk^[90,92]. Again, higher grade evidence in favor of or against full-dose anticoagulation in patients with CAT and reduced liver function due to pre-existing cirrhosis or advanced hepatic metastasis resulting in increased INR is lacking. Clinical experience suggests a cautiously balanced approach of 75% to 100% of the therapeutic LMWH

anticoagulation dose for acute VTE.

VTE recurrences are to be expected in more than 5% of patients within the first months despite CAT therapy according to guidelines with LMWH (Table 5). Again, there is sparse evidence on treatment of these patients. Based on a report from Carrier *et al.*^[93], a 20% increase in LMWH dose - without adjustment to laboratory parameters - is a practical, safe and effective approach.

NON-VITAMIN-K ANTAGONIST ORAL ANTICOAGULANTS IN THE PREVENTION OR TREATMENT OF CAT

A number of new oral anticoagulants (NOAC) have been introduced and licensed for prevention and treatment of VTE in recent years^[94]. These drugs work by direct inhibition of active coagulation factors direct oral anti-coagulants (DOAC), namely factor IIa/thrombin (dabigatran) or factor Xa (apixaban, edoxaban, rivaroxaban). The term non-vitamin K oral anticoagulant was created in order to maintain the acronym NOAC. Although these drugs have been successfully tested and licensed in trials investigating NOACs in the post-operative setting of elective hip or knee replacement, drug development for other surgical patients was never initiated and the programs for medical patients were stopped early^[50,51]. A placebo-controlled dose-finding phase II trial for primary prophylaxis of VTE in ambulatory cancer patients undergoing chemotherapy showed relevant activity^[95], but this indication has not yet been further developed. There

Table 6 Take home messages

Patients with gastrointestinal cancers are among those with the highest cancer-associated VTE risk (<i>e.g.</i> , pancreatic cancer, gastric cancer)
Primary prevention of VTE should be considered according to an individual risk-benefit estimation
Scoring systems help to identify patients at high VTE risk. These patients may benefit from prophylactic anticoagulation
Usual prophylactic dosages of LMWH may not be effective enough in patients with the highest risk (<i>e.g.</i> , pancreatic cancer)
Gastrointestinal cancer patients with VTE should have medical anticoagulation therapy with LMWH for at least three to six months
In patients with gastrointestinal cancers splanchnic vein thrombosis, portal hypertension, hepatopathy-associated coagulation defects (<i>e.g.</i> , decreased prothrombin time) and thrombocytopenia may complicate anticoagulation strategies

VTE: Venous thromboembolic event; LMWH: Low molecular weight heparins.

is therefore no evidence for the use of NOACs for primary prevention in hospitalized or ambulatory cancer patients undergoing surgery or any other kind of anticancer therapy.

The four drugs mentioned above have all been compared to VKA (warfarin) in randomized phase III trials for the treatment of DVT, PE and atrial fibrillation^[96-101]. All of these trials demonstrated that NOACs are at least as effective and safe as VKA. As confirmed by meta-analysis, the study data for these NOACs showed a clinically relevant reduction in bleeding, most pronounced for intracerebral bleeding events^[102]. Depending on the inclusion and exclusion criteria of the individual studies and on the definition of "patients with active cancer", all six trials included cancer patients, a subgroup adding up to 2.5% of more than 25000 patients^[102]. Meta-analysis of these patients suggests a potential role for NOACs in patients with CAT, with a similar risk/benefit relationship as demonstrated in non-cancer patients^[76]. There are major drawbacks to the use of NOACs for this indication, however. First of all, standard therapy of CAT is LMWH for several months and not LMWH followed by VKA - the standard treatment arm in the VTE trials. Secondly, "patients with active cancer" included in the phase III trials demonstrate a three-month-mortality of less than 15%^[103-105], whereas those included in the two pivotal CAT trials (CLOT, CATCH) recruiting cancer patients ten years apart, have a six-month-mortality of 40%^[80,81] (Table 5). Obviously these patient groups differ to a great extent. Thirdly, despite having less interactions with other drugs compared to VKA, NOAC still bear a largely unclear risk of interactions with cytotoxic or targeted drugs. A CLOT- or CATCH-like head to head comparison of NOACs vs LMWH, the current standard of care, is eagerly awaited, especially as the oral application of NOACs might provide an improvement in quality of life compared to s.c. administered LMWH. Unless study data are available, NOACs are not to be considered the first choice in patients with acute CAT. As an alternative to LMWH or VKA (Table 2), anticoagulant treatment with NOACs beyond six months may be reasonable in many CAT patients, as there are no studies defining the optimal drug in this period.

CONCLUSION

The risk for VTE is clearly elevated in patients with (gastrointestinal) cancer. This risk is highest for patients

with pancreatic cancer and those receiving anti-cancer therapies. VTE is the second leading cause of death in patients with cancer, and mortality is increased among patients with CAT^[10,106]. Whereas available guidelines usually refer to VTE in cancer patients without differentiating between types of cancer, those with gastrointestinal cancers are more likely to present with additional problems such as hepatopathy-associated low platelet counts and/or prolonged prothrombin times. Furthermore, symptomatic or incidental VTEs of the visceral veins may occur more often. Despite limited data, prophylactic anticoagulation must be endorsed in most hospitalized patients with malignancies. In ambulatory patients undergoing chemotherapy, an assessment of individual prothrombotic and prohemorrhagic factors may help transfer the beneficial effect of pharmacologic prophylaxis demonstrated in a number of trials to those patients with the highest VTE risk, in particular those suffering from pancreatic cancer. Treatment recommendations for CAT with LMWH have been reconfirmed by recent evidence. Further progress will help clarify the risk/benefit relationship of NOACs in this field, identifying economic and quality of life aspects as well (Table 6).

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Gastric cancer development after the successful eradication of *Helicobacter pylori*

Kaname Uno, Katsunori Iijima, Tooru Shimosegawa

Kaname Uno, Katsunori Iijima, Tooru Shimosegawa, Division of Gastroenterology, Tohoku University Hospital, Miyagi 981-8574, Japan

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Correspondence to: Kaname Uno, MD, PhD, Division of Gastroenterology, Tohoku University Hospital, 1-1 Seiryō-cho, Aoba-ku, Sendai, Miyagi 981-8574, Japan. kaname@wa2.so-net.ne.jp
 Telephone: +81-22-7177171
 Fax: +81-22-7177174

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Abstract

Gastric cancer (GC) develops as a result of inflammation-associated carcinogenesis due to *Helicobacter pylori* (*H. pylori*) infection and subsequent defects in genetic/epigenetic events. Although the indication for eradication therapy has become widespread, clinical studies have revealed its limited effects in decreasing the incidence of

GC. Moreover, research on biopsy specimens obtained by conventional endoscopy has demonstrated the feasibility of the restoration of some genetic/epigenetic alterations in the gastric mucosa. Practically, the number of sporadic cases of primary/metachronous GC that emerge after successful eradication has increased, while on-going guidelines recommend eradication therapy for patients with chronic gastritis and those with background mucosa after endoscopic resection for GC. Accordingly, regular surveillance of numerous individuals who have received eradication therapy is recommended despite the lack of biomarkers. Recently, the focus has been on functional reversibility after successful eradication as another cue to elucidate the mechanisms of restoration as well as those of carcinogenesis in the gastric mucosa after *H. pylori* eradication. We demonstrated that Congo-red chromoendoscopy enabled the identification of the multi-focal distribution of functionally irreversible mucosa compared with that of restored mucosa after successful eradication in individuals at extremely high risk for GC. Further research that uses functional imaging may provide new insights into the mechanisms of regeneration and carcinogenesis in the gastric mucosa post-eradication and may allow for the development of useful biomarkers.

Key words: *Helicobacter pylori* eradication; Gastric cancer; Congo-red chromoendoscopy; Biomarker

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Core tip: Since the indication for eradication therapy for *Helicobacter pylori* became widespread, the number of gastric cancer (GC) cases that emerge after eradication has continued to increase. However, the underlying mechanism of restoration/carcinogenesis in the gastric mucosa after eradication therapy has not been fully elucidated. We previously demonstrated that, instead of conventional endoscopy, Congo-red chromoendoscopy might be more precise in its ability to identify the multi-focal distribution of functionally irreversible mucosa that

may be present in individuals with high risk for GC even after successful eradication. Further research using functional imaging may provide new insights to address the mechanism of gastric regeneration/carcinogenesis, and subsequently, may allow for the development of biomarkers for GC in high risk individuals during post-eradication surveillance.

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INTRODUCTION

Gastric cancer (GC) is considered a major cause of cancer-related deaths worldwide^[1], and cancer prevention based on the knowledge of gastric carcinogenesis has been considered the most useful strategy. It has been shown that GC is closely associated with chronic inflammation due to persistent infection with *Helicobacter pylori* (*H. pylori*), a class I gastric carcinogen. The development of GC occurs through a multi-step sequence from chronic gastritis, chronic atrophic gastritis, intestinal metaplasia (IM), dysplasia, and finally, GC after an extended period of time^[2-4]. In a cohort study of 4655 healthy asymptomatic individuals with a 7.7 ± 0.9-year surveillance, the risk of GC was increased in a stepwise fashion from patients with *H. pylori*-positive chronic gastritis to those with *H. pylori*-positive chronic atrophic gastritis, and finally, to those with atrophic metaplastic gastritis (HR = 7.1; 95%CI: 1-53.3 vs HR = 14.9; 95%CI: 2-107.7 vs HR = 61.9; 95%CI: 5.6-682.6, respectively)^[5].

In fact, clinical outcomes are determined by a complex interaction of multiple factors, such as *H. pylori* virulence factors, genetic susceptibility of the host, and environmental factors^[6,7]. The bacterial virulence factors, such as cytotoxin-associated gene A antigen (CagA) and vacuolating cytotoxin, play an important role in *H. pylori*-associated gastric disorders^[8,9]. Notably, the *CagA* gene, which is part of the *cag* pathogenicity island, produces one of the most pivotal virulence factors of many carcinogenic signaling pathways^[10,11]. Moreover, the epithelial cells and inflammatory cells in the gastric mucosa are in direct contact with the outer environment. Therefore, the immune receptors on these cells, such as toll-like receptors and nucleotide-binding oligomerization domain-containing protein, recognize bacterial components of *H. pylori* and other oral microbes that reach the gut^[12,13]. Actually, the long-term colonization by *H. pylori* was reported to cause an environment of chronic inflammation together with reductions in acidity and in the level of antioxidant enzymes in the gastric juice. This results in the synergistic overgrowth of ingested outer bacteria in the intra-gastric environment, which sustains the inflammation^[12,14]. As a result, these reactions can produce large amounts of

inflammatory mediators, such as reactive oxygen/nitrogen species and inflammatory cytokines, which may facilitate the aberrant methylation of the cytosine ring preceding guanine (*i.e.*, CpG dinucleotide) islands in the promoters of DNA or microRNAs (miRNAs)^[15,16]. A theory of the field effect of carcinogenesis, which states that pathologically non-cancerous tissue that surrounds the tumor may also have genetic/epigenetic alterations, is currently accepted as a reliable mechanism of gastric carcinogenesis^[17]. For example, aberrant genetic methylation was reported to accumulate during the sequential development of GC^[18,19], and further quantitative methylation analyses of both driver and passenger genes showed that patients with GC had higher methylation levels in the background normal-appearing tissues than those without GC; additionally, patients with multiple gastric tumors had significantly higher methylation levels than those with a single gastric tumor^[20-22]. Thereafter, some epigenetic alterations were shown to be restored by the elimination of *H. pylori*^[23-31].

Accordingly, it is strongly believed that successful eradication of *H. pylori* may be an effective approach for the prevention of GC, and the numbers of subjects in whom *H. pylori* has been eradicated have continued to increase. However, it was also demonstrated that eradication therapy had only limited effects on the inhibition of GC development. Sporadic cases of GC that emerge after successful eradication have been observed in a clinical setting. These cases strongly suggest two possibilities for the development of sequential carcinogenesis after eradication: A point-of-no-return, where irreversible alterations have already occurred in spite of the eradication or another cause of carcinogenesis such as bile reflux or other microorganisms. Here, we review the results of clinical and laboratory studies on the preventive effect of *H. pylori* eradication on gastric carcinogenesis to establish future surveillance strategies.

CLINICAL EFFECTIVENESS OF ERADICATION THERAPY FOR THE PREVENTION OF GC OCCURRENCE

Eradication for patients with non-dysplastic gastritis

Although some studies have found that the *H. pylori* eradication gradually led to regression or complete resolution of chronic gastritis^[32-34], it is still unclear whether eradication therapy might truly prevent the development of premalignant lesions/GC in patients with chronic gastritis and several grades of inflammation, atrophy and metaplasia.

In a randomized control trial (RCT) that included a total of 435 *H. pylori*-infected subjects with/without eradication followed by an endoscopic biopsy after 5 years of surveillance, Leung *et al*^[35] showed that eradication was protective against the progression of premalignant gastric lesions in 52.9% of *H. pylori*-positive subjects. On the contrary, in a population-based RCT of 1630 healthy *H. pylori*-carriers in China, Wong *et al*^[36] showed that the incidence of GC was similar between participants who had

received eradication treatment and those who received placebo during an observation period of 7.5 years (7/817 vs 11/813, $P = 0.33$, respectively). However, eradication therapy significantly decreased the incidence of GC only in a limited group of 988 *H. pylori*-carriers who did not have premalignant lesions when they entered the study. A well-designed meta-analysis of 6 RCTs that were conducted primarily in Japan and China, demonstrated a significant decline of 1.6% (51/3294) in the occurrence of GC in patients who had received eradication therapy compared with 2.4% (76/3203) of all patients who did not receive eradication therapy during a period longer than 4 years [relative risk (RR) = 0.66; 95%CI: 0.46-0.95]. However, eradication therapy did not demonstrate a significant benefit in terms of the prevention of GC incidence in any of the subjects with or without premalignant lesions at base-line^[37,38]. These data not only provided evidence that successful eradication might reduce the incidence of GC in asymptomatic *H. pylori*-infected Asian individuals but also suggested the possibility of a point-of-no-return, beyond which eradication may be of no use for cancer prevention. Actually, some discrepancies exist about whether eradication therapy can reduce the risk of GC occurrence in subjects with pathologically defined premalignant changes. Such discrepancies may be caused by several factors, as follows: Selection of the study population, including age, dietary habits, smoking, alcohol abuse, and genetic factors that may influence the susceptibilities of the host; *H. pylori* strain; intra-gastric conditions with/without premalignant lesions at the time of eradication therapy; and methodological differences during surveillance, including biopsy protocols and the interval/duration of surveillance. A more recent study identified several risk factors for the failure of eradication therapy to prevent GC in a Chinese community-based randomized trial, and thus the sequential data from the long-term surveillance of these patients should provide promising data on the preventive effect of eradication on the incidence of GC^[39].

Eradication therapy for patients after endoscopic resection

Previous studies have demonstrated that eradication did not completely eliminate the risk of secondary cancer occurrence after curative endoscopic resection (ER) of the primary GC, although it was found that eradication might suppress or delay the development of cancer^[40]. Based on the theory of field cancerization, the remaining gastric mucosa after ER is thought to harbor endoscopically invisible micro-lesions with malignant potential^[41,42].

Uemura *et al.*^[43] first reported that eradication after ER significantly decreased the occurrence of metachronous GC^[44]. Similarly, an RCT in Japan also showed that eradication after ER had a protective effect with respect to the development of secondary GC during a 3-year follow-up period. In contrast, a RCT in South Korea revealed that eradication after ER for gastric neoplasms, including GC and low- or high-grade dysplasia, did not

reduce the incidence of metachronous GC^[45]. A Japanese retrospective study used longer-term follow-up data of 1.1 to 11.1 years (median 3.0 years) for 268 *H. pylori*-positive patients who had undergone ER for GC, which may or may not have been followed by successful eradication. This study showed that eradication did not reduce the incidence of metachronous GC more than 5 years after ER (8.5% vs 14.3%, respectively, $P = 0.262$, log-rank test) and that the existence of mucosal atrophy at the time of entry into the study, and not *H. pylori*-infection status, was an independent risk factor for the incidence of metachronous GC^[46]. Accordingly, the preventive effect of successful eradication on metachronous GC remains controversial, possibly because of selection bias, underpowered numbers and residual confounding factors that may have been present in the studies. However, in an up-to-date meta-analysis of 13 relevant studies including 3 prospective trials, Yoon SB *et al.*^[47] demonstrated a preventive effect of eradication on the incidence of metachronous GC after ER. In detail, compared with the patients who did not receive eradication therapy, the pooled odds ratio in those who received eradication therapy was 0.42 (95%CI: 0.32-0.56). The pooled analysis of 6237 patients in all 13 studies demonstrated a significantly lower incidence of metachronous GC in the patients who received eradication therapy compared with those who did not [4.7% (111/2382) vs 6.8% (263/3855, 6.8%), respectively]. From now on, it will be difficult to conduct further well-designed and practical studies with long-term surveillance due to the ethical considerations that result from the fact that Japanese and Korean clinical guidelines have already recommended *H. pylori* eradication in cases of chronic gastritis as well as in cases of post-ER gastric background mucosa^[48,49]. Instead, regular surveillance endoscopy after eradication is recommended as the most practical method because GC occurrence may be uninterrupted even after successful eradication. Therefore, the development of useful biomarkers for GC that emerges after successful eradication will be urgently needed to detect high risk groups among such a large number of individuals who have received eradication therapy.

THE EFFECT OF ERADICATION ON HISTOLOGY AND BIOLOGICAL MARKERS

The effect of eradication on histological alterations

Previous systematic reviews and meta-analyses have demonstrated the impact of *H. pylori* eradication on the gastric histology of precancerous lesions (e.g., atrophy and IM) according to the (updated) Sydney System, during a surveillance period of less than 10 years^[50-52]. Through an examination of one RCT and seven observational studies, Rokkas *et al.*^[50] found that eradication had beneficial effects on gastric atrophy, but not on IM, in both the antrum and corpus. After the inclusion of three RCTs and nine

observational studies, Wang *et al.*^[51] showed that gastric atrophy in the corpus only recovered after eradication, but atrophy in the antrum and IM of the corpus and antrum did not recover. On the contrary, some studies incorporated longer-term follow-up of the histological changes after successful eradication based on the updated Sydney System. These previous studies revealed that, after atrophy of any site within the gastric mucosa and IM in the lesser curvature of the corpus were gradually restored, scores at both 5 and 10 years after eradication were significantly decreased compared with those at baseline^[52-54].

Accordingly, controversial results have been obtained from limited surveillance periods and studies with various methodological flaws, and therefore, additional RCTs with a longer observational period are required.

The effect of eradication on genetic/epigenetic alterations

Genetic and epigenetic alterations have been implicated mainly in the activation of oncogenes and/or the inactivation of tumor suppressor genes that are involved in cell-cycle regulation, cell-cell adhesion, DNA repair, and telomerase activation during gastric carcinogenesis.

The effect of eradication on genetic alterations

The effect of eradication treatment on genetic abnormalities remains unclear. Ohara *et al.*^[55] showed that eradication might not have led to changes in chromosomal structures, such as loss of heterozygosity and microsatellite instability, in 13 patients with *H. pylori*-positive GC, 12 patients with *H. pylori*-negative GC and 8 patients with GC detected after eradication. On the contrary, Zhu *et al.*^[56] revealed that increased expression of human telomerase, including ribonucleoprotein/human telomerase reverse transcriptase and human telomerase RNA, in the *H. pylori*-infected mucosa was significantly diminished after successful eradication.

The effect of eradication on epigenetic abnormalities

Recent interest in oncology research has shifted from genetics toward epigenetics. Actually, 90% of heritable alterations in cancer cells have been reported to be epigenetic in nature^[57,58]. Additionally, the most important difference between genetic and epigenetic alterations is that epigenetic changes are reversible by therapeutic interventions. Previous studies demonstrated that the medical elimination of *H. pylori* infection might reverse the epigenetic alterations and thus restore the histological phenotype of the gastric mucosal tissue, in spite of some discrepancies.

Gene promoter methylation: Gene promoter methylation is one of the major epigenetic alterations that is responsible for gene silencing^[19]. In particular, the hypermethylation of CpG-dinucleotide islands within promoter regions of genes, such as CDH1, APC, COX2, CHFR, DAPK, DCC, GSTP1, MAGE, MLH1, p16, PTEN, p14,

RASSF1A, RUNX3, SNCG, THBS1, TIMP-3, and TSLC1, have been reported to be associated with aberrant transcriptional gene silencing and consequent loss of protein expression during the multistep development of gastric carcinogenesis. It is well-known that dense methylation of CpG islands through the accumulation of "non-core" and "scattered" DNA methylation causes permanent overall deregulation of carcinogenic signal transduction^[59-68]. These phenomena are strongly supported by the epigenetic field defect hypothesis, but previous studies have shown complex changes in DNA methylation in non-cancerous mucosa after eradication; these changes are possibly due to the complicated results of reversible and irreversible changes of aberrant methylation in the gastric mucosa with various grades of inflammation/atrophy/metaplasia.

Originally, two studies from groups in Hong-Kong found that eradication reversed CDH1 promoter methylation and caused a reduction of inflammatory cell infiltration in the non-cancerous gastric mucosa^[25,26]. The first RCT involved 81 patients with biopsy-proven *H. pylori*-positive chronic active gastritis who were treated with/without eradication therapy. In this study, Chan *et al.*^[25] demonstrated that the methylation status of CDH1 as well as inflammatory cell infiltration at 6 wk after eradication therapy were significantly reversed only in patients without IM. In the second RCT, Leung *et al.*^[26] showed that eradication significantly reduced the methylation density of the CDH1 promoter in biopsy specimens that were obtained from the antrum and corpus of 28 middle-aged *H. pylori*-infected subjects without GC.

Thereafter, interest in other target genes has increased. Perri *et al.*^[27] suggested that gene-specific alterations in the gastric mucosa after successful eradication resulted from promoter methylation of CDH1, APC, COX2, MLH1, and p16. This conclusion was based on an examination of three paired gastric biopsy specimens obtained from the antrum, angles, and corpus of 57 *H. pylori*-positive dyspeptic outpatients before and 1 year after eradication. Significant differences were observed in the mean numbers of methylated genes, which were 0, 1.1 ± 0.9 , and 1.6 ± 0.9 among the 12 *H. pylori*-/IM- patients, 28 *H. pylori*+/IM- patients, and 17 *H. pylori*+/IM+ patients, respectively. In the 17 patients who received eradication therapy out of 45 total *H. pylori*-positive patients, a significant reversal of DNA methylation in both CDH1 and p16, and to a lesser extent, in APC and COX2, was observed after eradication. Sepulveda *et al.*^[28] revealed that hyper-methylation of CpG islands within the promoter of the gene that encodes the DNA repair protein MGMT was significantly reduced at 6-8 wk after successful eradication in 19 patients with *H. pylori*-gastritis (from 70% to 48% of cases, $P < 0.039$); an increase in the expression of MGMT protein was only seen on the mucosal surface. Using data from real-time methylation-specific PCR for FLNc and THBD and the updated Sydney System, Nakajima *et al.*^[29] examined specimens from 35 post-ER patients and 11 *H. pylori*-

negative healthy volunteers who underwent successful eradication. They showed that the methylation levels were gradually decreased together with a decrease in inflammatory cell infiltration throughout the 1 year after eradication, but that the methylation levels in post-ER patients were persistently higher than those in healthy individuals. Shin *et al.*^[30] investigated DNA methylation of the tumor-suppressor genes *LOX*, *APC* and *MOS* over a time course of 26.0 mo in non-cancerous gastric mucosa after eradication in 221 subjects. They showed that successful eradication significantly decreased the methylation levels within the *LOX* promoter, but not in *APC*, while a reduction in the *MOS* methylation level after eradication was significant only in subjects without IM at base-line. In addition, hypo-methylation of CpG islands is also considered to be another major mechanism of DNA methylation at an early phase during *H. pylori*-associated gastric carcinogenesis, because many genes that are susceptible to aberrant hyper-methylation were reported to overlap with the target genes of global hypo-methylation^[69-71]. Using an immunohistochemical evaluation of 5-methylcytosine, a marker of global hypo-methylation, as well as Ki67 and p53, Compare *et al.*^[31] assessed the changes in global hypo-methylation during a 10-year follow-up period after successful eradication in 10 patients with pre-neoplastic gastric lesions (*i.e.*, atrophy and IM). They demonstrated that global DNA methylation was negatively correlated with a gradual decrease in proliferative/apoptotic markers, depending on the histological grade of the multi-step transformation into GC, but independently of the Cag-A status and treatment response to eradication therapy.

miRNA: MiRNAs, which are non-coding RNA sequences that are 18- to 25-nucleotides in length, are transcribed without translation into proteins and are involved in post-transcriptional gene silencing^[72,73]. Tissue-specific expression of miRNAs has been shown to be associated with apoptosis, proliferation, differentiation, metastasis, angiogenesis, and immune responses in inflammatory-associated carcinogenesis^[74,75]. With regard to gastric carcinogenesis, several studies have reported different phenotypes of miRNA expression in the normal mucosa, *H. pylori*-induced precancerous lesions and GC^[76-80], but the changes in miRNA expression after successful eradication are still not completely understood.

Matsushima *et al.*^[79] revealed that the down-regulation of 14 miRNAs in 4 *H. pylori*-positive individuals was increased at 4 wk after successful eradication, although the expression of oncogenic miRNAs was significantly diminished only in non-metaplastic mucosa, but not in cases of IM, at 1 year after the eradication. Specifically, hsa-miR-21, hsa-miR-25, hsa-miR-93, hsa-miR-194 and hsa-miR-196 were found to be overexpressed in GC in comparison with non-cancerous gastric tissues, and eradication therapy decreased the expression levels of these miRNAs only in cases of atrophic gastritis without metaplastic changes. Moreover, in a case-control study using endoscopic biopsy specimens from 20 patients

who were treated with ER for GC and 14 sex- and age-matched non-cancer controls, Shiotani *et al.*^[81] revealed that, although the expression of oncogenic miRNAs (miR-17/92 and the miR-106b-93-25 cluster, miR-21, miR-194, and miR-196) was significantly higher in the gastric mucosa of the post-ER group than in the controls, none of these miRNAs that were expressed in cases of IM were significantly changed after eradication in any of the groups. This indicated that eradication therapy might be effective in the restoration of the expression of oncogenic miRNAs only in patients with atrophic gastritis and not in those with IM. On the contrary, in a more recent multicenter prospective cohort study of 782 patients who were treated with ER for GC followed by eradication, Asada *et al.*^[82] demonstrated that the risk of metachronous GC after ER might be predicted *via* an assessment of epigenetic field defects using the methylation levels of three genes (*miR-124a-3*, *EMX1* and *NKX6-1*) by quantitative methylation-specific PCR. In the post-ER background gastric mucosa, which might be highly associated with metaplastic changes, the highest quartile of the miR-124a-3 methylation level had a significant univariate (HR = 2.2; 95%CI: 1.1-4.4; *P* = 0.03) and a multivariate-adjusted (HR = 2.3; 95%CI: 1.0-5.1; *P* = 0.04) for the incidence of metachronous GC^[82]. However, questions remain about whether those parameters are simultaneously expressed in the metaplastic lesions as well as in the non-metaplastic mucosa, both of which are categorized as background gastric mucosa with malignant potential; questions also remain as to whether some uniform phenotypes of IM with those parameters might develop into GC after eradication. Considering that the metaplastic mucosa is already regarded as a clinically malignant predictor, future biological predictors will provide valuable ways to overcome these clinical issues.

Furthermore, miRNAs have been reported to exist in cell-free stable forms in the plasma and serum, and therefore, serum miRNAs may serve as novel biomarkers during non-invasive surveillance for large numbers of individuals.

Summary of the histological and biological studies

Clinical and laboratory studies have found only limited usefulness of *H. pylori* eradication in the regression of DNA methylation and miRNA expression in the non-cancerous gastric mucosa, which seems to be dependent on the gene type and histological grade of the atrophy and metaplasia. In other words, successful eradication might reverse the malignant potential of pathologically non-cancerous gastric mucosa prior to the point-of-no-return.

The first limitation of the aforementioned studies is gene-specific restoration after eradication. Instead of genome-wide studies, the investigators targeted only a single gene or a few genes, whose role in gastric carcinogenesis had not yet been clarified^[17,30]. The second limitation may have originated from differences in the sensitivities of the histology and molecular biology techniques, such as methylation-specific PCR,

that were used to detect the abnormalities. In addition, the distribution of metaplastic/dysplastic lesions is uneven throughout the entire gastric mucosa, which results in sampling error by targeted biopsy, although the distribution as well as the phenotype of the IM were suggested to provide a higher predictive value of the cancer risk. Cassaro *et al.*^[83] showed that IM on the lesser curvature from the cardia to the pylorus, or throughout the entire stomach, was associated with a higher risk of GC than focal/antrum-predominant IM. Among the three phenotypes of IM [*i.e.*, complete IM (type I) or incomplete IM (types II, type III)], type III IM was reported to harbor more genetic/epigenetic changes, which are similar to those that were found in cases of gastric dysplasia^[84,85]. However, the distribution of molecular and morphological alterations in the gastric mucosal tissue after successful eradication are likely to be more complex than those that were present prior to eradication, where the multi-focal distribution of epigenetic events is related to the severity of inflammation or metaplastic changes. As previous studies have demonstrated the gradual and irregular occurrence of areas of gastric-acid secretion up to 2 years after successful eradication, the areas that are restored after eradication might be irregular/atypical^[86-88]. These phenomena may result in increased sampling errors by random biopsies under a conventional endoscopic inspection, especially those in the post-eradicated gastric mucosa. Since the updated Sydney System was originally established to assist pathologists in the formal recording of their findings in a clinical setting, but not in a research setting, a suitable scoring system to guide targeted biopsies of the gastric mucosa after successful eradication is needed^[89].

FUNCTIONAL IMPROVEMENT OF THE GASTRIC MUCOSA BY *H. PYLORI* ERADICATION THERAPY

Functional reversibility of the gastric mucosa after successful eradication will provide another important clue, similar to what was provided by the reversibility of the macroscopic/microscopic morphology. The reversibility might be caused by the reduced production of inflammatory cytokines that have inhibitory effects on gastric acid secretion or by the increased expression of H⁺/K⁺-ATPase, intrinsic factor, and M3 muscarinic receptor mRNA in the gastric mucosa, but is likely not caused by the numbers of parietal cells^[90,91].

Ji *et al.*^[92] used *in vivo* functional imaging as well as tissue morphological imaging by confocal laser endomicroscopy (CLE) and fluorescein tracers to investigate the reversibility of mucosal barrier defects after eradication in 42 *H. pylori*-positive patients. They demonstrated that gastric IM was associated with an impaired para-cellular barrier, which did not recover after eradication. Essentially, the para-cellular permeability in the setting of IM, despite *H. pylori* infection, was significantly higher than that in non-

IM tissue with *H. pylori* infection, which was in turn higher than that in non-IM tissue without *H. pylori* infection. Six months after successful eradication in 14 patients, the para-cellular barrier dysfunction of the non-IM mucosa was significantly improved, as shown by electron microscopy and CLE, in spite of a lack of significant changes in IM. The irreversible mucosal barrier dysfunction of IM may cause constant trans-epithelial penetration of various intra-gastric substances, which may promote an immune response and the further development of cancer. Accordingly, the CLE findings may be useful for the depiction of the functional and morphological irreversibility of the disrupted para-cellular barrier of IM as a reasonable cause of carcinogenesis in patients with IM, although CLE has point-sampling characteristics.

ROLE OF CONGO-RED CHROMOENDOSCOPY IN THIS RESEARCH FIELD AND FUTURE PERSPECTIVES

Congo-red chromoendoscopy is another functional test that is used to visualize the gastric areas that are capable of gastric acid secretion; this technique involves a pH-dependent color reaction, which may be performed during a conventional endoscopic inspection (Figure 1)^[93,94]. Instead of CLE and an endoscopic biopsy, this method provides a comprehensive view that distinguishes endoscopically normal-appearing gastric mucosa based on functional characteristics. Additionally, its reproducibility may be ideal for the measurement of functional and morphological changes in the mucosa during long-term surveillance after eradication.

Based on findings by Congo-red chromoendoscopy, Iijima *et al.*^[88] found an expansion of the acid-secreting mucosa during a mean follow-up of 62 mo after eradication in 24 *H. pylori*-positive patients. Subsequently, after an investigation of the histological features using a Ki67 labeling index in the biopsy samples that were obtained from both acid-secreting and non-acid-secreting areas, they showed that functionally irreversible gastric mucosa after eradication was associated with extensive IM and sustained hyper-proliferation. This indicates the importance of increased malignant potential in the genesis of GC. Specifically, during the surveillance periods, the entire area of the greater curvature became occupied exclusively by acid-secreting mucosa, whereas the expansion of the acid-secreting area in the lesser curvature was limited. With the exception of the score for neutrophil infiltration, the mean inflammation score in the non-acid-secreting area was significantly higher than in the acid-secreting area (1.3 ± 0.5 vs 0.8 ± 0.5 , respectively). Marked atrophic/metaplastic changes with high numbers of Ki67-positive cells were observed in non-acid-secreting areas, whereas none to mild atrophic/metaplastic changes with a low number of Ki67-positive cells were found in acid-secreting areas. Accordingly, this study showed that

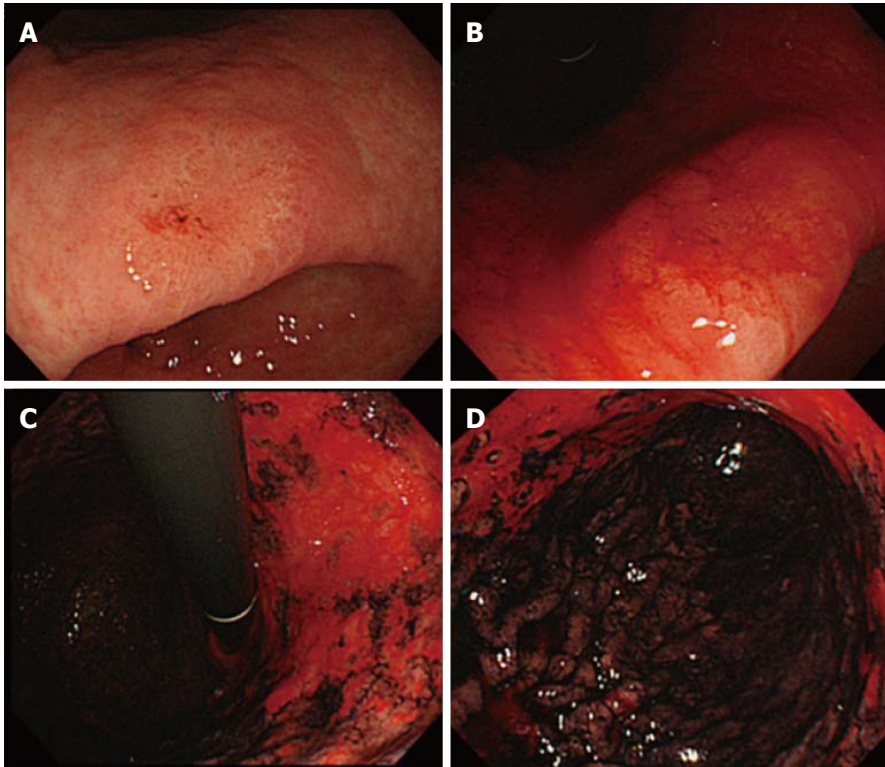


Figure 1 Representative photos of Congo-red chromoendoscopy in a 66-year-old female patient with gastric cancer that emerged after successful eradication of *Helicobacter pylori* infection. A: A lesion of well-differentiated adenocarcinoma located on the lesser curvature in the lower part of the stomach [FQ260Z (Olympus, Tokyo)]; B: Congo-red imaging showed GC in a non-acid secreting area (Red); C and D: Post-eradicated gastric background mucosa (Red: Non-acid secreting area; Black: Acid secreting area). GC: Gastric cancer.

even long after successful eradication, the histological degrees of residual inflammation infiltration, atrophy, IM, and epithelial proliferation in the functionally irreversible non-acid-secreting areas were significantly higher than those in the functionally recovered area. Another study that used Congo-red chromoendoscopy in a total of 19 GC lesions that emerged after more than 2 years post-successful eradication in 14 consecutive patients demonstrated that GCs were observed exclusively in non-acid-secreting areas with sustained hyper-proliferation and accumulation of p53 protein^[95]. Accordingly, the non-acid secreting areas that were observed by Congo-red chromoendoscopy might truly be at a high risk for gastric carcinogenesis after eradication. Surprisingly, these studies also demonstrated that histological evaluation of targeted biopsy specimens from both acid-secreting and non-acid-secreting areas based on Congo-red chromoendoscopic findings revealed striking differences in their histological manifestations, even if these areas were separated by only a few centimeters. Therefore, Congo-red chromoendoscopy enables us to identify high-risk areas in a site-specific manner and to determine the distribution of functionally and morphologically irreversible mucosa after successful eradication. With careful consideration of the balance between the risks and benefits, Congo-red chromoendoscopy can provide important clues to gastric carcinogenesis that occurs after successful eradication^[96]. Further research on biopsy specimens guided by functional imaging, such as Congo-red chromoendoscopy, may be a promising approach in the clarification of molecular-biological events in the functional and morphological restoration and carcinogenesis after successful eradication.

SUMMARY

In conclusion, we reviewed the limited effect of the *H. pylori* eradication on the reduction of GC incidence, although the indication for eradication therapy is clinically widespread. No useful biomarkers for individuals who are at a high risk for GC that emerges after successful eradication have been identified because previous studies by conventional methods have not yet fully elucidated the underlying mechanisms of mucosal restoration as well as of gastric carcinogenesis after eradication. In accordance with a proof-of-concept of the epigenetic field defect of gastric cancerization, we propose the usefulness of Congo-red chromoendoscopy to differentiate functionally irreversible mucosal areas that are at an extremely high risk for gastric carcinogenesis. In addition, another study that used CLE with tracers identified the functional irreversibility of the metaplastic mucosa in the setting of post-eradication carcinogenesis. Therefore, future research that focuses on functional aspects may provide new insights that address both the mechanisms of mucosal regeneration and cancer development after successful eradication.

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Emerging role of cystic fibrosis transmembrane conductance regulator - an epithelial chloride channel in gastrointestinal cancers

Yuning Hou, Xiaoqing Guan, Zhe Yang, Chunying Li

Yuning Hou, Xiaoqing Guan, Zhe Yang, Chunying Li, Department of Biochemistry and Molecular Biology, Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI 48201, United States

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Correspondence to: Chunying Li, PhD, Department of Biochemistry and Molecular Biology, Karmanos Cancer Institute, Wayne State University School of Medicine, 540 E. Canfield Avenue, 5312 Scott Hall, Detroit, MI 48201, United States. cl@med.wayne.edu
Telephone: +1-313-5774182
Fax: +1-313-5772765

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Abstract

Cystic fibrosis transmembrane conductance regulator (CFTR), a glycoprotein with 1480 amino acids, has been well established as a chloride channel mainly expressed in the epithelial cells of various tissues and organs such as lungs, sweat glands, gastrointestinal system, and reproductive organs. Although defective CFTR leads to cystic fibrosis, a common genetic disorder in the Caucasian population, there is accumulating evidence that suggests a novel role of CFTR in various cancers, especially in gastroenterological cancers, such as pancreatic cancer and colon cancer. In this review, we summarize the emerging findings that link CFTR with various cancers, with focus on the association between CFTR defects and gastrointestinal cancers as well as the underlying mechanisms. Further study of CFTR in cancer biology may help pave a new way for the diagnosis and treatment of gastrointestinal cancers.

Key words: Gastrointestinal cancer; Protein interaction; Cystic fibrosis transmembrane conductance regulator; Nuclear factor κ B; Signaling molecule

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Core tip: The present review aimed to analyze most published data regarding the emerging role of cystic fibrosis transmembrane conductance regulator (CFTR), an epithelial chloride channel in many tissues and organs, in various cancers, with the focus on the link between CFTR dysfunction and gastrointestinal cancers. The possible underlying mechanisms have also been discussed.

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INTRODUCTION

Cystic fibrosis transmembrane conductance regulator (CFTR), a glycoprotein with 1480 amino acids, is a cAMP activated anion channel expressed in the epithelial cells of a wide variety of tissues including lung, pancreas, liver, sweat gland, reproductive system, and intestine^[1,2]. Mutations or dysregulation of CFTR will cause several pathological conditions such as cystic fibrosis (CF), a common life threatening autosomal recessive disease in the Caucasian population^[3]. The *CFTR* gene, identified in 1989, is approximately 230kb containing 27 exons^[4]. CFTR protein contains 12 hydrophobic membrane-spanning regions, 2 cytoplasmic nucleotide binding domains (NBD) involved in ATP binding and hydrolysis, and one cytosolic regulatory domain which can be phosphorylated by PKA and PKC^[5,6]. Even though it was identified initially as a chloride channel, other functions of CFTR have also been characterized over the past decades. CFTR performs as a modulator of other ion channels and transporters^[7-10], and it can also cooperate with other proteins by forming macromolecular complexes to regulate important cellular processes^[11-14]. Recently, it has been reported that CFTR is likely to interfere with the expression of proteins involved in the signaling pathways of inflammation^[15,16]. Additionally, it has been reported that CFTR is associated with apoptosis in proximal renal failure^[17]. Therefore, the defect in CFTR function results in multi-system disorders affecting airways, pancreatic ducts, bile ducts, intestines^[18,19].

Accumulating research has demonstrated that aberrant expression or function of CFTR may lead to various pathological conditions, especially inflammatory diseases and inflammation-associated cancers^[20-22]. Recently, a large cohort study which recruited 41188 cases of CF patients conducted in the United States revealed an elevated risk of digestive tract cancers among CF patients^[23]. In addition, another group also reported an increased risk of malignancies for the kidney, thyroid, endocrine, lymphoma, skin and prostate in CF patients^[23]. Moreover, some groups showed that the *CFTR* gene is hypermethylated in several cancer cell lines and resected tumors^[22,24-26], which indicates that defects in the expression or function of CFTR may have a pivotal role in the pathogenesis of various cancers. It has also been well established that CFTR is critical in maintaining epithelial cell polarity and integrity^[27]. Consequently, the observed association between cancer risk and CFTR, as well as the connection between CFTR and cell junction molecules, has led to the hypothesis that CFTR plays important roles in cancer progression^[28,29]. In this review, we focus on the recent findings that associate CFTR with various cancers, especially gastrointestinal cancers.

CFTR AND GASTROINTESTINAL CANCER

Focus has been placed on the association between pancreatic cancer risk and CFTR deficiency since the early 1990s^[30] when cohort studies were performed to investigate how CFTR variants affect the risk of pancreatic cancer^[31,32]. CF patients suffer from chronic pancreatic damage and inflammation, which may predispose them to malignancy^[33]. Even though the overall cancer risk in CF patients is similar to that of the general population, CF patients are at elevated risk (6.5 fold) of developing gastrointestinal cancers including pancreatic cancer^[32]. The results from the cohort studies indicate an increased risk for pancreatic cancer among CF patients, which is supported by two recent reports^[33,34], in which multiple CFTR mutations were analyzed. These studies also found that patients who are CFTR mutant carriers develop pancreatic cancer earlier than those who carry normal CFTR. While there are reports that do not support the view that CFTR mutations increase the risk of pancreatic cancer^[35,36], Maisonneuve *et al.*^[23] concluded that there is a consistent increased risk of pancreatic cancer in CF patients, which is supported by retrospective studies that recruited a large amount of cases and screened multiple CFTR mutations^[33,34]. The difference between the reported associations of pancreatic risk and CFTR variation could be due to multiple reasons such as limited case number, different parameters regarding selection of the control population, mutations investigated in the study, and environmental factors including smoking and alcohol consumption. This requires regional or even global collaboration to adjust the possible biases that could occur as a result of the mentioned parameters^[37].

Different interpretations have been presented by investigators based on the observations of epidemiological studies and laboratory research to reveal the underlying mechanism of the association between CFTR and cancer. One mechanism is that CFTR dysfunction in the exocrine pancreas results in an ion transport defect, giving rise to obstruction of the pancreatic duct and consequently chronic inflammation, which is closely related with cancer initiation^[34]. It has been reported that wild-type CFTR suppresses tumor progression by negatively regulating MUC4, a protein involved in tumor growth and metastasis, providing a potential mechanism for CFTR inhibiting pancreatic cancer progression^[38]. Another group reported that increased PKC activity is associated with degradation of CFTR in pancreatic ductal cells^[39]. Altogether, these studies indicate that the microenvironment and cell signaling change in tumor cells could be implicated in the down-regulation of CFTR expression and the up-regulation of certain cancer promoting proteins in pancreatic cancer.

There is an increased risk of gastrointestinal cancers in CF patients, of which colon cancer is the most prevalent^[32]. Case reports have described CF patients developing colon cancer at relatively young ages^[40-42]; meanwhile, the mechanism behind this phenomenon has also been investigated, and several speculations have been

proposed. According to reports from different groups, Ras, PKC and interferon-gamma (IFN- γ) are involved in the down-regulation of CFTR in colonic ductal cells^[39,43-45]. More interestingly, the down-regulation of CFTR is correlated with drug resistance in colon cancer^[46]. When HT-29 cells, a human colorectal adenocarcinoma cell line, were treated with an increasing amount of colchicine, an alkaloid with anti-cancer activity^[47], the down-regulation of CFTR was accompanied by the acquisition of a drug resistant phenotype, and the restored expression of CFTR was observed when the drug was removed^[46]. Although the mechanism is not fully understood, it is possible that CFTR may transport certain unidentified metabolites or factors other than ions. Recently, Sun *et al.*^[48] demonstrated that the interaction between CFTR and AF-6/afadin, an adherens junction molecule, is important in the process of colon cancer migration and metastasis. In their study, the expression of CFTR and the interaction between CFTR and afadin were linked to epithelial-mesenchymal transition (EMT), an important process during tumor metastasis, indicating that functional CFTR may act as a tumor suppressor, while the deficiency of CFTR would facilitate tumor progression in colon cancer^[48].

The involvement of CFTR deficiency was also studied in other gastrointestinal malignancies such as gastric cancer^[49]. It is reported that serum CFTR is significantly associated with a cancer biomarker, carbohydrate antigen 199 (CA199), and it is significantly associated with gastric cancer staging. In addition, the combination of CFTR and CA199 yields a higher receiver operating characteristic (ROC) curve, which is used in the diagnosis of gastric cancer^[49]. Although this research did not discuss the association between CFTR and gastric cancer, it has provided a potential biomarker for the diagnosis of not only gastric cancer but also other types of cancer.

CFTR AND OTHER CANCERS

A large cohort study conducted by Neglia *et al.*^[32] demonstrated that the genetic variation of the CFTR gene is associated with a moderate increase (1.4 fold) in the risk of lung cancer, and CFTR gene mutation has been reported in lung cancer patients^[50,51]. Epigenetic modifications such as DNA methylation regulate gene expression to maintain cell growth and development. However, aberrant DNA methylation of the cytosine preceding guanosine (CpG) sites of the promoter regions of many genes plays a critical role in cancer by silencing the tumor suppressor genes, which is one of the earliest alternations in cancer^[52,53]. Hypermethylation of the CFTR promoter gene has been reported, and corresponding clinical data also indicates that CFTR promoter gene hypermethylation is associated with significantly poorer survival among younger lung cancer patients^[22]. On the other hand, CFTR gene expression was restored when A549 cells, a human lung adenocarcinoma epithelial cell line, were treated with demethylation reagents^[22]. Similarly, the results of a retrospective study from another group demonstrated that low CFTR expression is correlated with advanced stage

and metastasis as well as poor prognosis in non-small cell lung cancer^[54]. Similar to the findings in colon cancer^[48], investigators linked the expression of CFTR to EMT in lung cancer and demonstrated that CFTR suppresses EMT *via* inhibiting the uPA/uPAR-mediated signaling pathway^[54]. These findings indicate that functional CFTR may play an inhibitory role in the initiation and progression of lung cancer.

Based on the results obtained from different groups, the protective role of CFTR on tumor progression is reflected by the inhibitory effect on tumor metastasis, which seems unrelated to its ion transportation activity. It has been reported that CFTR physically interacts with multiple proteins including ZO-1^[55], E-cadherin^[56], multidrug resistance protein-2 (MRP2)^[14], MRP4^[13], and afadin^[48]. These protein-protein interactions may be important in the inhibition of tumorigenesis. On the other hand, functional CFTR has been shown to down-regulate tumor promoting proteins such as NF- κ B, which plays a pivotal role in inflammation and cancer progression^[57].

The role of CFTR has also been examined in the pathogenesis of other cancers such as prostate cancer^[24,58], hepatocellular cancer^[26,59] as well as bladder^[25] cancer with the focus mainly on DNA methylation. CFTR promoters are hypermethylated in these cancers compared with normal tissues. Moreover, researchers proposed that it is possible that methylation of the CFTR promoter may be an early event in the process of tumorigenesis^[26]. Additionally, it is also important to point out that more frequent hypermethylation of CFTR promoter was observed in prostate cancer patients with a high Gleason score and Ki-67 index^[24], indicating that CFTR hypermethylation could be used to predict prognosis in prostate cancer. Based on the study of CFTR hypermethylation in lung cancer, it is possible that CFTR may function as a tumor suppressor in these cancers as well, which is partly supported by the findings in prostate cancer^[28] that the CFTR knockdown promoted cancer cell proliferation, invasion and migration.

The role of CFTR in breast cancer has been investigated since the 1990s, but the findings are controversial. According to the cohort study conducted by Neglia *et al.*^[32] breast cancer is less common in CF patients. In 1996, Abraham *et al.*^[60] reported that the CF phenotype with elevated blood ATP concentration yielded decreased tumor growth and implantation in mice. The authors believed that dysfunctional CFTR in the digestive duct failed to excrete ATP into gut lumen resulting in elevated blood ATP, which is cancer suppressive. However, recently Zhang *et al.*^[29] reported that the down-regulation of CFTR promoted cell migration and invasion *in vitro* as well as metastasis *in vivo* while over-expressing CFTR reversed these activities. Although this seems controversial, the findings indicate that the role of CFTR in cancer depends, at least partially, on the pathological conditions of the individuals. Under the CF condition, in which the CFTR and other CFTR-regulated ion channels/transporters are dysfunctional, the de-regulated transport activity due to dysfunctional CFTR may

be the main factor that contributes to the pathogenesis of cancer. When the CFTR is functional, it is possible ion transportation activities do not account for the main aspect of the tumorigenesis.

CFTR has also recently been related to cervical cancer^[61-63]. A series of studies from Peng *et al.*^[61] demonstrated that the expression of CFTR is gradually increased from normal to pre-cancerous cervical tissue and cervical cancer tissues, and it is also positively correlated to histological grades, tumor stage, and metastatic grades. In addition, further investigation by the Peng *et al.*^[61] indicated that the upregulated CFTR in pre-cancerous and cervical cancer tissues resulted from aberrantly increased NF- κ B p65 translocation^[62]. RNA sequencing results obtained from samples co-existing with pre-cancerous lesions and adjacent normal cervical tissue demonstrated that CFTR was also significantly up-regulated in cervical cancer^[63], which is in agreement with the findings from Peng *et al.*^[61] and Wu *et al.*^[62]. These findings are opposite of those from other types of cancers which demonstrated the tumor suppressive roles of CFTR, indicating that the role of CFTR in cancer is organ/system specific. Although CFTR has been originally identified as a chloride channel, accumulating evidence has demonstrated it has different functions in different models, suggesting that the function of CFTR is organ/system dependent, and consequently the role of CFTR in different cancers may also be organ/system specific.

MOLECULAR MECHANISMS LINKING DYSFUNCTIONAL CFTR AND CANCERS

Investigations have been performed to propose molecular mechanisms to link defect found in CFTR to cancer, the underlying mechanism of which can be divided into two major subgroups depending on the different aspects of the CFTR function.

One subgroup is based on the transporter feature of CFTR. As a transporter located on the epithelial membrane, CFTR is able to transport ATP across the membrane^[64]. On the other hand, it is demonstrated that extracellular ATP processes an inhibitory effect on tumor growth both *in vitro* and *in vivo*^[65,66]. In gastrointestinal cancers, CFTR dysfunction or dysregulation leads to decreased luminal ATP, which may account for the elevated cancer incidence. However, as a result of decreased ATP secretion into gut lumen, the blood concentration of ATP increased, probably resulting in decreased malignancy such as breast cancer^[60]. This explains, to some degree, why CFTR dysfunction promotes the risk of certain types of cancer while suppresses the risk in others. In addition, the transporting property of CFTR is also linked to the apoptosis and survival of cells. CFTR is reported to be able to transport glutathione (GSH), an important antioxidant protecting cells from excessive oxidative stress, out of cells during apoptosis^[67]. This activity is hampered when CFTR is dysfunctional or the expression of CFTR is too low, which are common in several types of cancers. In addition, metabolites such

as CO₂ and lactate are generated by fast tumor growth, resulting in an acidic and hypoxic microenvironment around cancer cells. Functional CFTR facilitates the survival of cancer cells *via* transporting bicarbonate out of the cells^[68]. Therefore, CFTR seems to possess both cancer promoting and suppressing properties, but the exact role it plays in a particular cancer may depend on the cancer type and tumor microenvironment.

The other subgroup, which is more complicated and independent of the transporter feature of CFTR, is based on the signaling and protein-protein interactions that CFTR is involved in. It is well established that NF- κ B pathway, an important player in cancer proliferation and cell survival^[69,70], is negatively regulated by functional CFTR^[57,71]. Meanwhile, decreased NF- κ B activity was also observed in CFTR over-expressed lung cancer cells^[72]. Recently, CFTR was found to be associated with tumor metastasis in multiple cancers in which uPA/uPAR pathway is involved^[28,54]. Although the mechanisms are not fully understood, these investigations have shed light on the importance of the NF- κ B related signaling. Moreover, Ras, PKC, IL-1 β , and IFN- γ are involved in the down-regulation of CFTR in cancer cells^[43-45,73]. In addition, CFTR has been shown to physically interact with other proteins such as ZO-1, afadin, and E-cadherin contributing to various cellular activities^[48,55,56], and the disruption of these interactions leads to enhanced cell invasion and migration, indicating that CFTR plays more complicated roles than previously thought. Further, CFTR promoter hypermethylation is also observed in multiple types of cancer including hepatocellular cancer, lung cancer, bladder cancer, and prostate cancer^[22,24-26,58]. It is still not clear whether this epigenetic modification occurs at early stages or is a result of tumorigenesis, although a study in hepatocellular cancer suggests that the methylation may be an early event of cancer progression^[26]. Conclusively, CFTR functions not only as an ion channel/transporter but also as a signaling hub involved in multiple signaling pathways and macromolecular complexes, contributing to seemingly opposite cellular functions: Either promoting or suppressing cancer progression.

CONCLUSION

CFTR, a glycoprotein with 1480 amino acids, was originally identified as a chloride channel that transports chloride ions in and out of cells. Emerging evidence suggests that CFTR possesses both tumor promoting and tumor suppressing properties as shown in Table 1. However, the exact role it plays in cancer depends on the cancer type and the microenvironment of the cancer cells. Since the expression of CFTR differs from one organ to another, it is possible that the functions of CFTR in different organs are also different. For instance, in the pancreas CFTR is mainly responsible for excretion^[74] while in macrophages it contributes to controlling cytokine production^[75]. In addition, the microenvironment of cancer cells also dictates the role of CFTR. There is variation in proteins expressed in different cancer cells as well as cytokines

Table 1 Summary of impact of cystic fibrosis transmembrane regulator on the malignancies of different organs

Organs	Impact	Ref.
Pancreas	Wildtype CFTR suppresses tumor progression	[23,32-34,38,39]
	CFTR dysfunction my results in obstruction of pancreatic duct and chronic inflammation	
Colon	CFTR dysfunction increases the risk of colon cancer	[40-46,48,73]
	Wildtype CFTR may act as a tumor suppressor in colon cancer	
Stomach	Serum CFTR is associated with gastric cancer staging	[49]
Lung	CFTR mutation is associated with increased risk of lung cancer	[22,32,50,51,54]
	CFTR expression is also associated with tumor progression and poor prognosis	
Prostate	CFTR gene was hypermethylated in prostate cancer cells	[24,58]
Liver	CFTR gene was hypermethylated in hepatocellular cancer cells	[26,59]
Bladder	CFTR gene was hypermethylated in bladder cancer cells	[25]
Breast	Suppresses breast cancer by elevating blood ATP but promotes cancer metastasis by enhancing EMT	[29,32,60]
Cervix	Promotes cancer progression	[61-63]

CFTR: Cystic fibrosis transmembrane regulator; EMT: Epithelial-mesenchymal transition.

or chemokines produced in different types of cancers, generating different microenvironment around the cells. Consequently, CFTR may react differently according to environmental stimulants. For example, IFN- γ and TNF- α down-regulate the expression of CFTR, while cAMP up-regulates the expression of CFTR in cancer cells^[76,77]. Even though the biology of CFTR has been investigated for decades, its function is still not fully understood, especially in cancer cells. Based on the literature to date, it will be helpful to study the protein interactome and the signaling pathways associated with CFTR in cancer cells. In addition, previous studies of CFTR dysfunction were largely based on CF related CFTR gene variations, and most of the studies usually only covered very small amounts of mutations, though over 1900 CFTR mutations have been documented so far. This may limit the findings and/or conclusions from these studies. In order to obtain a more comprehensive knowledge of CFTR biology and its role in cancer, interdisciplinary collaborations using system biology and integrative approaches are encouraged.

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Role of genetic detection in peritoneal washes with gastric carcinoma: The past, present and future

Hyun-Dong Chae

Hyun-Dong Chae, Department of Surgery, School of Medicine, Catholic University of Daegu, Daegu 705-718, South Korea

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Correspondence to: Hyun-Dong Chae, MD, PhD, Department of Surgery, School of Medicine, Catholic University of Daegu, 3056-6, Daemyung-4-Dong, Namgu, Daegu 705-718, South Korea. hdchae@cu.ac.kr
Telephone: +82-53-6504429
Fax: +82-53-6247185

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Abstract

The most frequent cause of treatment failure following surgery for gastric cancer is peritoneal dissemination, mainly caused by the seeding of free cancer cells from the primary gastric cancer, which is the most common

type of spread. Unfortunately, there is no standard modality of intraperitoneal free cancer cells detection to predict peritoneal metastasis until now. We reviewed English literature in PubMed was done using the MeSH terms for gastric cancer, peritoneal wash, and reverse transcriptase polymerase chain reaction. All the articles were reviewed and core information was tabulated for reference. After a comprehensive review of all articles, the data was evaluated by clinical implication and predictive value of each marker for peritoneal recurrence. There are still many limitations to overcome before the genetic diagnosis for free cancer cells detection can be considered as routine assay. To make it a reliable diagnostic tool for detecting free cancer cells, the process and method of genetic detection with peritoneal washes should be standardized, and the development of simple diagnostic devices and easily available kits are necessary. Herein, we reviewed the past, present and future perspectives of the peritoneal lavage for the detection of intraperitoneal free cancer cells in patients with gastric cancer.

Key words: Gastric cancer; Peritoneal metastasis; Free cancer cells; Reverse transcriptase polymerase chain reaction; Genetic detection

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Core tip: The most common cause of treatment failure after gastric cancer surgery is peritoneal metastasis, mainly caused by free cancer cells from primary cancer. Genetic detection using reverse transcriptase polymerase chain reaction analysis has been used for the detection of free cancer cells. The process and method of genetic detection with peritoneal washes should be standardized, and the development of simple diagnostic devices and easily available kits are necessary in the future. In this article, we summarize the current evidence of genetic detection in peritoneal washes from gastric cancer patient.

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INTRODUCTION

Although the incidence of gastric cancer has been declining in western countries, gastric cancer is still the fourth most common cancer and the second leading cause of cancer deaths worldwide^[1]. Peritoneal dissemination of gastric cancer, which is caused by free cancer cell seeding from the primary tumor, is the most common cause of treatment failure after surgery. In general, the frequency of peritoneal dissemination is increased with depth of invasion of the gastric wall^[2]. Intraperitoneal free cancer cells detected in peritoneal washes from gastric cancer patients have been reported to be significant and independent prognostic factor for recurrence and survival after surgery.

To predict peritoneal metastasis, several methods have been used for many studies, which are conventional cytology^[3,4], ThinPrep^[5], and molecular markers^[4,6-8]. Conventional cytology had been regarded as the gold standard for detecting cancer cells in peritoneal washes. However, the usefulness of conventional cytology for prediction of peritoneal metastasis has controversy because of its low sensitivity^[3,4,7,9].

Recently, reverse transcriptase polymerase chain reaction (RT-PCR) analysis has been used for the genetic detection of free cancer cells^[10-12]. Several target genes have been used, which are carcinoembryonic antigen (CEA), heparanase, matrix metalloproteinase-7 (MMP-7), cytokeratin 20 (CK-20), telomerase, and melanoma-associated gene (*MAGE*)^[2,13]. The sensitivity of RT-PCR is higher than that of conventional cytology^[14-16]. Several studies reported that the results of RT-PCR analysis from peritoneal washes correlate strongly with peritoneal recurrence and prognosis after curative surgery in patients with advanced gastric cancer^[6,14-20].

In this reason, we reviewed the present status and future perspectives of peritoneal lavage for the detection of intraperitoneal free cancer cells in patients with gastric cancer (Figure 1).

DETECTION OF INTRAPERITONEAL FREE CANCER CELL WITH GASTRIC CARCINOMA

The past

Conventional cytology from the peritoneal washes had been widely used to detect free cancer cells and to predict peritoneal metastasis^[3,9], based on several studies reporting a correlation between peritoneal recurrence and presence of intraperitoneal free cancer cells^[21-24].

In patients with serosal involvement, 50% of patients develop peritoneal recurrence even if curative resection is performed^[25,26]. Bando *et al*^[3] reported that 24% of positive cytology from 1297 gastric cancer patients. This result presented higher positive rate than other studies, probably because many patients with advanced stage of gastric cancer were included in the study (296 patients had peritoneal metastasis). Ribeiro *et al*^[27] reported much higher incidence of positive cytology (41%) from their study from 49 patients with advanced gastric cancer including metastatic disease. Other studies that evaluated patients with gastric cancer underwent curative resection showed approximately 5% of positive rate from peritoneal washes cytology^[3,8-10,25,27,28].

In many past studies, intraperitoneal free cancer cells detected in peritoneal washing cytology have been demonstrated as significant and independent prognostic factor, influencing both recurrence free survival and overall survival of patients with gastric cancer. Therefore, peritoneal wash cytology is recommended in the Japanese Classification of Gastric Carcinoma from 1998^[29]. Recently, the American Joint Committee on Cancer tumor node metastasis staging system classified the positive peritoneal cytology in gastric cancer as metastatic disease (M1) in the 7th edition^[1].

However, the conventional cytology is often criticized for its relatively low sensitivity to detect intraperitoneal free cancer cells and to predict peritoneal metastasis^[3,4,7,9]. Furthermore, previous studies reported that the conventional cytology in patient without any macroscopic peritoneal metastasis (P0) after curative resection had very low sensitivity (5%-15%)^[3,4,7]. Although obtaining peritoneal cytology has been advocated by Japanese and Dutch investigators^[30,31], this is not a uniform practice in other Western centers. Immunocytochemical methods that used a panel of monoclonal antibodies, directed to gastric cancer-associated antigens had improved detection of peritoneal cytology by providing more sensitive and may have a higher specificity than conventional cytology^[32].

The present

General principles: The high sensitivity of RT-PCR analysis has made it possible to detect micrometastasis on the cancer tissue specific messenger RNA (mRNA) expression in peripheral vein, bone marrow, lymph nodes, and peritoneal cavity^[19,33-35]. Although RT-PCR analysis of peritoneal washes is a more sensitive than conventional cytology, the result variations in RT-PCR between different laboratories have been reported. Therefore standardization and quality control of the process of RT-PCR is very important.

For the detection of gastric cancer micrometastases in peritoneal washes, RT-PCR technique has been used from the twenty-first century^[10-12]. The primer sequences, which are used in previous studies, for RT-PCR of peritoneal lavage in patients with gastric cancer are summarized in Table 1. The sensitivity of RT-PCR is higher than that of conventional cytology^[10-12]. Based on several studies, There is strong correlation between

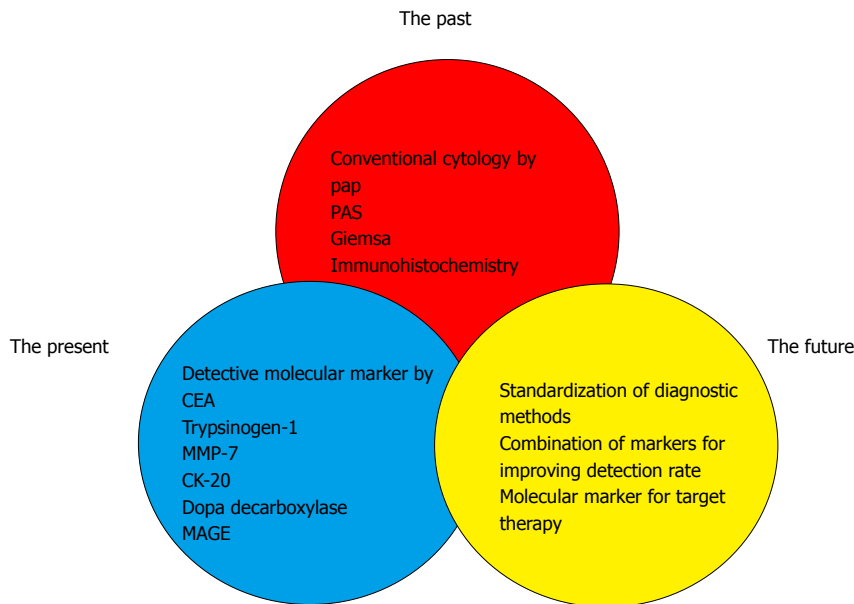


Figure 1 Detection of free cancer cell in peritoneal washes of gastric cancer patients: The past, present and future. CEA: Carcinoembryonic antigen; MMP-7: Matrix metalloproteinase-7; CK-20: Cytokeratin-20; MAGE: Melanoma associated gene.

the results of RT-PCR analysis from peritoneal washes and prognosis, including peritoneal recurrence rate, after curative resection in patients with advanced gastric cancer^[6,14,16-20] (Table 2).

Although many investigators have demonstrated that molecular techniques using RT-PCR may serve as a useful method for the detection of free cancer cells, there are still several problems. These are time-consuming, expensive, relatively laborious compared with conventional cytology, and the accuracy is widely variable between laboratories and the methods of processing the peritoneal washes is not yet standardized. Current experimental studies which aim to identify a rapid, accurate, and cost-effective detection method are proposed. The transcription-reverse transcription concerted reaction system and the LightCycler system have shown promise in intraperitoneal free cancer cell detection.

Molecular markers: (1) CEA: CEA is the most commonly studied tumor marker and is predominantly used clinically in patients with gastrointestinal cancer. Structurally, it is a glycoprotein with a molecular weight of 200 kd and is a component of the glycocalyx, located on the luminal side of the cell membrane of normal epithelial intestinal cells. Although its exact function is unknown, CEA has been shown to be involved in cell adhesion and is able to inhibit apoptosis induced by loss of anchorage to the extracellular membrane.

In 1997, Nakanishi *et al*^[16] had proposed the first study that more sensitive detection of free cancer cells could be achieved through amplification of CEA mRNA by means of the RT-PCR, and reported a high sensitivity of the CEA RT-PCR assay for the detection of peritoneal micrometastasis and a 20% improvement in the positive rate, as compared with that of cytology alone. CEA is presented in all of gastric cancer cell lines examined irrespective of the differentiation degree,

and it is absent in blood and mesothelium. That is indicating the specificity of CEA RT-PCR for detection of carcinoma cells in peritoneal lavage fluid.

Since this report, many investigators have been used CEA as the target gene of RT-PCR for the patients with gastric cancer. From 2001, many other studies have reported that quantitative CEA mRNA detection using RT-PCR is an accurate method for predicting the risk of peritoneal recurrence in patients with gastric cancer^[36-42].

Many studies reported that the detection of CEA mRNA using RT-PCR of peritoneal washes was the most reliable prognostic marker for recurrence and peritoneal carcinomatosis in patient with gastric cancer, and more sensitive than conventional cytology^[8,17]. Kodera *et al*^[43] found that the use of RT PCR for CEA mRNA resulted in a detection rate of free cancer cells of 28% (41 of 148), with a 14% higher detection rate than for peritoneal wash cytology. However, the sensitivity of the CEA RT-PCR assay for the detection of peritoneal micrometastasis still remains low. Furthermore, false-positive results, caused by expression of CEA in non-cancer cells like mesothelial cells and lymphocytes, is remained as main problem of this technique^[20].

(2) Trypsinogen-1: Trypsin is a member of the serine protease family composed of three trypsinogen genes (*trypsinogen 1, 2 and 4*) with a potential role in cancer invasion^[44,45]. These family members are sharing same nucleotide structures above 90% with each other.

Trypsin has potent proteolytic activity, and its inappropriate activation may lead to peritoneal dissemination of infiltrative gastric cancer by defoliation of cancer cells from the surface of advanced primary tumor, attachment and invasion to extracellular matrix under the mesothelium^[46].

(3) MMP-7: Recently, MMPs are gaining attention and attracted by many investigators who are interested in new biomarkers for cancer spread and metastasis^[47].

Table 1 Primer sequences used for reverse transcriptase polymerase chain reaction

Gene	Sequence of primer pair (5'→3')	Tm (°C)	Amplicon length (bp)
CEA	F: TCTGGAAGTCTCTCTCTCTCAGCTGG R: TGTAGCTGTGCAAAATGCTTTAAGGAAGAAGC	69	160
CK-20	F: GGTGCGGACTACAGTGCATATTACA R: CCTCAGCAGCCAGTTTAGCATTATC	72	121
Trypsinogen	F: ACCACCATGAATCCACTCCTG R: GCTTTAGCTATTGGCAGCTAT	62	
MMP-7	F: ATGTTAACTCCCGCGTCATA R: CAGCATACAGGAAGTTAATCC	72	418
DDC	F: AAGCACAGCCATCAGGATTCA R: TGGACATGCTTGCGGATATAAG	70	
L3PP	F: GATGCTGTGTGTTTGTAT GTTGAC R: CTTGACTGTGCTGATCACATT	95	
MAGE A1-6	F: CTGAAGGAGAAGATCTGCC R: CTCCAGGTAGTTTCTCTGCAC	60	855

Tm: Melting temperature; F: Forward; R: Reverse; CEA: Carcinoembryonic antigen; CK-20: Cytokeratin-20; MMP-7: Matrix metalloproteinase-7; DDC: Dopa decarboxylase; L3PP: L-3 phosphoserine phosphatase heparanase; MAGE: Melanoma associated gene.

Table 2 Reports on molecular marker with peritoneal washes in gastric cancer

Ref.	Year	Method	Molecular marker	Outcome
Nakanishi <i>et al</i> ^[17]	1997	RT-PCR	CEA	
Fujimura <i>et al</i> ^[46]	1998	RT-PCR	Trypsinogen	
Kodera <i>et al</i> ^[20]	2002	RT-PCR	CEA	Peritoneal recurrence, survival
Nakanishi <i>et al</i> ^[17]	2000	Q-RT-PCR	CEA	Peritoneal recurrence, survival
Yonemura <i>et al</i> ^[6]	2001	RT-PCR	MMP-7	
Sugita <i>et al</i> ^[14]	2003	Q-RT-PCR	CEA and CK-20	Peritoneal recurrence, survival
Sakakura <i>et al</i> ^[62]	2002	Q-RT-PCR	Dopa decarboxylase	
Shimomura <i>et al</i> ^[66]	2004	Q-RT-PCR	L3-PP	
Kodera <i>et al</i> ^[7]	2006	RT-PCR	CEA	Overall survival
Jeon <i>et al</i> ^[13]	2010	RT-PCR	MAGE	Overall survival
Jeon <i>et al</i> ^[71]	2014	RT-PCR	MAGE and CEA	3-yr survival

CEA: Carcinoembryonic antigen; CK-20: Cytokeratin-20; MMP-7: Matrix metalloproteinase-7; L3-PP: L-3 phosphoserine phosphatase heparanase; MAGE: Melanoma associated gene.

MMPs have key role in degrading extracellular matrix by proteolytic activity, as well as regulating other enzymes, chemokines and cell receptors^[48,49]. MMP-7, also called Matrilysin, is a distinct family member in MMPs family because its highest proteolytic activity for wide range of molecules and pivotal role in activating other MMPs for cell degradation^[50-53]. Another specific characteristic of matrilysin in contrast to other MMPs is that it is mainly expressed by tumor cells and not by stromal cells^[54-56].

Yonemura *et al*^[6] reported that MMP-7 mRNA was not expressed by fibroblasts, peripheral blood, mesothelial cells, normal gastric mucosa, or the peritoneal lavage fluid of patients with benign disease. In contrast, it was expressed by all of the examined specimens of peritoneal dissemination in gastric cancer. By combining cytology and MMP-7 RT-PCR, the sensitivity rate for the prediction of peritoneal dissemination was improved to 11% over the prediction by routine cytology.

(4) CK-20: The keratins are intermediate filament proteins responsible for the structural integrity of epithelial cells. Recently, many investigators identified CK-20 as

one of the potential cancer-related biomarkers used for detecting peritoneal free cancer cells in gastric cancer^[57].

CEA had been reported that there are strong positivity of mRNA expression in differentiated gastric carcinoma, but relatively weak or undetectable in poorly differentiated type^[57]. Thus, CK-20 has been the use as option for multiple marker assay with CEA for the detection of nodal metastasis^[58]. Also for the peritoneal recurrence, many studies reported the usefulness of multi-marker assay, employing both CEA and CK-20 RT-PCR^[14,15,59,60].

(5) Dopa decarboxylase (DDC): DDC is an enzyme for the metabolism of dopamine, and also has a role for the synthesis of important neurotransmitters including serotonin. Although DCC is investigated as cancer-related marker with its high expression in lung cancer and neuroblastoma^[61], the role of this marker in gastric cancer is still unclear.

Sakakura *et al*^[62] have analyzed in their studies for the global gene expression of a gastric cancer cell line, and approximately 21168 genes were established from the tumor and metastatic site of gastric cancer patients.

Among them, they found that 24 genes were upregulated and 17 genes downregulated, and DCC was one of these upregulated genes. DCC-specific RT-PCR is potentially a novel marker for peritoneal dissemination of gastric cancer and reliable and efficient for the prediction of peritoneal recurrence of gastric cancer.

(6) L-3 phosphoserine phosphatase heparanase (L3-PP): L3PP is one of the up-regulated genes and hundreds of protein phosphatases are known. It is an intermediary enzyme in both amino acid biosynthesis and gluconeogenesis, and is an enzyme that acts specifically in the synthesis of serine from 3-phosphoserine^[63,64]. It has been reported that activity of the enzyme L3-PP increases when cell multiplication and frequency of mitosis increases^[65].

Shimomura *et al*^[66] had reported that L3-PP over-expression in gastric cancer cells from peritoneal metastasis by cDNA microarray as well as RT-PCR suggest that overexpression of L3-PP is probably involved in peritoneal metastasis of gastric cancers. When they had used combination RT-PCR of CEA and L3-PP to reduce false-negative result of CEA mRNA, the sensitivity of peritoneal dissemination was improved from 71.4% to 85.7%.

(7) Melanoma associated gene (*MAGE*): *MAGE* has been shown to be a cancer-specific marker that suppresses apoptosis and plays an important role in carcinogenesis^[67]. *MAGE* gene expression in RT-PCR of gastric cancer is relatively higher than that of other markers^[68,69]. Although the rate of expression is different according to their subtype, expression of *MAGE-4*, *MAGE-6*, *MAGE-8*, *MAGE-9*, *MAGE-10*, and *MAGE-12* genes was very high as 82% in gastric cancer tissue^[70]. Furthermore, previous studies reported that *MAGE* showed no expression in normal gastric tissue^[69,70]. In this reason, *MAGE* has been attracted for the new target gene for predicting prognosis of gastric cancer, and expected as a marker for target therapy because of its specific expression^[69,70].

Jeon *et al*^[71] had reported, in their trial on the comparison of the 2 markers (CEA and *MAGE*) after long-term follow-up, that *MAGE* RT-PCR showed better specificity and more significant associations with peritoneal recurrence than CEA RT-PCR, and *MAGE* RT-PCR results was proved to be the most important prognostic factor for recurrence in patients with gastric cancer after curative resection.

The future

Genetic detection in peritoneal washes with gastric carcinoma can be performed only at university hospitals and large volume cancer centers. Furthermore, there is no standard for processing of peritoneal fluid and the method of genetic detection. To make it a reliable diagnostic tool for detecting free cancer cells, the process and method of genetic detection with peritoneal washes should be standardized, and the development of simple diagnostic devices and easily available kits are necessary.

Also, effective modality of treatment for the patient with a positive peritoneal molecular diagnosis should be needed. If the molecular marker for peritoneal free cancer cell is used not only for the diagnosis but also for the therapeutic modality with the development of target therapy, it might be one of useful method for the treatment of advanced gastric cancer and the prevention of peritoneal recurrence.

CONCLUSION

The methods of detecting intraperitoneal free cancer cells represent are still area of evolution. In past, conventional cytology was regarded as the only method for detecting peritoneal free cancer cells from gastric cancer. However low sensitivity of this method had been criticized, and many studies about various method with peritoneal washes were performed for better prediction of peritoneal metastasis. In present, genetic detection using RT-PCR analysis has been used for improving the detection rate. It has been suggested that these tools have better sensitivity in detecting intraperitoneal free cancer cells with better correlation to peritoneal recurrence. But it can be performed only at university hospitals and large volume cancer centers. Furthermore, there is no standard for processing of peritoneal fluid and the method of genetic detection and effective modality of treatment for the patient with a positive peritoneal molecular diagnosis. In near future, standardization of diagnostic methods, combination of markers for improving detection rate, and development of molecular marker for target therapy could provide us with being relevant in clinical decision-making, detection methods need to be accurate, reliable, cost-effective and effective modality of treatment for the patient with a positive peritoneal diagnosis.

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Is metastatic pancreatic cancer an untargetable malignancy?

Hampig Raphael Kourie, Joseph Gharos, Fadi Elkarak, Joelle Antoun, Marwan Ghosn

Hampig Raphael Kourie, Joseph Gharos, Fadi Elkarak, Joelle Antoun, Marwan Ghosn, Department of Oncology, Faculty of Medicine, Saint Joseph University, Beirut, Lebanon

Author contributions: Kourie HR and Ghosn M initiated the review; Kourie HR, Gharos J and Ghosn M performed the review, wrote and analyzed the data; Kourie HR, Gharos J, Elkarak F, Antoun J and Ghosn M reviewed the paper.

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Correspondence to: Marwan Ghosn, MD, Department of Oncology, Faculty of Medicine, Saint Joseph University, Monot St, PO Box 166830, Beirut, Lebanon. marwanghsonmd@yahoo.com
Telephone: +961-1-3226842
Fax: +961-1-1613397

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Abstract

Metastatic pancreatic cancer (MPC) is one of the most aggressive malignancies, known to be chemo-resistant and have been recently considered resistant to some targeted therapies (TT). Erlotinib combined to gemcitabine is the

only targeted therapy that showed an overall survival benefit in MPC. New targets and therapeutic approaches, based on new-TT, are actually being evaluated in MPC going from immunotherapy, epigenetics, tumor suppressor gene and oncogenes to stromal matrix regulators. We aim in this paper to present the major causes rendering MPC an untargetable malignancy and to focus on the new therapeutic modalities based on TT in MPC.

Key words: Pancreatic cancer; Tumor suppressor genes; Targeted therapies; Immunotherapy; Epigenetics

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Core tip: This paper will report on the most recent updates in the treatment of metastatic pancreatic cancer (MPC). We present the major causes rendering MPC an untargetable malignancy and we focus on the new therapeutic modalities based on targeted therapies in MPC.

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INTRODUCTION

Pancreatic cancer (PC) is one of the most aggressive and devastating solid tumors, with less than 5% of patients still alive at 5 years^[1]. Its poor prognosis is also due to the late diagnosis of PC and the absence of early detection tools or markers.

The incidence of this cancer is increasing worldwide; it represents actually the seventh most diagnosed cancer in Europe and the fifth leading cause of cancer mortality^[2]. More than 80% of these cancers are locally advanced

or metastatic at diagnosis. The only curative treatment remains surgery, possible in less than 20% of the patients with PC, diagnosed at an early stage^[1].

Adenocarcinomas represent the majority of PC, less than 5% are neuroendocrine tumors. Pancreatic neuroendocrine tumors have specific features and different treatment modalities^[3]. In this paper, we will focus only on metastatic pancreatic adenocarcinomas.

Many risk factors and inherited syndromes are incriminated in the development of this cancer. PC represents a genetically complex and heterogeneous tumor; it results from successive accumulation of gene mutations. PC tumor cells harbor more than 60 genetic alterations that affect more than twelve signaling pathways^[4].

According to the national cancer institute dictionary, targeted therapies (TT) are defined as a type of treatment that uses drugs or other substances to identify and attack specific types of cancer cells with less harm to normal cells^[5]. Till the end of the last century, hormonal therapies were considered one of the rare routinely used drugs respecting the definition of TT in oncology. After hormonal therapies, monoclonal antibodies and tyrosine kinase inhibitors (TKIs) were widely being tested and approved in the management of different cancers during the last 15 years.

TT can be divided into two major groups: Therapies targeting the cancer cell itself and therapies targeting the tumor microenvironment. The first group includes hormonal therapies, small molecules (TKIs) and monoclonal antibodies; antibody drug conjugates and oncolytic viruses were recently added to this category. The second group includes mainly angiogenesis inhibitors and immunotherapy.

In the emerging era of TT, this new concept was widely introduced in the management of different tumors; however, PC remains resistant to available TT. Is metastatic pancreatic cancer (MPC) an untreatable malignancy? This review focuses on the major clinical trials evaluating TT in MPC and the potential new targets and new approaches in MPC.

APPROVED TREATMENTS IN MPC

Before Gemcitabine era in PC, 5-FU was the most frequently used chemotherapy in the treatment of MPC. In 1997, gemcitabine, as monotherapy, was approved by the Food and Drug Administration (FDA) as the first line treatment in MPC after a trial comparing gemcitabine to 5-FU in untreated MPC; less than two months of difference in overall survival were good enough for the approval of gemcitabine as a first line therapy in MPC^[6]. During 14 years, two new combinations associating gemcitabine with either erlotinib or cisplatin were considered potential regimens in the treatment of MPC. The first was approved based on a minimal overall survival benefit when added to gemcitabine compared to gemcitabine alone, knowing that this regimen is only beneficial to the subgroup of patients that develop rash^[7], while the approval of the second regimen was based on

two meta-analyses^[8,9].

The turning point in the natural history of MPC was in 2011 with the approval of the FOLFIRINOX regimen. FOLFIRINOX was the first chemotherapeutic regimen that surpasses the barrier of one year of survival in MPC. It became the standard of care in patients having MPC with good performance status. The major side effects of this regimen are neutropenia, fatigue diarrhea and sensory neuropathy^[10]. Another regimen, associating gemcitabine with nab-paclitaxel, showed a better overall survival when compared to gemcitabine alone, and was therefore approved in the treatment of MPC. Its toxicity profile was different compared to FOLFIRINOX: Less febrile neutropenia and fatigue and more peripheral neuropathy^[11].

TT IN MPC

After the approval of gemcitabine as standard of care in MPC in 1997 and the emergence of TT during the same period, many trials tried to associate gemcitabine to one of the TT. All these trials had negative results; only Gemcitabine-erlotinib combination demonstrates statistically significantly improved survival in advanced PC^[7]. Although the practical implication and benefit of the use of this drug remains debatable, erlotinib is currently the only FDA approved targeted therapy drug for pancreatic adenocarcinoma.

The results phase III trials associating TT to gemcitabine were disappointing. Thus, cetuximab^[12], bevacizumab^[13], aflibercept^[14], axitinib^[15], sorafenib^[16] and ganitumab^[17] failed to show any benefit when added to gemcitabine and compared to gemcitabine alone in the treatment of MPC. The results of these studies (PFS-OS) and mechanisms of action of TT are summarized in the Table 1.

Associating two TT with gemcitabine also failed to show any positive results, the association of erlotinib and bevacizumab to gemcitabine was not superior to erlotinib-gemcitabine^[18]. Another phase I trial evaluated the combination of gemcitabine-erlotinib-cixitumumab, but without any promising results^[19]. Associating two chemotherapies (gemcitabine and capecitabine) and two TT (bevacizumab and erlotinib) showed promising results with an overall survival exceeding one year in a phase I / II trial^[20]. Clinical trials in MPC associating two TT to chemotherapeutic agents are summarized in the Table 2. Many TT are tested as single agent in second line treatment of MPC; selumetinib (anti-MEK) and everolimus (mTOR inhibitor) are two examples^[21,22]. Combining two TT without chemotherapy is also being tested actually in new ongoing trial in a second line treatment of MPC with afatinib and selumetinib compared to capecitabine (NCT02450656) and sorafenib plus everolimus (NCT 00981162).

MECHANISMS OF RESISTANCE OF PC TO CHEMOTHERAPY AND TT

PC was known to be resistant to chemotherapy before

Table 1 Phase III trials evaluating gemcitabine with a targeted therapy in advanced or metastatic pancreatic cancer

Ref.	Regimen	Mechanism of action	PFS	OS
Moore <i>et al</i> ^[7]	Gemcitabine/erlotinib	Anti-EGFR	3.7	6.2
	Gemcitabine		3.5	5.9
Philip <i>et al</i> ^[12]	Gemcitabine/cetuximab	Anti-EGFR	3.4	6.3
	Gemcitabine		3.0	5.9
Fuchs <i>et al</i> ^[17]	Gemcitabine/ganitumab	Anti-IGF1R	3.7	7.2
	Gemcitabine		3.6	7.0
Ioka <i>et al</i> ^[15]	Gemcitabine/axitinib	TKI	NA	5.1
	Gemcitabine		NA	5.4
Gonçalves <i>et al</i> ^[16]	Gemcitabine/sorafenib	TKI	5.7	9.2
	Gemcitabine		3.8	8.0
Kindler <i>et al</i> ^[13]	Gemcitabine/bevacizumab	Anti-VEGF	3.8	5.8
	Gemcitabine		2.9	5.9
Rougier <i>et al</i> ^[14]	Gemcitabine/aflibercept	Anti-VEGF	3.7	6.5
	Gemcitabine		3.7	7.8

PFS: Progression free survival; OS: Overall survival; EGFR: Epidermal growth factor receptor; IGF1R: Insulin-like growth factor receptor 1; TKI: Tyrosine kinase inhibitor; VEGF: Vascular endothelial growth factor; NA: Not available.

the era of FOLFIRINOX; many hypotheses tried to elucidate this chemo-resistance. Thus, alterations in key pathways involved in cell cycle control, namely apoptosis, were largely incriminated. NF κ B, pro-inflammatory and anti-apoptotic factor, seemed to be a key link between inflammation and cancer chemo-resistance in PC. The genetic complexity and heterogeneity of PC is also a challenge in the treatment of this cancer, since more than 60 genetic alterations affecting more than twelve signaling pathways are involved in its pathogenesis. The most commonly affected signaling pathways in PC are apoptosis, DNA damage repair, G1/S transition (CDKN2A/p16, CyclinD), cell-cell adhesion, regulation of invasion, embryonic signaling (Notch pathway, Hedgehog pathway and Wnt pathway) and MAPK signaling [c-Jun N-terminal kinase, ERK and transforming growth factor beta (TGF- β) signaling]^[23].

Actually, recent data revealed that tumor stroma is a major extrinsic mechanism of resistance to chemotherapy and targeted therapy of PC. The tumor stroma of PC is limiting the drug delivery to pancreatic tumor cells at therapeutically relevant concentrations^[24].

Next to intrinsic resistance to TT due to the multitude of pathways incriminated in the pathogenesis of PC, extrinsic resistance seems to be as important in the mechanism of non-response to TT. This extrinsic resistance seems to be particular to PC, rendering it a hardly targetable malignancy.

NEW TREATMENT APPROACHES AND TARGETS IN MPC

PC represents one of the least targetable malignancies with the present available drugs. Despite the huge efforts in research and promising results in some phase I and II trials, TT have not been approved yet in this

Table 2 Clinical trials in metastatic pancreatic cancer associating two targeted therapies to chemotherapeutical agents

Ref.	Regimen	Phase	PFS	OS
Van Cutsem <i>et al</i> ^[18]	Gemcitabine/erlotinib/ bevacizumab	III	4.6 3.6	7.1 6.0
Philip <i>et al</i> ^[19]	Gemcitabine/erlotinib/ cixutumumab	I	3.6 3.6	7.0 6.7
Watkins <i>et al</i> ^[20]	Gemcitabine/capecitabine/ erlotinib/bevacizumab	I / II	8.4	12.6
NCT02450656 (ongoing trial)	Afatinib/selumetinib Capecitabine	II	NA	NA

PFS: Progression free survival; OS: Overall survival; NA: Not available.

indication, neither influenced the natural history and the evolution of PC. New approaches and modalities are necessary to counteract the resistance to treatment of this malignancy, from immunotherapy and epigenetics to oncogenes and tumor suppressor genes regulation (Table 3).

Immunotherapy

Several immunotherapy approaches for PC have shown promising results in early clinical trials. Checkpoint inhibitors represent actually the most expanding and booming novel therapeutic approach in oncology; many new agents were recently approved in melanomas^[25-27] and lung cancer^[28]. Adoptive T cell transfers are also being largely evaluated and studied with encouraging results in some cancers including PC.

Many trials are testing different checkpoint inhibitors in advanced solid tumors including MPC. Check point inhibitors are new immunologic agents, that block inhibitory receptors of immune system elements, such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), programmed cell death protein -1 (PD-1) and its ligand PD-L1, leading to the activation of tumor-specific T cells with effector function against tumor cells^[29]. An ongoing phase I trial is evaluating a checkpoint inhibitor exclusively in advanced PC by combining gemcitabine and ipilimumab (anti-CTLA4).

Adoptive cell therapy is a new therapeutic approach based on the modification and selection of autologous T cells *in vitro* from the patient's tumor in order to have more potent and efficient T-cells, either by collecting tumor infiltrating lymphocytes (TILs) or by developing genetically engineered cells. These modified and selected T cells are re-infused to the patient. Two different mechanisms are implicated in the concretization of the concept of genetically engineered cells, the first by modifying genetically a T-cell receptor for cancer antigen (transgenic TCR) and the second by adding a chimeric antigen receptor (CAR), which recognizes a specific cancer antigen^[30]. Two specific phase I / II trials for PC are adopting this new approach. The first one is a phase I clinical trial exploring the potential of CAR T cells modified to recognize mesothelin, which is expressed in all PCs but not in healthy pancreatic cells (NCT01897415,

Table 3 New treatment modalities based on targeted therapies in metastatic pancreatic cancer

Treatment modality	Mechanism of action	Target
Immunotherapy	Check point inhibitors	CTLA4
	Adoptive cell therapy	T-cells
Epigenetics	Histone acetylation	Histones
Stromal	Hyaluronidase	Hyaluronan
extracellular matrix		
Tumor suppressor genes regulation	miRNA inhibitors	TP53-SMAD4- CDKN2A
	Anti-PARP	BRCA1-BRCA2

CTLA4: Cytotoxic T-lymphocyte-associated antigen 4; TP53-SMAD4-CDKN2A: Tumor protein p53 - cyclin-dependent kinase inhibitor 2A; BRCA: Breast cancer; PARP: PolyADP ribose polymerase.

NCT02159716). The second one is a phase I /II trial also testing CAR T cells modified to recognize mesothelin in patients with PC at the National Cancer Institute (NCT01583686). Many other trials including patients with solid tumors (including PC) are evaluating the effect of reinfusing TILs (NCT01174121) and genetically reengineered T cells to target the NY-ESO-1 antigen in patients with NY-ESO-1-positive cancers (NCT01967823).

Toll-like receptors (TLRs) recognize distinct pathogen-associated molecular patterns and play a critical role in innate immune responses. They participate in the first line of defense against invading pathogens and play a significant role in inflammation, immune cell regulation, survival, and proliferation. Likewise, TLRs can start immunological reactions against endogenous molecules released into the extracellular compartment under due to stress or tissue damage^[31]. TLRs are expressed in the PC tissue, whereas they are not expressed in the normal pancreas^[32,33]. Thus, they appear to play a role in the pathophysiology of PC and may thereby also represent targets for intervention. Many ongoing phase I /II trials combining TLRs with chemotherapy or radiation therapy are being evaluated in breast cancer, sarcomas and melanomas are being evaluated and some preclinical trials in PC showed promising results^[34].

Epigenetics

Epigenetic modifications are independent from the changes in the DNA sequence. They are due to variations in DNA methylation and histone acetylation. Many new drugs, targeting methylation of DNA and acetylation of histones have been mainly approved in hematology. This new approach is also being evaluated in PC. Ongoing phase I and II trials in PC are associating vorinostat, gemcitabine, bortezomib (anti-proteasome) and radiation therapy (Clinical Trial.gov. NCT00983268, NCT00243100).

Targeting tumor suppressor genes and oncogenes

As mentioned before, PC is caused by multiple genetic sequential changes. This subtype of cancer is associated also with chromosomal instability, since more than 90% of PC is aneuploidy: Presence of losses and gains of large portions of chromosomes or whole chromosomes

leading to abnormal karyotype. The accumulation of genetic instability mainly higher number of mutations of oncogenes and tumor suppressor genes is a part of the early event of the development of PC. More than 80% of ductal PCs exhibit KRAS mutations. Furthermore, 90% of the tumors exhibit deletions, mutations or epigenetic alterations in the *CDKN2A* gene. Nearly 50% have mutations in the tumor suppressor p53 and also approximately 50% exhibit mutations or homozygous deletions in the *SMAD4* gene^[2]. Ten percent of sporadic PCs present one of these mutations present BRCA1 or BRCA2 mutations^[35].

MicroRNAs, single-stranded chains of non-coding RNA of small number of nucleotides, that inhibit gene expression at the post-transcriptional level, regulate CDK2NA, TP53 and SMAD4 tumor suppressor genes^[36]. For example, CDK2NA or p16 inhibits cycline -dependant kinase 1, 4 and 6 and also help to stabilize p53; CDKN2A itself is regulated by a microRNA, miR-10b^[37]. TGFβ is a potent tumor suppressor that signals *via* the SMAD pathway and intersects with the WNT beta-catenine signaling pathway; it regulates the cell cycle by inhibiting cyclin-dependant kinases, E2F and histone deacetylase during the G1 phase of the cell cycle. TGFβ itself is regulated by different miRNA including mi-RNA 15/16 and mi-RNA 224^[38,39].

Some of these microRNAs inhibiting these tumor suppressor genes are in relation with the resistance to chemotherapy (gemcitabine), the poor prognosis of PC and the higher potential to rapid progression and metastasis development; inhibiting these microRNAs can be a promising strategy in TT. Other microRNAs are in relation with radio-resistance of PC; in a recent study evaluating a radio-resistant pancreatic cell line, miRNA-216a was significantly down regulated, whereas the autophagy activity was controlled. Using bioinformatics analysis, it was concluded that forced expression of microRNA-216a enhances the radio-sensitivity of pancreatic cells by inhibiting beclin-1 mediated autophagy^[40].

BRCA1 and *BRCA2* are two genes implicated in the DNA repair process. A mutation in one of these genes can cause breast, ovarian, and PCs. Recently another gene of the same family, the *PALB2*, was incriminated in the development of PC^[41]. Many studies are evaluating anti-PARP drugs in patients carrying BRCA1 and BRCA2 diagnosed with breast and ovarian cancers with promising results. Anti-PARP drugs cause multiple double strand breaks in the DNA and in tumors carrying one of these three mutations, these DNA breaks cannot be efficiently repaired, leading to the death of the cells. The FDA has recently approved Olaparib (anti-PARP) in BRCA-mutated ovarian cancers^[42]. Thus, the presence of one of these mutations can be a predictive biomarker of the use of these new drugs alone or in combination with gemcitabine in patients with PC. Many ongoing trials are studying this treatment options. NCT00515866 trial is evaluating the safety and tolerability of a PARP inhibitor in combination with gemcitabine in PC. Another randomized, phase II trial (NCT01585805) is testing the veliparib (anti-PARP) in

combination with gemcitabine hydrochloride and cisplatin compared to gemcitabine hydrochloride and cisplatin alone in patients with pancreatic adenocarcinoma having a known BRCA/PALB2 mutation.

Targeting stromal extracellular matrix

One of the important reasons of resistance to treatment in PC is the difficulty to deliver drug to tumor cells, because of the extensive deposition of extracellular matrix components and low vascularization of tumor environment. Targeting stromal extracellular matrix components seems to be an interesting approach to counteract this mechanism of resistance. Many trials targeting matrix metalloproteinases failed to show any benefit^[43,44], actually new targets are being tested using Hyaluronidase next to gemcitabine with promising results^[45]. Hyaluronidase acts by depleting pancreatic tumors of their high hyaluronan content in preclinical trials; hyaluronan being a glycosaminoglycan, one of the major components of extracellular matrix throughout the pancreatic tumor.

Targeting oxidative stress, chronic inflammation and targeting programmed cell death pathway

Oxidative stress has been shown to participate in the process of PC. Evidences supporting the role of reactive oxygen species and cytokines, as factors in the development of PC have been proposed. The concept of antioxidant supplementation as a preventive approach for PC has been evaluated^[46]. Curcumin, resveratrol, and genistein have antioxidant activities and demonstrated anti-cancer effects against PC *in vitro* and *in vivo* experiments^[47-49].

Chronic inflammation seems also to be incriminated in the development of PC. Many studies showed an increased incidence of pancreatic in patients with chronic pancreatitis. Prolonged inflammation may precede the onset of frank malignancy by a significant interval, and that once malignancy is established, the resulting inflammation occurred may act as a continued driving force in accelerating further malignant change. Targeting this chronic inflammation may prevent or postpone the process of PC. NFκB, cyclooxygenase 2 (COX2), lipoxygenase and inducible nitric oxid (NO) are the main targetable components of the chronic inflammation in PC^[50]. A study of 28283 participants, over a 7-year period, found that women who took regular aspirin had a 43% lower risk of PC than women who did not use aspirin^[51].

PC is characterized by an important resistance to apoptosis, which is associated by high expression levels of multiple prosurvival proteins of the extrinsic and intrinsic apoptosis signaling cascades and/or reduced expression or function of pro-apoptotic proteins. Many components of programmed cell death signaling pathways are being studied as targets for cancer therapies, for example the TRAIL system, IAP proteins or anti-apoptotic Bcl-2 proteins^[52].

DISCUSSION

The molecular and genetic complexity of MPC is one

of the major barriers causing the failure of TT in this indication; more than 60 genetic alterations that affect more than twelve signaling pathways are involved in the development of PC rendering this tumor resistance to TT. Inhibiting one pathway by a specific targeted therapy is not sufficient to block to the cellular proliferation and will probably induce the activation of another pathway. Many other factors make MPC difficult to treat: The aggressive molecular and cellular features, the late diagnosis and absence of tools of early detection the stromal proliferation forming a drug's barrier, the reduced vascular density and the immune suppression.

Detecting PC at an early stage remains the most rationale and solid perspective in the future management of this disease. At present, serum CA-19-9 (carbohydrate antigen 19-9) is the only FDA-approved biomarker for PDA, and it has utility marker of disease recurrence and surveillance. There has been a recent explosion in the PC biomarker field with more than 2000 biomarker studies implicating thousands of informative genes as candidate biomarkers^[53].

Many markers of early detection of PC are being evaluated in blood, pancreatic cyst fluid, pancreatic juice and stool based on the new advances in technology for whole genome, methylome, ribonucleome and proteasome interrogation. Many promising results are being reported for different markers of early detection of PC^[54]. Circulating tumor cells are one of the promising markers in blood used to the early detection of PC; the detection of a mutation, as KRAS for example, in the cells of pancreatic juice can help the early diagnosis of the PC^[55,56]. Glycpan-1 circulating exosomes were detected in the serum of patients with PC with absolute specificity and sensitivity; this new diagnostic and screening marker may serve as a potential non-invasive tool to detect early stages of PC and consequently, to facilitate possible curative surgical therapy^[57]. Recently, a new non-invasive urinary biomarker, based on a set of three urinary proteins (LYVE-1, REG1A, and TFF1) was identified, able to distinguish patients with early-stage PDAC from healthy individuals^[58].

To conclude, associating many TT, based on well-defined molecular biomarkers leading to a specific profile, can surpass some of those obstacles aiming to offer the best treatment to the appropriate patient. Combining different approaches in the same patient according to his molecular profile can be the best treatment option.

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Basic Study

Expression of p-STAT3 and vascular endothelial growth factor in MNNG-induced precancerous lesions and gastric tumors in rats

Xiao-Yan Wang, Lou-Lei Wang, Xuan Zheng, Li-Na Meng, Bin Lyu, Hai-Feng Jin

Xiao-Yan Wang, Lou-Lei Wang, the First Affiliated Hospital of Zhejiang University, Hangzhou 310003, Zhejiang Province, China

Xuan Zheng, Li-Na Meng, Bin Lyu, Hai-Feng Jin, the First Affiliated Hospital of Zhejiang Chinese Medicine University, Hangzhou 310006, Zhejiang Province, China

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Correspondence to: Hai-Feng Jin, MD, Resident Physician of Gastroenterology, the First Affiliated Hospital of Zhejiang Chinese Medicine University, Youdian Rd. 54, Hangzhou 310006, Zhejiang Province, China. jinhaifeng0908@163.com
Telephone: +86-571-87077785
Fax: +86-571-87077785

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Abstract

AIM: To investigate the dynamic expression of p-signal transducer and activator of transcription 3 (STAT3) and vascular endothelial growth factor (VEGF) in the formation of gastric tumors induced by drinking water containing N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in Wistar rats.

METHODS: One hundred and twenty Wistar rats were randomly divided into two groups (60 in each group): Control group and Model group. The rats in each group were then randomly divided into three groups (20 in each group): C/M15, C/M25 and C/M40 (15, 25 and 40 represent the number of feeding weeks from termination). Rats in the control group received normal drinking water and rats in the model group received drinking water containing 100 µg/mL MNNG. Stomach tissues were collected at the end of the 15th, 25th and 40th week, respectively, for microscopic measurement using hematoxylin and eosin staining. The expression of p-STAT3 and VEGF in different pathological types of gastric tissue, including normal, inflammation, atrophy, hyperplasia and gastric stromal tumor, was observed by immunohistochemistry and Western blot, and the correlation between p-STAT3 and VEGF was analyzed.

RESULTS: (1) The expression of p-STAT3 in tissue with gastritis, atrophy, dysplasia and gastric stromal tumor were significantly increased in the model group compared with the control group (2.5 ± 1.0 , $2.75 \pm$

0.36, 6.2 ± 0.45 , 5.67 ± 0.55 vs 0.75 ± 0.36 , $P = 0.026$, 0.035, 0.001, 0.002, respectively); the expression of p-STAT3 in tissue with dysplasia was higher than that in samples with gastritis or atrophy (6.2 ± 0.45 vs 2.5 ± 1.0 , $P = 0.006$; 6.2 ± 0.45 vs 2.75 ± 0.36 , $P = 0.005$, respectively); however, the expression of p-STAT3 in gastritis and atrophy was not significantly different ($P > 0.05$); (2) the expression of VEGF in tissue with gastritis, atrophy, dysplasia and gastric stromal tumor was significantly increased in the model group compared with normal gastric mucosa; and the expression of VEGF in tissue with dysplasia was higher than that in tissue with inflammation and atrophy (10.8 ± 1.96 vs 7.62 ± 0.25 , $P = 0.029$; 10.8 ± 1.96 vs 6.26 ± 0.76 , $P = 0.033$, respectively); similarly, the expression of VEGF in tissue with gastritis and atrophy was not significantly different ($P > 0.05$); and (3) the expression of VEGF was positively correlated with p-STAT3.

CONCLUSION: p-STAT3 plays an important role in gastric cancer formation by regulating the expression of VEGF to promote the progression of gastric tumor from gastritis.

Key words: Wistar rat; Precancerous gastric lesions; Gastric tumor; Vascular endothelial growth factor; p-signal transducer and activator of transcription 3; N-methyl-N'-nitro-N-nitrosoguanidine

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Core tip: The results show that signal transducer and activator of transcription 3 (STAT3) is partially responsible for the progression from chronic gastritis to gastric carcinoma induced by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and is significantly related to the expression of vascular endothelial growth factor (VEGF) during this process. It is considered that STAT3 induces an abnormal level of VEGF expression to promote the formation of gastric carcinoma. To the best of our knowledge, this is the first report to show that p-STAT3 is persistently activated during the progression of chronic gastritis to gastritis carcinoma induced by the administration of MNNG in rats, and was positively associated with the expression of VEGF.

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INTRODUCTION

Gastric cancer is the second leading cause of cancer-related death worldwide, its prevalence rate and mortality are very high, especially in some developing

countries, and the high incidence of gastric cancer is a major public health problem worldwide^[1]. The formation and development of gastric cancer are complex and are associated with a multifactorial etiology^[2]. *Helicobacter pylori*, high-salt diet, smoking, obesity and Epstein-Barr virus are factors which increase gastric cancer risk^[3-7]. In addition, environmental factors significantly contribute to the etiology of cancer^[2]. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) is commonly used to induce gastric tumors in basic research^[8,9], therefore we chose MNNG to induce gastric precancerous lesions and gastric tumors in this study. Consistent with other tumors, the pathogenesis of gastric tumors is still unclear.

Signal transducer and activator of transcription 3 (STAT3), a key transcription factor^[10] was shown to play a vital role in human gastric cancer angiogenesis^[11]. It was reported that activation of STAT3 and the microbial environment were important for gastric tumor initiation and development in the gp130 (757F/F) mouse^[11], and it is believed by some investigators that STAT is directly involved in gastric tumorigenesis^[12].

Vascular endothelial growth factor (VEGF) plays a very critical role in cancer metastasis due to its function in the generation of vascular tissue^[13,14]. Recently, VEGF was reported to be closely associated with gastric cancer, which may provide additional prognostic information for the preoperative evaluation of gastric cancer invasion and tumor type^[15], thus anti-VEGF may be a targeted therapy for the treatment of gastric cancer^[16]. Several researchers demonstrated that the expression of STAT3 was potentially associated with the expression of VEGF-C in various malignant diseases^[17,18], and Judd *et al.*^[11] reported that constitutive STAT3 activation promoted VEGF expression and stimulated tumor angiogenesis. However, to our knowledge, the roles of STAT3 and VEGF in the formation of gastric precancerous lesions and gastric cancer induced by MNNG are still unknown.

The aim of this study was to investigate the dynamic expression of p-STAT3 and VEGF as well as the relationship between them in the formation of gastric tumors from gastritis induced by MNNG in male Wistar rats.

MATERIALS AND METHODS

Animal preparation

One hundred and twenty male Sprague-Dawley (SD) rats aged 4 wk and weighing 125-150 g were provided by the Animal Center of Zhejiang Chinese Medical University. The animals were housed in groups of five per cage under controlled illumination (12:12 h light/dark cycle, lights on/off: 6 h/18 h), humidity (60%) and temperature ($22^{\circ}\text{C} \pm 2^{\circ}\text{C}$) for one week to acclimatize to the environment. The animals were then randomly divided into two groups (60 in each group): Control group (the rats were able to eat regular food and drinking water) and the model group (the rats were able to eat regular food and drinking water containing 100

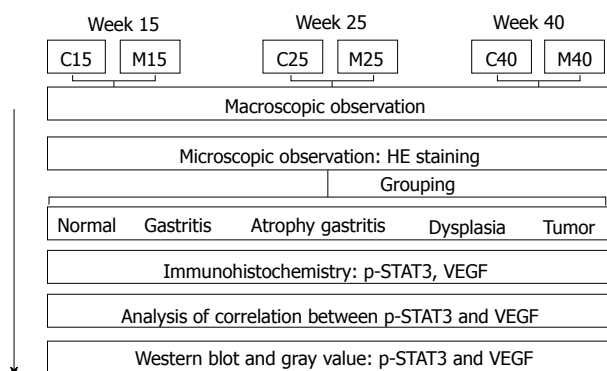


Figure 1 The experimental protocol. VEGF: Vascular endothelial growth factor; STAT3: Signal transducer and activator of transcription 3.

μg/mL MNNG); each group was then randomly divided into three groups (20 rats in each group): C/M15, C/M25 and C/M40 (15, 25 and 40 represent the number of feeding weeks). The Animal Care and Use Committee of the Zhejiang Chinese Medical University approved the protocol.

Experimental protocols

Rats were fed normal food and drinking water or drinking water with MNNG. Rats in C/M15 were sacrificed at the end of the 15th week in both the control group (C15) and the model group (M15) and the gastric tissue was collected immediately and stored at -80 °C or was perfused with cold PBS on ice and then placed in a tube with 4% paraformaldehyde to obtain various measurements as follows. Similarly, rats in C/M25 or C/M40 were sacrificed at the end of the 25th week or 40th week and gastric tissue was collected. The experimental protocol is described in Figure 1.

Measurement of morphological changes

The rats were killed at the end of the 15th week, 25th week and 40th week respectively. The stomachs were separated immediately and were opened along the long axis on ice to record the following conditions: Edema, hyperemia, erosion, ulcer or mass, and photographs were taken by two pathologists who were unaware of the treatment groups. The stomach tissue was then divided into two parts, one was stored at -80 °C and the other was placed in tubes with 4% paraformaldehyde (PFA).

Histology measurements

Gastric tissue was perfused with PBS followed by 4% PFA in 0.1 mol/L phosphate buffer and cryoprotected in 30% sucrose in PBS overnight at 4 °C. Samples were embedded in paraffin wax, and 5 μm specimens were stained with hematoxylin and eosin. The results of histology were divided into four types according to the following histological features: Chronic superficial gastritis, chronic atrophic gastritis, dysplasia and tumor.

Gastritis was defined as inflammation of the stomach lining associated with mucosal injury. Atrophic gastritis was characterized by chronic inflammatory processes

of gastric mucosa leading to the loss of appropriate glands^[19] and gastric dysplasia was diagnosed according to the classification of gastrointestinal tract epithelial neoplasia by the World Congress of Gastroenterology in Vienna in 1998^[20]; gastric tumor was diagnosed by the presence of tumor cells.

Tissue collection and immunohistochemical processing

The expression of p-STAT3 and VEGF in gastric mucosa in the different groups was evaluated using immunohistochemistry. Specimens were cut into 1-cm blocks transversely oriented to the hippocampal long axis. Blocks were placed in buffered PFA (Sigma, St Louis, MO, United States). After 48 h, the specimens were paraffin-embedded for immunohistochemistry as described by Jung *et al.*^[21] and Meng *et al.*^[22]. Briefly, endogenous peroxidase activity was blocked using 3% H₂O₂ in methanol for 15 min. The sections were washed, and stained with a rabbit polyclonal antibody against p-STAT3 and VEGF (Santa Cruz Biotechnology, TX, United States) diluted 1:50. Primary antibody binding was detected using the Bond Polymer Refine Detection Kit (Leica, Wetzlar, Germany). The sections were counterstained with hematoxylin, dehydrated and mounted.

Evaluation of immunohistochemical staining

Scoring of immunohistochemical results was performed according to the semi-quantitative method as described by Meng *et al.*^[22]. Briefly, the percentage of immunopositive cells was scored using a four-point system: 0 points, < 5% of positive cells; 1 point, 5%-25% of positive cells; 2 points, 26%-50% of positive cells; 3 points, 51%-75% of positive cells; 4 points, > 75% of positive cells. The staining intensity was scored similarly, with 0 points for negative staining, 1 point for weak staining (light yellow), 2 points for moderate staining (brown), and 3 points for strong staining (dark brown). The immunoreactivity score for each lesion was calculated as (the score for the percentage of immunopositive cells + the score for the staining intensity)/2. Based on the immunoreactivity scores, immunohistochemical staining was considered negative (< 0.5), weakly positive (0.5-1.5), or strongly positive (> 1.5).

Extraction of proteins for Western blot analysis

Frozen gastric tissue samples (whole layer) were homogenized in homogenization buffer (50 mmol/L Tris-HCl, pH 7.2) containing Na₃VO₄ and a protease inhibitor cocktail (Sigma-Aldrich) using an OmniTH homogenizer (Omni International, Marietta, GA, United States). After sonication, the homogenate was centrifuged at 2000 rpm for 5 min. The resulting supernatants were collected as total proteins and protein concentrations were measured using the Bio-Rad Protein Assay (Bio-Rad Inc., Hercules, CA, United States)^[23].

SDS-PAGE and Western blot analysis

Western blots were performed as previously described by Li *et al.*^[24] with minor modifications. Briefly, the proteins were separated on 12.5% SDS polyacrylamide gels and

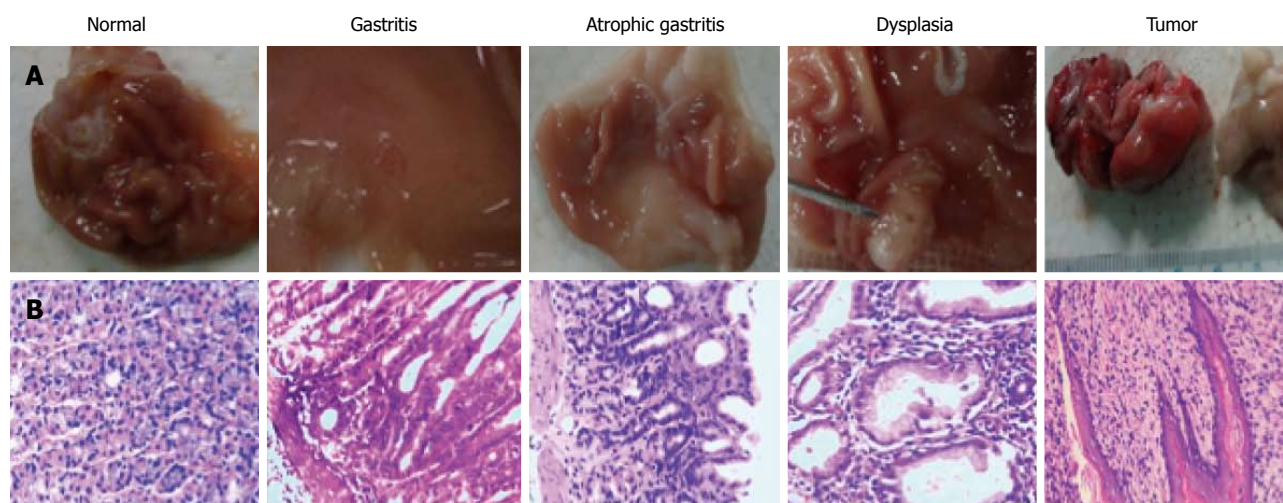


Figure 2 Administration of N-methyl-N'-nitro-N-nitrosoguanidine induced macroscopic changes: Gastritis, atrophic gastritis, dysplasia and gastric tumor in rats (A) and respective microscopic changes were observed by hematoxylin and eosin staining (B).

blotted onto nitrocellulose membranes. These membranes were incubated in PBST-milk (0.1% Tween-20 and 5% milk), followed by primary antibodies (2 h) for p-STAT3 and VEGF and β -actin (Santa Cruz Biotechnology Inc., TX, United States). The blots were then washed with PBST 3 times, and subsequently incubated for 1 h with corresponding fluorescently labeled secondary antibodies (Rockland Immunochemicals, Inc., Gilbertsville, PA, United States) diluted in PBST-milk. The blots were washed 3 times and the fluorescence intensity of the detected protein bands was quantified using the Quantity One system (Bio-Rad Inc., Hercules, CA, United States).

Statistical analysis

Statistical analyses were performed using SigmaStat 17.0 (SPSS, Chicago, IL, United States). Data are reported as means \pm SE. The Student's *t*-test was used to analyze the difference in measurements between the ES group and sham-ES group. Analysis of variance (ANOVA) was used for multiple comparisons. A *P* value of < 0.05 was considered statistically significant.

RESULTS

Macroscopic and microscopic changes in the different groups

Administration of MNNG induced significant changes in macroscopy and microscopy in the model group compared with the control group. Little hyperemia and sporadic erosion were observed in gastric tissue at the end of the 15th week (A15 group); and ulcer, reduction in mucosal folds, gray mucosa and even some mucosal hyperplasia in addition to hyperemia and erosion were observed at the end of the 25th week (A25 group); obvious masses were observed at the end of the 40th week (A40 group) (Figure 2A). Respective changes in microscopy were observed in these treatment groups. Inflammatory cells were observed in gastritis samples in the A15 group, loss of appropriate glands in some samples in the A25 group and

A40 group and tumour cells were noted in some samples in the A40 group (Figure 2B). These changes were not observed in the control group.

Histological changes in the different groups

Only 20% of rats had gastritis (4/20) in the C15 group, while 42.1% of rats had gastritis (8/19), 10.53% of rats had atrophic gastritis (2/19) and 5.3% of rats had dysplasia (1/19) in the M15 group (Table 1). In the C25 group, 30% of rats had gastritis (6/20), 10% of rats had atrophic gastritis (2/20) and none of the rats had dysplasia or tumour; however, in the M25 group, 31.58% of rats had gastritis (6/19), 26.32% of rats had atrophic gastritis (5/19) and 15.79% of rats had dysplasia (3/19) although no rats had tumours (Table 1). At the end of the study, 16.67% of rats had gastritis (3/18) in the M40 group (vs 35% in the C40 group, 7/20), 27.78% of rats had atrophic gastritis (5/18) (vs 20% in the C40 group, 4/20) and 27.78% of rats had dysplasia (5/18) (vs 5% in the C40 group, 1/20). It was observed that 27.78% of rats had tumours (5/18) in the M40 group (vs 0% in the C40 group, 0/20) (Table 1).

Expression of p-STAT3 and VEGF in different pathological types of stomach tissues measured by immunohistochemistry

The expression of p-STAT3 and VEGF in the model groups measured by immunohistochemistry increased significantly compared with the control groups. The expression of p-STAT3 in gastric tissue was mainly in the nucleus (Figure 3A), and the immunochemical score for p-STAT3 in tissues with gastritis, tissues with atrophy and tissues with dysplasia were significantly higher than those in the control groups (all *P* values < 0.05), and the expression of p-STAT3 in tissues with dysplasia was higher than that in tissues with gastritis and tissues with atrophy ($P < 0.001$, $P < 0.001$, respectively). The expression of p-STAT3 in tissues with tumor was

Table 1 Histological findings

Group	n	Pathological type				
		Normal	Gastritis	Atrophic gastritis	Dysplasia	Tumour
A15 group						
Control	20	80.00% (16/20)	20.00% (4/20)	0	0	0
A15 group	19	42.11% (8/19)	42.11% (8/19)	10.53% (2/19)	5.26% (1/19)	0
A25 group						
Control	20	60.00% (12/20)	30.00% (6/20)	10.0% (2/20)	0	0
A25 group	19	26.32% (5/19)	31.58% (6/19)	26.32% (5/19)	15.79% (3/19)	0
A40 group						
Control	20	40.00% (8/20)	35.00% (7/20)	20.00% (4/20)	5.00% (1/20)	0
A40 group	18	0	16.67% (3/18)	27.78% (5/18)	27.78% (5/18)	27.78% (5/18)

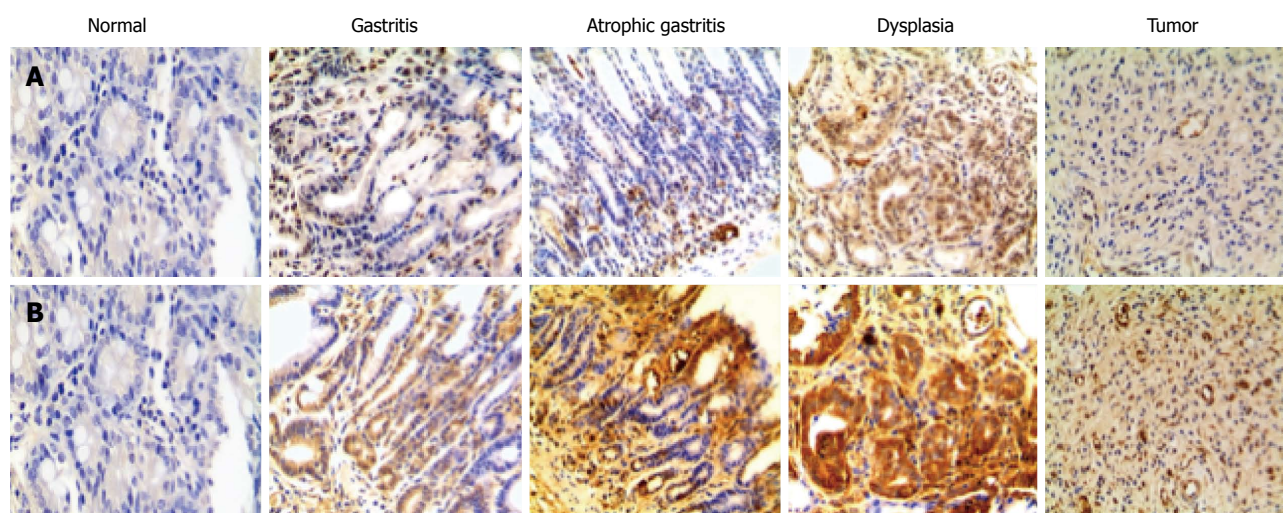


Figure 3 Expression of p-signal transducer and activator of transcription 3 and vascular endothelial growth factor in the model groups. A: The expression of p-signal transducer and activator of transcription 3 (STAT3) measured by immunohistochemistry. From a normal sample to samples with tumor induced by the administration of N-methyl-N'-nitro-N-nitrosoguanidine, the expression of p-STAT3 was more obvious; B: Similar results for the expression of vascular endothelial growth factor were observed.

substantially higher than that in tissues in the control group ($P < 0.001$); however, the difference in expression of p-STAT3 between tissues with gastritis and tissues with atrophy was not significant ($P > 0.05$) (Figure 4A).

Similarly, the expression of VEGF, mainly in the cytoplasm (Figure 3B), was higher in precancerous lesions than in the control group ($P < 0.05$) (Figure 4B). Consistent with p-STAT3, the expression of VEGF in tissue with dysplasia was higher than that in tissue with gastritis, tissue with atrophy and the control group ($P < 0.001$, $P < 0.001$, respectively); the expression of VEGF in tissue with stromal tumor was substantially higher than tissue in the control group ($P < 0.001$) (Figure 4B), however, the difference between the tissue with gastritis and tissue with atrophy was not significant ($P > 0.05$).

Expression of p-STAT3 and VEGF in different pathological types of stomach tissue measured by Western blot

To further determine whether the observed p-STAT3 and VEGF changes were consistent with the changes shown by immunohistochemistry, proteins were extracted from scraped freshly excised stomach tissue, and the samples

were analyzed by SDS-PAGE and Western-blot using primary antibodies for p-STAT3 and VEGF. As shown in Figure 5: (A) both p-STAT3 and VEGF protein expression were significantly higher in tissue with gastritis, atrophy, dysplasia or tumor than in the control group ($P < 0.05$); (B) both p-STAT3 and VEGF protein expression were significantly higher in tissue with dysplasia or tumor compared with that in tissue with gastritis or atrophy ($P < 0.05$); and (C) there was no significant difference in p-STAT3 and VEGF protein expression between tissue with dysplasia and tumor ($P > 0.05$).

The relationship between the expression of p-STAT3 and VEGF

A strong correlation was observed between the expression of p-STAT3 and VEGF both in the immunohistochemistry and Western Blot results. In order to further examine the relationship between the expression of p-STAT3 and VEGF, we performed a relativity analysis and found that there was a strong positive correlation in the immunohistochemical score between the expression of p-STAT3 and VEGF ($R^2 = 0.86$, $P < 0.001$) as well as protein expression measured by Western Blot ($R^2 = 0.90$,

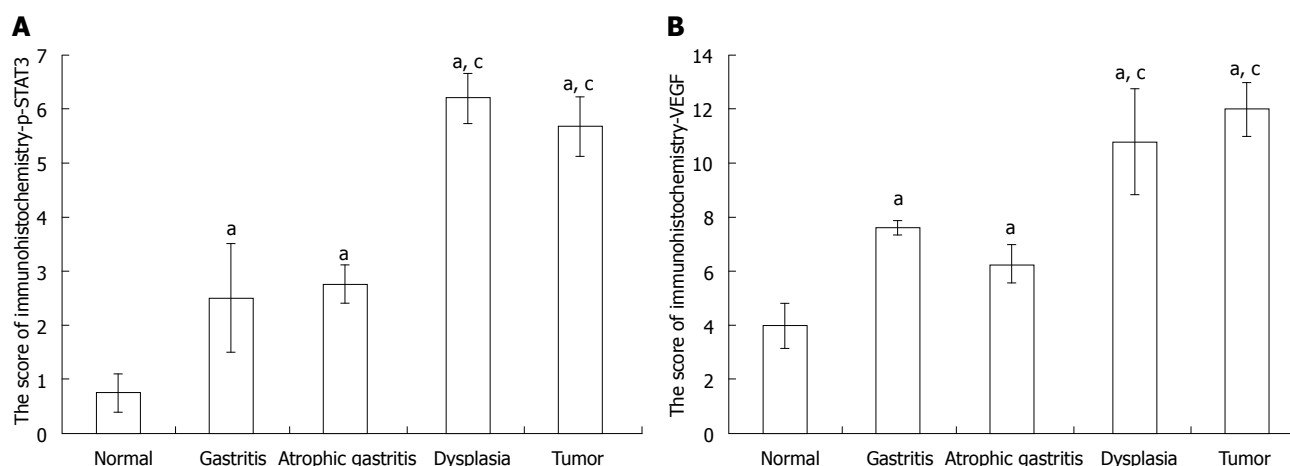


Figure 4 Immunohistochemical score of p-signal transducer and activator of transcription 3 and vascular endothelial growth factor. A: The immunohistochemical score of p-signal transducer and activator of transcription 3 (STAT3) was significantly increased by N-methyl-N'-nitro-N-nitrosoguanidine during the progression from gastritis to gastric tumor. The p-STAT3 score in precancerous lesions and tumor samples was significantly higher than that in the normal sample; and the p-STAT3 score in dysplasia samples was significantly higher than that in gastritis and atrophic gastritis, which was comparable to that in tumor samples; B: Similar results were observed for the vascular endothelial growth factor score (^a $P < 0.05$ vs normal group; ^c $P < 0.05$ vs gastritis or atrophic gastritis).

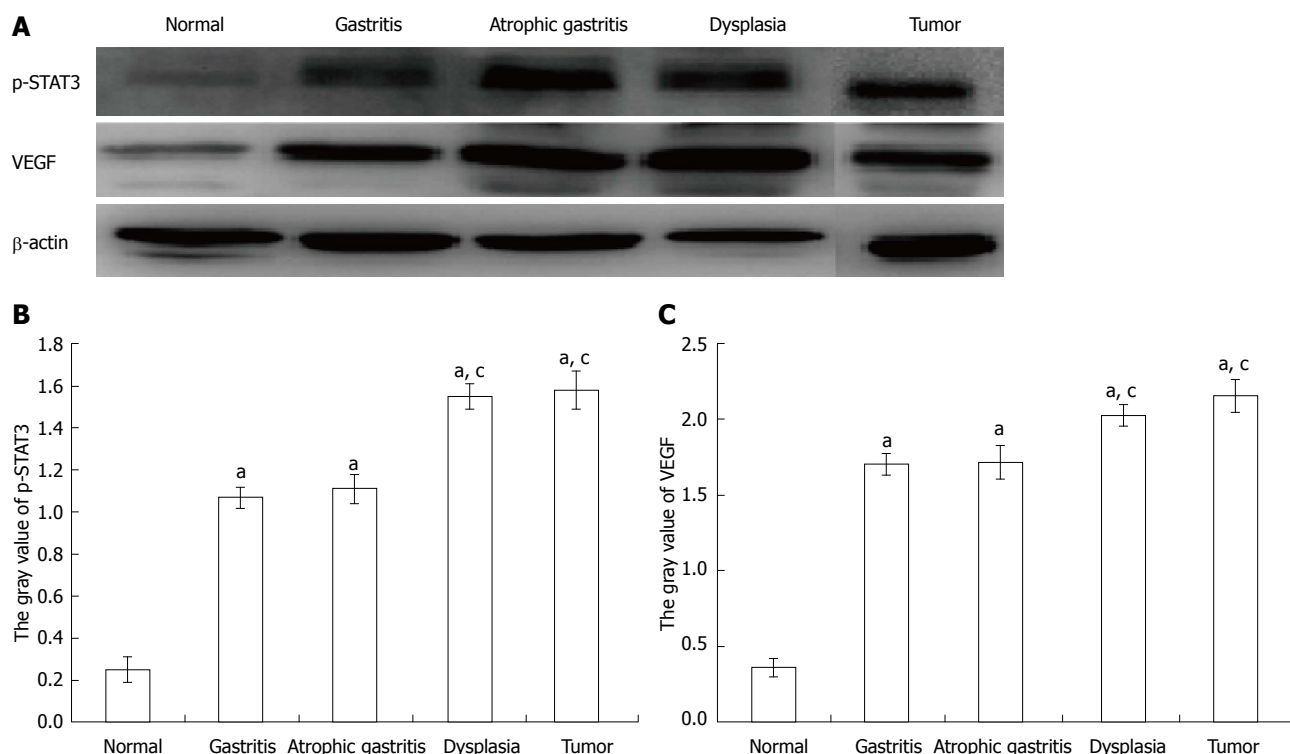


Figure 5 Protein expression of p-signal transducer and activator of transcription 3 and vascular endothelial growth factor. A: The protein expression of p-signal transducer and activator of transcription (STAT3) in dysplasia and tumor samples was more obvious than that in the other samples; B: The protein expression of p-STAT3 in dysplasia and tumor samples was significantly higher than that in gastritis and atrophic gastritis samples, but the difference between the dysplasia and tumor samples was not significant. The same results were observed for gastritis and atrophic gastritis samples; C: Consistent results were found for the protein expression of vascular endothelial growth factor in samples (^a $P < 0.05$ vs normal group; ^c $P < 0.05$ vs gastritis or atrophic gastritis).

$P < 0.001$), suggesting that p-STAT3 may regulate VEGF to promote the formation and development of gastric cancer (Figure 6).

DISCUSSION

In this study, we examined the significance of p-STAT3 and VEGF expression in the development of gastric

cancer from gastritis, atrophic gastritis and dysplasia induced by the administration of MNNG in rats. The results showed that STAT3 is partially responsible for the development of gastric carcinoma from chronic gastritis induced by MNNG and was significantly related to the expression of VEGF during this process. Although overexpression of p-STAT3 in primary tumor sites has been recognized as a predictor of poor survival in many

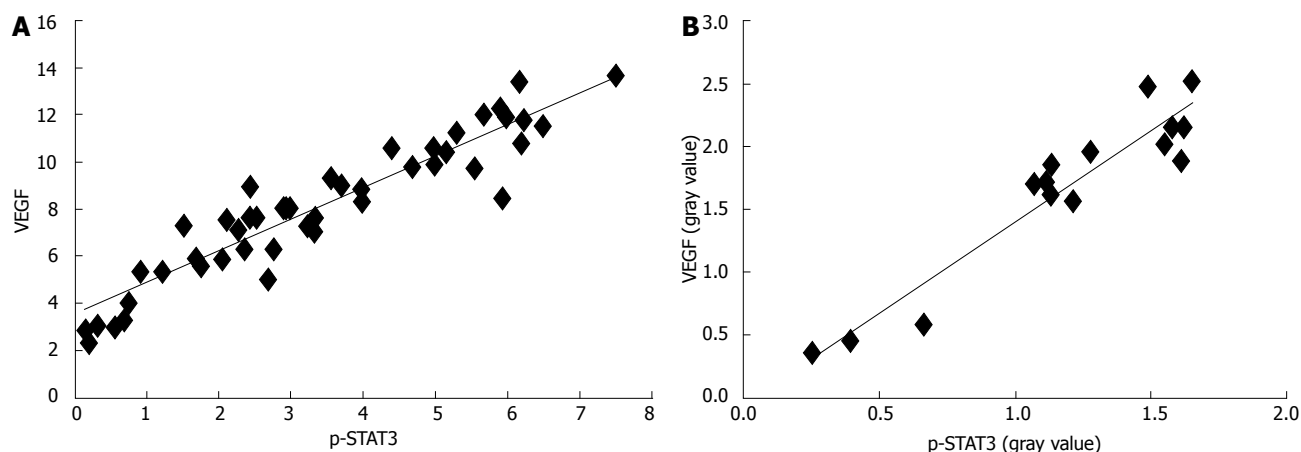


Figure 6 Correlation of immunochemical score between p-signal transducer and activator of transcription 3 and vascular endothelial growth factor. A: There was a strong correlation in immunochemical score between p-signal transducer and activator of transcription (STAT3) and vascular endothelial growth factor (VEGF). The immunochemical score of p-STAT3 was positively related to that of VEGF ($R^2 = 0.86$, $P < 0.001$); B: Similar results were observed for the protein expression of p-STAT3 and VEGF ($R^2 = 0.90$, $P < 0.001$).

malignancies, including gastric cancer^[25,26], to the best of our knowledge, this is the first report to show that p-STAT3 is also persistently activated in the progression of chronic gastritis to gastric carcinoma induced by MNNG in rats, and was positively associated with the expression of VEGF.

STAT3, plays important roles in the development of inflammation and carcinogenesis as well as tumor metastasis^[27,28]. Normally, STAT3 is activated for a short time and then is deactivated to maintain homeostasis; however, under abnormal conditions, STAT3 activation continues, which triggers oncogene transcription^[29]. Only a few studies have reported the difference in expression of p-STAT3 between normal gastric mucosa and precancerous lesions. In a clinical study^[30], it was reported that p-STAT3 expression was significantly higher in chronic atrophic gastritis (60.0%), intestinal metaplasia (77.1%), dysplasia (68%) and gastric cancer (60.1%) than in normal gastric mucosa (41.1%, $P < 0.05$). Consistent with these findings, we found that p-STAT3 expression in samples with chronic gastritis or atrophic gastritis was significantly higher than that in normal mucosa and was substantially increased in samples with dysplasia or tumor compared with normal mucosa, and even higher than that in samples of chronic gastritis or atrophic gastritis, further indicating the potential role of p-STAT3 not only in the invasion and metastasis of gastric cancer, but also in the development of gastric cancer from chronic gastritis. However, there was no significant difference in the expression of p-STAT3 between chronic gastritis and atrophic gastritis samples. Similar results were observed between dysplasia and tumor samples, indicating the important effect of p-STAT3 on the progression from atrophic gastritis to gastric carcinoma.

VEGFs are glycoproteins secreted by tumor cells and are the most important factors in angiogenesis and tumor metastasis^[31]. The VEGF family includes VEGF-A to F and placental growth factor. It has been reported

that VEGF-A and B play a key role in blood vessel growth, whereas VEGF-C and D are important for the growth of lymphatic vessels^[32,33]. VEGFs, particularly VEGF-A, C, and D promote angiogenesis and metastasis of many cancers including gastric cancer^[34].

Some clinical studies have shown the potential effects of VEGF inhibitors in the treatment of multiple cancers including gastric cancer^[35]. Raica *et al.*^[36] investigated the immunohistochemical expression of VEGF on 80 patients with intestinal-type gastric carcinoma and found that VEGF was positive in 52 of 80 cases (70%), indicating that the expression of VEGF could signify an early angiogenic switch during tumorigenesis. Consistent with this finding, we also found that the expression of VEGF in dysplasia and tumor samples was higher than that in chronic gastritis and atrophic gastritis samples. However, consistent with the expression of p-STAT3, both the difference in expression of VEGF between chronic gastritis and atrophic gastritis samples and the difference between dysplasia and tumor samples were not significant. According to these results, we analyzed the correlation between the expression of p-STAT3 and VEGF and found that the expression of VEGF was significantly and positively related to p-STAT3. To the best of our knowledge, this is the first report on the positive relationship between p-STAT3 and VEGF in precancerous lesions of gastric cancer induced by MNNG.

Some published papers support the findings in our study regarding the relationship between the expression of p-STAT3 and VEGF in gastric cancer. Deng *et al.*^[37] reported that STAT3, p-STAT3, and VEGF-D expression in gastric cancer tissue was significantly higher than those in adjacent non-tumor tissue, and both STAT3 and VEGF-D mRNA expression was much higher in many gastric cancer cell lines than in the GES-1 cell line. Using STAT3 siRNA transfection, these authors found that VEGF-D expression significantly decreased in HGC-27 cells, representing the potential STAT3 regulation of VEGF-D expression^[37]. Similar results were observed

by Zhu *et al.*^[38] who found that (-)-epigallocatechin-3-gallate inhibited IL-6-induced VEGF expression and angiogenesis by suppressing STAT3 activity in human gastric cancer cells. However, the relationship between p-STAT3 and VEGF in precancerous lesions of gastric cancer is still unclear. In the current study, we found that even in chronic gastritis and atrophic gastritis samples, the expression of both p-STAT3 and VEGF were higher than that in normal mucosa and showed a positive relationship, suggesting that VEGF may be activated on initial damage of gastric mucosa induced by MNNG via the p-STAT3 pathway and contributes to the development and progression of gastric cancer, highlighting the potential for anti-p-STAT3 as a therapeutic target for the prevention of gastric cancer.

In conclusion, STAT3 activation plays an important role in the changes in gastric mucosa from chronic gastritis to gastric tumor via the STAT/VEGF pathway, confirming the potential value of targeted therapy focusing on STAT/VEGF in protecting against gastric cancer in clinical applications.

COMMENTS

Background

Signal transducer and activator of transcription 3 (STAT3) has been shown to play a vital role in human gastric cancer angiogenesis. Vascular endothelial growth factor (VEGF) plays a very critical role in cancer metastasis due to its function in vascular formation. Several researchers have demonstrated that the expression of STAT3 is potentially associated with the expression of VEGF-C in various malignant diseases; Judd *et al* reported that constitutive STAT3 activation promoted VEGF expression and stimulated tumor angiogenesis. However, to the best of our knowledge, the role of STAT3 and VEGF in the formation of gastric precancerous lesions and gastric cancer induced by "N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) is still unknown.

Research frontiers

The effects of STAT3 and VEGF on gastric cancer have been reported. The present study showed that STAT3 activation plays an important role in the changes in gastric mucosa from chronic gastritis to gastric tumor via the STAT/VEGF pathway.

Innovations and breakthroughs

To the best of our knowledge, this is the first report to show that p-STAT3 is also persistently activated during the progression from chronic gastritis to gastric carcinoma induced by the administration of MNNG in rats, and was positively associated with the expression of VEGF.

Applications

It was shown in this study that STAT3 is partially responsible for the progression from chronic gastritis to gastric carcinoma induced by MNNG and was significantly related to the expression of VEGF during this process. It is considered that STAT3 can induce an abnormal level of VEGF expression to promote the formation of gastric carcinoma.

Peer-review

Previous studies have established that STAT3 has close relationship with VEGF in cancers. However, this is the first time to report that p-STAT3 is also persistently activated during chronic gastritis to gastritis carcinoma induced by administration of MNNG in rats, which is positively associated with the expression of VEGF.

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Retrospective Study

Clinical and epidemiologic variations of esophageal cancer in Tanzania

Jaime V Gabel, Robert M Chamberlain, Twalib Ngoma, Julius Mwaiselage, Kendra K Schmid, Crispin Kahesa, Amr S Soliman

Jaime V Gabel, Robert M Chamberlain, Amr S Soliman, Department of Epidemiology, College of Public Health, University of Nebraska Medical Center, Omaha, NE 68198, United States

Robert M Chamberlain, M. D. Anderson Cancer Center, University of Texas, Houston, TX 78712, United States

Twalib Ngoma, Julius Mwaiselage, Crispin Kahesa, Ocean Road Cancer Institute, Dar es Salaam 3592, Tanzania

Kendra K Schmid, Department of Biostatistics, College of Public Health, University of Nebraska Medical Center, Omaha, NE 68198, United States

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Informed consent statement: Because this study utilizes medical records of those who gave permission for their records to be used for research purposes, most of whom were deceased at the time of the study, the IRB waived informed consent.

Conflict-of-interest statement: No authors for this manuscript have any conflicts of interest for this project.

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Correspondence to: Amr S Soliman, MD, PhD, Professor and Chair, Department of Epidemiology, College of Public Health, University of Nebraska Medical Center, Omaha, NE 68198, United States. amr.soliman@unmc.edu
Telephone: +1-402-5593976
Fax: +1-402-5523863

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Abstract

AIM: To estimate the incidence of esophageal cancer (EC) in Kilimanjaro in comparison to other regions in Tanzania.

METHODS: We also examined the clinical, epidemiologic, and geographic distribution of the 1332 EC patients diagnosed and/or treated at Ocean Road Cancer Institute (ORCI) during the period 2006-2013. Medical records were used to abstract patient information on age, sex, residence, smoking status, alcohol consumption, tumor site, histopathologic type of tumor, date and place of diagnosis, and type and date of treatment at ORCI. Regional variation of EC patients was investigated at the level of the 26 administrative regions of Tanzania. Total, age- and sex-specific incidence rates were calculated.

RESULTS: Male patients 55 years and older had higher incidence of EC than female and younger patients. Of histopathologically-confirmed cases, squamous-cell carcinoma represented 90.9% of histopathologic types of tumors. The administrative regions in the central and

eastern parts of Tanzania had higher incidence rates than western regions, specifically administrative regions of Kilimanjaro, Dar es Salaam, and Tanga had the highest rates.

CONCLUSION: Further research should focus on investigating possible etiologic factors for EC in regions with high incidence in Tanzania.

Key words: Esophagus; Cancer; Tanzania; Epidemiology; Distribution

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Core tip: In the manuscript "Clinical and epidemiologic variations of esophageal cancer in Tanzania", the authors describe the geographic distribution of esophageal cancer in Tanzania. The regions of Dar es Salaam and Kilimanjaro have higher incidences of esophageal cancer in comparison to other regions of Tanzania. There were also higher incidences found in the eastern and central parts of Tanzania compared to Western Tanzania. The authors did not find any differences in the age, sex, and histopathologic distribution of esophageal cancer when grouping the regions based on incidence rates.

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INTRODUCTION

Globally, esophageal cancer (EC) is the 8th most common incident cancer and the cancer with the 6th highest mortality^[1,2]. There are two histologic types of EC that are commonly diagnosed, squamous cell carcinoma (SCC) and adenocarcinoma (AC). SCC has been linked to tobacco and alcohol use while AC has been linked to diet and esophageal reflux; both are associated with older age^[3-5]. The incidence of esophageal AC is often significantly higher in developed than developing countries while the incidence of SCC is usually higher in developing countries, including Africa, than developed countries^[6,7]. In certain developed countries in East Asia, such as China and Japan, the incidence of SCC is much higher than AC incidence^[8].

Approximately 80% of all EC occur in developing countries^[2]. Africa specifically has a much higher incidence of EC in the Eastern and Southern regions^[6]. The highest risk populations in Africa tend to be poorer, malnourished, and consume alcohol and tobacco^[6,7,9,10]. There is also a large variation of risk for EC based on geographic region in Africa. Specifically, West Kenya and the Rift Valley Province of Kenya have shown high

rates^[6,11-15]. These regional differences may reflect risk and etiologic factors other than tobacco and alcohol^[6,11].

Tanzania, located on the southern border of Kenya, is one of the poorest countries in the world, and a location where EC is commonly found^[6]. Globocan estimates show an age-standardized rate of EC incidence in Tanzania for both sexes of 9.2 per 100000, which is the second highest cancer in Tanzania^[16]. This rate is similarly high among other East African countries^[16].

The Ocean Road Cancer Institute (ORCI), located in Dar es Salaam is the only specialized cancer treatment center in Tanzania, and the only facility in the country where radiation therapy is available^[17]. The majority of EC patients in Tanzania are diagnosed at advanced disease stages (stage III and IV) and the main lines of treatment for such advanced stages include chemotherapy and radiation therapy^[18]. It is also important to note that although AC and SCC develop from different cell types in the esophagus, they have similar treatment options^[19]. Therefore, the majority of EC patients in Tanzania are treated at ORCI because of its radiation therapy facility^[17].

Oncologists at ORCI have noted a higher number of EC patients referred from the Kilimanjaro region than other regions of Tanzania, despite similarities of population between regions, distance between the consultant hospital in the region and ORCI, and quality of roads between the consultant hospital and ORCI. This study aimed to estimate the incidence of EC in Kilimanjaro in comparison to other regions in Tanzania. The study also aimed to examine the demographic, clinical, and geographic distribution of EC patients in Tanzania during the period 2006-2013.

MATERIALS AND METHODS

Study population and data collection

This study was conducted at ORCI, the only specialized cancer treatment center in Tanzania. The majority of EC patients treated at ORCI are referred from a few major hospitals in Tanzania after initial histopathologic confirmation or clinical diagnosis. ORCI keeps medical records for all patients treated by chemotherapy and/or radiotherapy, including EC. These medical records and patient logbooks were utilized for this study, for the period 2006-2013. Logbooks were used as the original source for identifying medical record numbers of EC patients. Logbooks contain the names, medical record numbers, and type of cancer for each patient and were used to identify all patients with EC. Logbooks for 2007 were not available so manual search was performed to identify the EC medical records of 2007 without the initial list from the logbooks.

Abstracted information from the medical records included age, sex, residence, smoking status, alcohol consumption, tumor site, histopathologic type of tumor, treatment received, date and place of diagnosis, and date of beginning treatment at ORCI were abstracted from the medical records. This procedure was used for all EC patient records treated at ORCI during the period

2006-2013. There were a total of 1277 patient records collected and recorded. There were a total of 112 records of EC patients that were found in ORCI logbooks, but no medical record could be recovered (8.1%). Of the 1277 patients, 15 were removed because they were from a country other than Tanzania ($n = 13$) or had no recorded place of residence ($n = 2$), leaving a total of 1262 patients from ORCI records.

Surgical resection of EC is a major procedure that is mainly done at Muhimbili National Hospital (MNH) and the Kilimanjaro Christian Medical Centre (KCMC), and these hospitals are among the only in the country that provide histopathology diagnosis. Medical records for patients referred from MNH are included in the medical records of patients when they receive treatment at ORCI. For EC patients referred from KCMC, we created a list of their names and year of diagnosis and cross-referenced this information with all EC patients diagnosed and/or treated at KCMC during the period of 2006-2013. Additional information of patients from KCMC who were not included in the ORCI records were added to the final study dataset that included 1332 EC patients.

Statistical analysis

Regional variation in geographic distribution was investigated at the level of the 26 administrative regions of Tanzania. For calculating incidence rates, the total, age-, sex-specific population data of each region was obtained from the 2002 and 2012 Census of Tanzania^[20,21]. The annual projected growth rate for each region was used to estimate the annual population for years 2006-2013^[20,21].

Based on the incidence rates that were calculated, the 26 administrative regions and Zanzibar were grouped into 5 groups. This was done to compare risk factors between different incidence groups. The very high incident group, with a rate of over 1.00 cases per 100000 person-years, was made up of the Dar es Salaam and Kilimanjaro regions. The high incident group, with an incidence of 0.75-0.99 cases per 100000 person-years was comprised of the region Tanga. The mid-range group ranged from 0.50-0.74 cases per 100000 person-years was made up of the administrative districts of Morogoro, Pwani, and Iringa. The regions of Ruvuma, and Dodoma represented the group with low incidence range, 0.25-0.49 cases per 100000 person-years. All other regions, Lindi, Mtwara, Njombe, Mbeya, Rukwa, Katavi, Tabora, Singida, Tabora, Kigoma, Manyara, Arusha, Mara, Simiyu, Kagera, Geita, Shinyanga, Zanzibar, and Mwanza, had an incidence of less than 0.25 cases per 100000 person-years and made up the very low incidence group. These groups will now be referred to as Group 1, Group 2, Group 3, Group 4, and Group 5, respectively.

Data were analyzed using χ^2 tests to examine differences in the distribution of patient characteristics between the 5 incidence groups. These included differences based on demographic, clinical, and histopathologic variation of EC by age and sex groups and possible sex, age, and treatment differences among the two histologic

types. The analysis for this paper was generated using SAS software, Version 9.3 of the SAS System. Copyright © 2011 SAS Institute Inc^[22].

The study was approved by the University of Nebraska Medical Center IRB and the ORCI Academic, Research, Publications and Ethics Committee.

RESULTS

Figure 1 categorizes all of the administrative regions in Tanzania based on the incidence rate per 100000 person-years. The administrative regions of Kilimanjaro and Dar es Salaam were found to have the highest incidence rates for EC in Tanzania for years 2006-2013, at 1.20 and 1.65 cases per 100000, respectively.

Table 1 illustrates the age, sex, year of treatment, histopathologic type, and smoking and alcohol history of the study population. Male patients comprised slightly over 2/3 of the study population (68%) and over half (60%) of the EC patients were aged 55 years and older. Over 50% of the EC patients smoked tobacco or consumed alcohol, however this rate may be much higher as over 20% had unknown alcohol and tobacco status. Over 90% of histopathologically-confirmed EC cases were SCC (Table 1).

Table 2 displays the sex, age, and treatment distribution of both histopathologic types. The distribution of age group, sex, and treatment was not significantly different among SCC and AC patients. Males comprised nearly 2/3 of both SCC (67.1%) and AC (64.9%) patients. Sex distribution was not significantly different among SCC and AC patients ($P = 0.73$). Patients aged 55 and older were the majority of both SCC (63.2%) and AC (75.4%) patients. The distribution of age groups did not differ significantly between SCC and AC patients ($P = 0.1702$). The treatment received among SCC and AC patients also was not significantly different ($P = 0.13$).

Table 3 illustrates the distribution of histopathologic type, sex, and age group among the different incident groups. Males comprised the majority of patients in every incident group (64.7%-73.6%). The distribution of sex was not significantly different among the different incident groups ($P = 0.12$). SCC was the majority histopathologic type among all groups (86.6%-98.2%). The histopathologic type composition did not differ significantly among incident groups ($P = 0.13$). Those aged 55 and older comprised over half of all incident groups (52.3%-70.5%). The age group distribution among the incident groups was not significantly different ($P = 0.14$).

DISCUSSION

In this study, the eastern and central parts of Tanzania had higher incidence rates compared to western parts of the country. This can be observed for all age groups and sexes. This is an interesting observation, as the Western part of Kenya has been noted to have a higher incidence

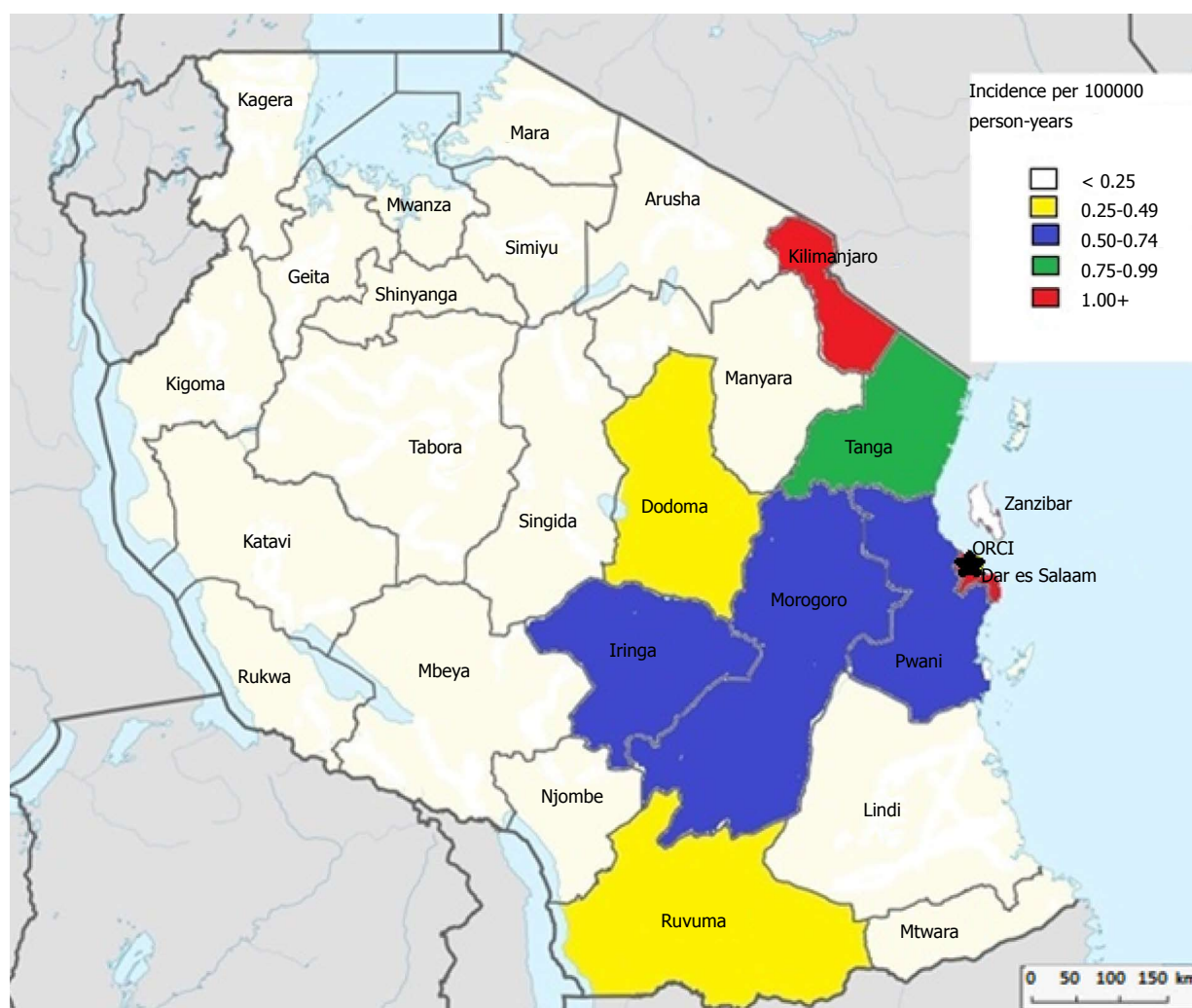


Figure 1 Geographic distribution of overall esophageal cancer incidence in Tanzania, 2006-2013.

compared to other areas in Kenya^[6,10-14]. Because our study hospital (ORCI) is on the eastern side of Tanzania, the higher rates of the disease from eastern Tanzania might be related to more accessibility to care. It is worth noting that some regions with the lowest incidence rates are not a significantly farther distance than some of the regions with a higher incidence. For example, Manyara, a region in the lowest incidence group, is not significantly farther from ORCI than Kilimanjaro, a region in the highest incidence group.

Males had a higher incidence in all administrative regions, and in total comprised 68% of the EC patients included in this study. The male-to-female ratio reported in this study is consistent with other studies on EC from developed and developing countries^[3,23,24].

SCC represented over 88% of all histopathologically confirmed patients in every age and sex group. SCC also made up the majority of confirmed patients who received treatment. This finding is not surprising, as SCC has been noted to be the more prominent histopathologic type in developing countries^[6,7,23]. The proportion of SCC and AC did not change significantly among administrative regions when grouped by incidence.

In this study, patients 55 years and older constituted over 64% of all EC patients in the study population. Older age was also found to be a risk factor for EC in a variety of studies from developing and developed countries^[3,23,24]. The age distribution of patients did not change significantly among administrative regions when grouped by incidence.

The etiology of EC is important when considering factors that may contribute to a higher incidence in different regions of Tanzania. Some known risk factors for EC include age, sex, tobacco smoking, alcohol intake, obesity, and reflux^[3-5]. No significant differences between regions with respect to age and sex exist. There is no available information detailing possible regional differences in lifestyle factors related to EC such as tobacco smoking, alcohol intake, viral infections, or customs.

It is worth noting that over 20% of SCC and AC patients who identified from the medical records, had no treatment information recorded. Lack of availability of treatment information in the medical records could be due to death of patients before starting treatment or seeking alternative medicine outside ORCI.

Table 1 Descriptive information of the study population of 1332 esophageal cancer patients at Ocean Road Cancer Institute, Tanzania 2006-2013

	<i>n</i> (%)
Age group (<i>n</i> = 1329)	
15-34 yr	78 (5.9)
35-44 yr	141 (10.6)
45-54 yr	246 (18.5)
55-64 yr	374 (28.1)
65+ yr	489 (36.8)
Sex (<i>n</i> = 1332)	
Male	906 (68.0)
Female	426 (32.0)
Year starting treatment at Ocean Road Cancer Institute (<i>n</i> = 1332)	
2006	135 (10.1)
2007	140 (10.5)
2008	174 (13.1)
2009	202 (15.2)
2010	211 (15.8)
2011	171 (12.8)
2012	139 (10.4)
2013	160 (12.0)
Histopathologic diagnosis (<i>n</i> = 626)	
Squamous cell carcinoma	569 (90.9)
Adenocarcinoma	57 (9.1)
Smoking and alcohol history (<i>n</i> = 1332)	
Status	
Alcohol and smoking	396 (29.7)
Alcohol only	150 (11.3)
Smoking only	137 (10.3)
Neither alcohol nor smoking	371 (27.9)
Unknown	278 (20.9)

This study has several strengths. First, the large number of cases and several years of study population were assets for this study. Second, availability of reliable census data for Tanzania allowed us to calculate the incidence rates. Third, the limited number of treatment facilities in Tanzania also contributed to capturing most patients that had EC in Tanzania, who are usually diagnosed at advanced disease stages. Fourth, the availability of clinical information within ORCI and MNH records also contributed to our ability to accurately abstract information and cross-reference patients between the two hospitals.

The study also has a few limitations. While we included all the patients who were seen at ORCI for treatment, it is still a possibility that some patients might have died before reaching ORCI, and therefore contributed to possible underestimation of the incidence. There may also be additional missing cases due to difficulty in journey because of roads, climate, and terrain, patients not believing that treatments in the hospital can treat cancer, or because traveling to the hospital is too expensive. It is also possible that certain geographic areas of Tanzania experience these barriers to treatment and other lifestyle factors related to EC more strongly than others leading to a greater underestimation in those areas, however this information is not available and is a limitation of the study. Patients wishing to escape enumeration due to the large amount of people seeking treatment at ORCI also have the potential to seek treatment outside of Tanzania and contribute to

Table 2 Characteristics of esophageal cancer patients at Ocean Road Cancer Institute, Tanzania by histopathologic types *n* (%)

	Squamous cell carcinoma (<i>n</i> = 569)	Adenocarcinoma (<i>n</i> = 57)	<i>P</i> value
Sex			0.7338
Male	382 (67.1)	37 (64.9)	
Female	187 (32.9)	20 (35.1)	
Age group			0.1702
15-34 yr	34 (6.0)	4 (7.0)	
35-44 yr	64 (11.2)	1 (1.8)	
45-54 yr	111 (19.5)	9 (15.8)	
55-65 yr	139 (24.4)	15 (26.3)	
65+ yr	221 (38.8)	28 (49.1)	
Treatment			0.1349
Radiation	170 (29.9)	16 (28.1)	
Chemotherapy	13 (2.3)	4 (7.0)	
Both	263 (46.2)	22 (38.6)	
None	123 (21.6)	15 (26.3)	

Unknown histology and histology reports categorized as "other" were removed for this analysis.

possible underestimation of the incidence; however, this is unlikely because these patients would no longer receive the free universal medical care that is provided to citizens of Tanzania. Cross-referencing KCMC helped identifying patients not included in the ORCI records. However, this opportunity of utilizing the large hospital of KCMC with histopathologic resources was not available at small hospitals in Tanzania. The country has the goal of establishing more cancer centers in the future to avoid patients missing treatment. Finally, missing data was another limitation for this study^[25,26]. HIV status is not commonly recorded for EC patients in Tanzania, as they receive the same treatment regardless of HIV condition. Other studies have reported that HIV is a risk factor for EC^[27,28]. The missing data for tobacco smoking, alcohol intake, non-HIV viral infections, and other lifestyle factors, which are risk factors for EC, was also a limitation of this study^[3-5].

This study confirmed the clinical impressions of physicians at ORCI of a higher proportion of EC patients from the Kilimanjaro region. The study also revealed higher incidence in patients from the Central and Eastern regions compared to Western Tanzania. This study confirmed previous findings about higher rates among older patients, higher male-to-female ratio, and higher SCC compared to AC on EC in East Africa^[4,5].

Future studies should further address the difference in geographic distribution of EC in Tanzania. The studies should examine the etiological factors that may contribute to the higher incidence in Kilimanjaro and the Central and Eastern regions. Also, future studies should examine the possibility of underestimation of EC in Tanzania.

COMMENTS

Background

Esophageal cancers (ECs) are a top ten cancer both for incidence and

Table 3 Difference in proportion of histopathologic type, sex, and age group between different incident groups in Tanzania *n* (%)

	Group 1	Group 2	Group 3	Group 4	Group 5	P value
Histology						0.1269
Squamous cell carcinoma	276 (92.0)	54 (98.2)	90 (89.1)	39 (90.7)	110 (86.6)	
Adenocarcinoma	24 (8.0)	1 (1.8)	11 (10.9)	4 (9.3)	17 (13.4)	
Sex						0.1233
Males	425 (65.6)	75 (64.7)	159 (73.6)	71 (66.4)	176 (71.8)	
Females	223 (34.4)	41 (35.3)	57 (26.4)	36 (33.6)	69 (28.2)	
Age group						0.1441
15-34 yr	41 (6.3)	6 (5.2)	13 (6.0)	5 (4.7)	14 (5.7)	
35-44 yr	65 (10.0)	12 (10.4)	28 (13.0)	13 (12.1)	23 (9.4)	
45-54 yr	110 (17.0)	16 (13.9)	46 (21.3)	33 (30.8)	41 (16.8)	
55-64 yr	196 (30.3)	34 (29.6)	57 (26.4)	20 (18.7)	67 (27.5)	
65+ yr	235 (36.3)	47 (40.9)	72 (33.3)	36 (33.6)	99 (40.6)	

Group 1-Group 5 reflect decreasing incidence of EC by region, *i.e.*, Group 1 were regions with highest incidence and Group 5 were regions with lowest incidence. Unknown histology and histology reports categorized as "other" were removed for histology analyses, unknown sex were removed for sex analyses, and unknown age were removed for age group analyses.

mortality, globally. ECs are very common in developing countries. Previous studies have observed high rates of EC in western Kenya and in the Rift Valley. Oncologists at the Ocean Road Cancer Institute in Dar es Salaam, Tanzania noted what they believed to be a large number of patients being referred from the Kilimanjaro region in Tanzania for EC compared to other regions. Because of this, medical records were used to determine the geographic distribution of EC in Tanzania for 2013-2006.

Research frontiers

This study brings to light the possibility of geographic differences in EC in Tanzania. This could lead to additional investigations to determine what etiologic factors may contribute to these differences. Additionally, the possibility of underestimation of EC in Tanzania should be further explored.

Innovations and breakthroughs

This is the first study to our knowledge to look at the distribution of EC in Tanzania. This study found that the regions of Dar es Salaam and Kilimanjaro had the highest incidences of EC in Tanzania. There were also generally higher incidences in the Eastern and Central parts of Tanzania compared to Western Tanzania.

Applications

This study brings to light differences in geographic distribution and potential access to care and underestimation of cancer patients in Tanzania.

Terminology

The histology of the cancer is determined in a laboratory and is based on which cell type the cancer derives from.

Peer-review

Peer-reviewers noted that the incidence of EC in Tanzania was estimated in this study and clinical, epidemiologic, and geographic distribution from medical record information was described. Previous findings on EC were confirmed in this study and a difference in geographic distribution was revealed. There is potential for underestimation of EC in this study and the information is limited to Tanzania.

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Myeloid sarcoma presenting as a colon polyp and harbinger of chronic myelogenous leukemia

Robert Rogers, Mark Ettel, Margaret Cho, Alexander Chan, Xiao-Jun Wu, Antonio G Neto

Robert Rogers, Mark Ettel, Margaret Cho, Antonio G Neto,
Department of Pathology, New York University School of
Medicine, New York, NY 10003, United States

Alexander Chan, Department of Pathology, Memorial Sloan
Kettering Cancer Center, New York, NY 10065, United States

Xiao-Jun Wu, Department of Pathology, George Washington
University School of Medicine, Washington, DC 20037, United
States

Author contributions: All authors equally contributed to this
paper.

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involving human subjects was employed in this case, rendering it
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Correspondence to: Xiao-Jun Wu, MD, PhD, Department of
Pathology, George Washington University School of Medicine,
2120 L Street NW, Suite 200, Washington, DC 20037,
United States. xwu@mfa.gwu.edu
Telephone: +1-202-6776600
Fax: +1-202-6776601

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Abstract

Myeloid sarcoma, also known as granulocytic sarcoma or chloroma is an unusual accumulation of malignant myeloid precursor cells in an extramedullary site, which disrupts the normal architecture of the involved tissue. It is known to occur more commonly in patients with acute myelogenous leukemia and less commonly in those with myelodysplastic syndrome and myeloproliferative neoplasm, such as chronic myelogenous leukemia. The most common sites of involvement include bone, skin and lymph nodes. However, rare cases have been reported in the gastrointestinal tract, genitourinary tract, or breast. Most commonly, a neoplastic extramedullary proliferation of myeloid precursors in a patient would have systemic involvement of a myeloid neoplasm, including in the bone marrow and peripheral blood. Infrequently, extramedullary disease may be the only site of involvement. It may also occur as a localized antecedent to more generalized disease or as a site of recurrence. Herein, we present the first case in the English literature of a patient presenting with an isolated site of myeloid sarcoma arising in the form of a colonic polyp which, after subsequent bone marrow biopsy, was found to be a harbinger of chronic myelogenous leukemia.

Key words: Myeloid sarcoma; Granulocytic sarcoma; Chloroma; Chronic myelogenous leukemia

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Core tip: Myeloid sarcoma rarely presents in the gastrointestinal tract. Rarer still, does myeloid sarcoma manifest in the gastrointestinal tract without systemic involvement

by a myeloid neoplasm. This case report documents the first instance in the English literature wherein an isolated extramedullary site of myeloid sarcoma adopting the form of a colonic polyp was found to be a harbinger of chronic myelogenous leukemia.

Rogers R, Ettl M, Cho M, Chan A, Wu XJ, Neto AG. Myeloid sarcoma presenting as a colon polyp and harbinger of chronic myelogenous leukemia. *World J Gastrointest Oncol* 2016; 8(3): 321-325 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v8/i3/321.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v8.i3.321>

INTRODUCTION

Myeloid sarcoma (MS) is defined as an extramedullary tumor composed of myeloid precursors which efface the normal architecture of the involved tissue^[1-3]. There is a predilection for males and the median age at diagnosis is 56 years^[1-3]. MS occurring *de novo* is considered the equivalent of a diagnosis of acute myelogenous leukemia (AML)^[1-3]. It may precede or coincide with AML, signify a relapse in AML or constitute an acute blastic transformation of myelodysplastic syndrome or myeloproliferative neoplasms, such as chronic myelogenous leukemia (CML) (663, 742)^[1-3]. In this case report, we illustrate a novel presentation of this lesion, discuss its histologic appearance and hematopathologic workup, and review the pertinent literature.

CASE REPORT

A 55-year-old man with hematochezia for an unknown period of time underwent colonoscopy. A polyp (< 1 cm) was seen in the colon, 20 cm from the anal verge, and was retrieved. No other endoscopic findings were identified. HE slides and accompanying immunostained slides from the colon polyp were evaluated at our institution after referral from the outside institution. HE sections showed polypoid colonic mucosa markedly expanded by an infiltrate of sheets of large neoplastic cells exhibiting fine chromatin, prominent nucleoli and moderately irregular nuclear contour (Figure 1). The background stroma was expanded by a mixed cellular infiltrate. Immunohistochemistry revealed that the large, immature appearing neoplastic cells were diffusely positive for myeloperoxidase, lysozyme, CD43, CD15, and CD33 (Figure 2), while negative for B-cell markers (CD20, CD79a, Pax5), T/NK-cell markers (CD3, CD56, granzyme), and plasma cell marker (CD138). Few scattered neoplastic cells were positive for CD34 and CD117 (Figure 2). In addition, the neoplastic cells were negative for CD30, CD61 and glycophorin. The findings support the diagnosis of myeloid sarcoma (MS). The neoplastic cells were limited to the polyp with adjacent fragments of colonic mucosa in the biopsy showing unremarkable findings. During subsequent workup, the patient was noted to have a leukocytosis with a white blood cell count of 221 k/ μ L, Hgb 11.7 g/dL, MCV 89.1 fL,

and platelets 366 k/ μ L. The bone marrow biopsy showed hypercellular marrow with myeloid hyperplasia (Figure 3). There was no evidence of increased blasts (Figure 3). Cytogenetics reported a t(9;22) translocation in the bone marrow. A diagnosis of chronic myeloid leukemia, Ph+ was rendered. A retrospective FISH analysis for t(9;22) performed on the colonic polyp was negative. Because Ph chromosome + CML with MS in the colon is considered equivalent to a blast crisis, the patient received systemic chemotherapy, with protein kinase inhibitors (PKIs) and Dasatinib. Repeat colonoscopies two and three years later showed no residual or recurrent disease. Follow up bone marrow biopsies over a five-year period demonstrated a cytogenetic and morphologic complete response with minimal residual CML by molecular analysis. A bone marrow biopsy in 2014 showed that the number of cells carrying the t(9;22) translocation had risen from < 1% previously to 13%. The most recent bone marrow biopsy in March 2015 was stable with the prior biopsy. The patient did not receive bone marrow transplantation.

DISCUSSION

MS has been referred to as chloroma because of the green hue imparted to the cut surface of the tumor due to the presence of myeloperoxidase, an enzyme present in granulocytic cells. MS is known to occur in patients with various myeloid neoplasms. It occurs predominantly in four clinicopathological situations: (1) as a harbinger of AML in the non-leukemic patient; (2) as a sign of impending blast crisis in CML or leukemic transformation in myelodysplastic disorders; (3) as an additional manifestation in patients with known AML; and (4) as an isolated event, preceding the onset in the marrow by months to years^[4-9].

Our case showed an unusual presentation of hematochezia resulting from an isolated colonic polyp which subsequently revealed Ph+ CML upon workup. This unexpected diagnosis of MS, an AML equivalent, prompted the systemic chemotherapy and evaluation for potential bone marrow transplant as a future therapy modality. Recognition of MS is clinically critical for the choice of treatment modality. However, the majority of MS do not produce clinical signs and symptoms, hence the difficulty in making the diagnosis either clinically or histologically; it is most often discovered at autopsy^[4]. Soft tissue, bone/spine, skin, lymph nodes and the periosteum are the commonest sites of involvement. MS infrequently involves the gastrointestinal tract^[7-12]. Neiman *et al.*^[13] reported 61 such tumors and found gastrointestinal involvement in only 4 (7%) of the tumors^[3]. Choi *et al.*^[7] reported 8 cases where the small bowel and large intestine were affected. It has been reported that the ileum is the most affected area in the intestine^[14]. Isolated primary involvement of the colon and rectum is exceedingly rare with secondary extension from the peritoneum being more common^[7]. Several reported cases of MS presented as an intraluminal polypoid mass, diffuse polyposis^[7], or rarely, coexisting

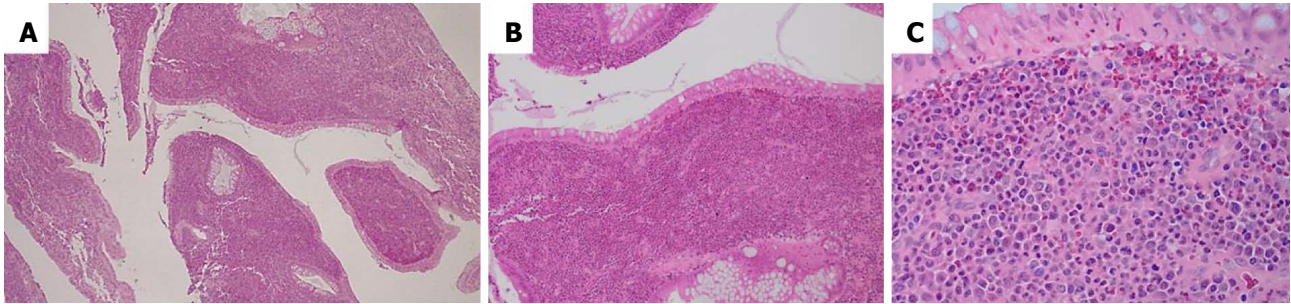


Figure 1 Fragments of polypoid colonic mucosa (A) markedly expanded by a hypercellular infiltrate (B) composed of large immature-appearing neoplastic cells with prominent nucleoli (C) (HE stain; 4 ×, 10 ×, 40 ×).

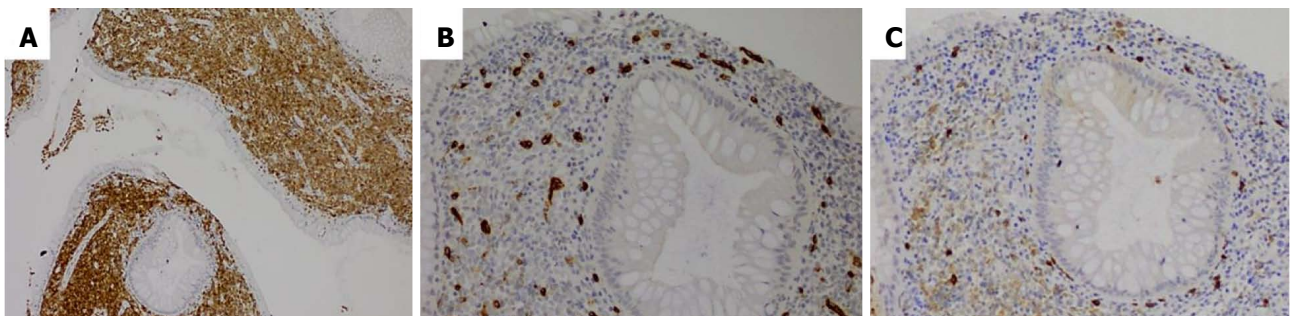


Figure 2 Immunohistochemical studies demonstrate diffuse positivity for CD33 signifying myeloid origin (A), weak, granular CD34 staining highlighting immature cells (B) (strong CD34 staining highlights endothelial cells), and CD117 highlighting scattered large, immature cells (C) (immunohistochemistry; 10 ×, 20 ×, 10 ×).

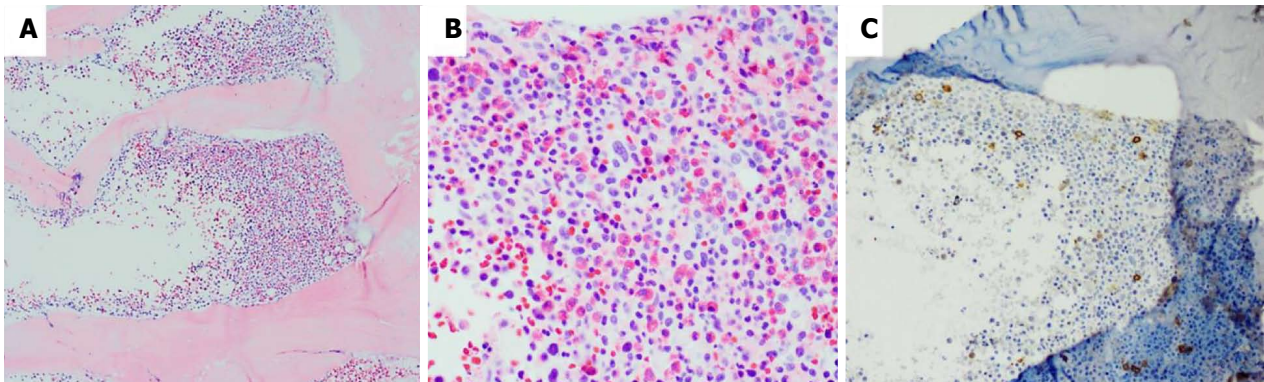


Figure 3 Bone marrow biopsy shows a hypercellular marrow (A) with myeloid hyperplasia (B) without an increased number of blasts by CD117 (C) (HE; 10 ×, 40 ×; immunohistochemistry; 20 ×).

with adenoma^[12]. Our case is one of the few cases that presented as polyp in the large intestine without any other manifestation of a leukemic process and the first case to reveal Ph+ CML upon subsequent workup.

When present in the gastrointestinal tract, particularly in the intestine, the most common complication of leukemic involvement is massive hemorrhage, perforation, necrosis, acute or intermittent abdominal pain from partial to complete bowel obstruction and intussusceptions^[4,15,16]. Nevertheless, MS of the gastrointestinal tract can be also an isolated manifestation in the absence of any hematologic disorder, thereby precluding an overt diagnosis of MS^[4,7,9,17,18]. The diagnosis of MS can be challenging and confused with other malignant tumors by

histomorphologic evaluation. The morphologic features of the tumors can be poorly differentiated or that of nondistinct hematolymphoid neoplasms with little or no evidence of myeloid differentiation by morphology. To that end, a significant proportion of tumors (47%-56%) are initially misdiagnosed as malignant lymphoproliferative disorders including Hodgkin lymphoma, lymphoblastic lymphoma, Burkitt's lymphoma, large cell non-Hodgkin lymphomas, small round blue cell tumors (Ewing sarcoma, rhabdomyosarcoma, neuroblastoma, medulloblastoma), or poorly differentiated carcinoma^[14,19]. An accurate diagnosis of MS is of great clinical importance in the ongoing management of hematologic malignancies, and must be distinguished from extranodal lymphoma

and other entities aforementioned, in order to achieve optimal therapeutic benefit and avoid detrimental outcome^[12,19]. If left untreated, the majority (88%) of MS patients progress to AML within 11 mo whereas a much longer disease free interval is noted in patients receiving upfront treatment with AML chemotherapy^[12]. Of note, patients with isolated MS of the GI tract who are treated with standard induction chemotherapy appear to have a substantially lower probability of subsequent development of leukemic form of AML^[7,20].

The current World Health Organization classification system states that MS most commonly consists of myeloblasts with or without maturation that partially or totally efface the tissue architecture in an extramedullary site^[1]. Tumors with other hematopoietic elements such as erythroid precursors or megakaryocytes are rare and may occur in conjunction with transformation of myeloproliferative neoplasms^[1,3]. In a significant proportion of cases, MS displays myelomonocytic or pure monoblastic phenotypes^[1-3]. The common cytogenetics abnormalities include trisomy 8, t(8;21), inv(16), and rearrangement involving MLL^[1]. Of note, it bears mentioning that extramedullary hematopoietic tumor (EMT) is not considered the same tumor, has a different prognosis, warrants different treatment strategy and is not covered here.

In our patient with Ph+ CML, myeloid sarcoma in the colon polyp failed to demonstrate the presence of t(9;22) translocation by FISH analysis. There have been reported cases of development of clonal unrelated AML or myeloid sarcoma in CML patients. These patients usually had a prolonged history of CML with interferon and busulphan treatment or tyrosine kinase inhibitor (TKI) treatment^[21,22], which was not the case with our patient. The possibility of a false negative result exists with FISH analysis. However, given the limited material, further confirmatory testing by cytogenetic analysis or molecular sequencing studies could not be performed.

As a general rule, systemic chemotherapy is recommended, given that MS has a dismal prognosis with a median survival of 9 mo from the time of diagnosis^[23,24]. Because most cases of MS progress to AML, the majority of investigators recommend the treatment for AML^[4,7,25]. Most of the tumors respond to chemotherapy or external radiation. Patients with MS should also be considered as high risk AML with a poor outcome and an early and intensive therapy according to AML protocols, possibly including allogenic/autologous bone marrow transplantation in the first remission, is strongly suggested in order to avoid systemic involvement which could occur with the use of only local surgical or radiation therapies^[4,26]. The clinical behavior and response to therapy seem not to be influenced by any of the following factors: Age, sex, anatomical site(s) involved, *de novo* presentation, histological features, immunophenotype, cytogenetic findings or clinical history related to AML, myelodysplastic syndrome, and myeloproliferative neoplasms^[1,3]. Patients who undergo allogenic or autologous bone marrow

transplantation seem to have a higher probability of prolonged survival or cure^[1,3,27]. Our patient is still living at the time of this report, six years from his initial presentation.

In summary, MS presenting as a colonic polyp is infrequent. We describe an unusual presentation and emphasize the need to be aware of granulocytic sarcomas presenting in the GI tract, as they may precede or occur concurrently with acute myeloid leukemia, or reveal blastic transformation of chronic myeloproliferative disorders or myelodysplastic syndromes. MS can be easily misdiagnosed in this site, especially without a previous history of such disease. Indeed, experienced pathologists encounter difficulty with this lesion as incorrect initial diagnosis occurs in up to 50% of cases, most of which are diagnosed as high grade non-Hodgkin's lymphoma. Therefore careful evaluation of morphology for evidence of myeloid differentiation, use of an immunohistochemistry panel, and high index of suspicion when confronted with a less differentiated neoplasm are required to avoid diagnostic pitfalls. Awareness and prompt recognition of this disease entity is of great clinical importance given that an early start of anti-leukemic therapy could lead to a longer survival for these patients. The prognosis is in general poor and comparable to that of other acute leukemias^[4].

COMMENTS

Case characteristics

A 55-year-old man with no significant medical history presented with hematochezia.

Clinical diagnosis

Colonoscopy revealed a subcentimeter colon polyp located 20 cm from the anal verge.

Differential diagnosis

Hyperplastic polyp, adenomatous polyp, malignant polyp.

Laboratory diagnosis

White blood cell count was elevated (221 k/ μ L).

Pathological diagnosis

Myeloid sarcoma forming a colon polyp with subsequent cytogenetic studies of a bone marrow biopsy revealing chronic myelogenous leukemia (CML).

Treatment

The patient received systemic chemotherapy including protein kinase inhibitors and a tyrosine inhibitor, Dasatinib.

Related reports

There are infrequent reports of isolated primary involvement of the colon and rectum by myeloid sarcoma. This case is one of a few documented instances of myeloid sarcoma presenting as a colon polyp without any manifestations of a leukemic process and the first to reveal Ph+ CML upon subsequent workup.

Term explanation

Myeloid sarcoma is an extramedullary tumor composed of myeloid blasts with or without maturation which effaces the normal architecture of the involved

tissue.

Experiences and lessons

This case report serves to illustrate a unique manifestation of myeloid sarcoma. CML with myeloid sarcoma is considered equivalent to a blast crisis, and should be treated accordingly.

Peer-review

The paper is well-written. This is an interesting case.

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Rare case of entero-enteric intussusception caused by small bowel metastasis from a cardiac liposarcoma

Gustavo Gomez, Mohammad Bilal, Paul Klepchick, Kofi Clarke

Gustavo Gomez, Kofi Clarke, Department of Gastroenterology, Hepatology and Nutrition, Allegheny General Hospital, Pittsburgh, PA 15229, United States

Mohammad Bilal, Department of Internal Medicine, Allegheny General Hospital, Pittsburgh, PA 15229, United States

Paul Klepchick, Department of Radiology, Allegheny General Hospital, Pittsburgh, PA 15229, United States

Author contributions: Gomez G and Bilal M were involved in care of patient and performed literature review and wrote majority of the manuscript; Klepchick P provided insight into important radiographic findings; Clarke K was involved in editing the manuscript and provided expert opinion.

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Correspondence to: Mohammad Bilal, MD, Department of Internal Medicine, Allegheny General Hospital, 320 East North Avenue, Pittsburgh, PA 15229, United States. mbilal@wpahs.org
Telephone: +1-412-6603624
Fax: +1-412-3598439

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Abstract

Primary cardiac liposarcoma is exceedingly rare and its metastatic potential varies based on the actual tumor subclass. Intestinal intussusception is also an uncommon cause of abdominal pain and bowel obstruction in adults and it usually generates at a malignant lead point in this age group. We report a case of a primary cardiac dedifferentiated liposarcoma in a pregnant woman causing small bowel seeding leading to bowel intussusception.

Key words: Liposarcoma; Intussusception; Small bowel metastasis; Enteroenteric; Cardiac

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Core tip: Primary cardiac liposarcoma is exceedingly rare and its metastatic potential varies based on the actual tumor subclass. Intestinal intussusception is also an uncommon cause of abdominal pain and bowel obstruction in adults and it usually generates at a malignant lead point in this age group. We report a case of a primary cardiac dedifferentiated liposarcoma in a pregnant woman causing small bowel seeding leading to bowel intussusception.

Gomez G, Bilal M, Klepchick P, Clarke K. Rare case of entero-enteric intussusception caused by small bowel metastasis from a cardiac liposarcoma. *World J Gastrointest Oncol* 2016; 8(3): 326-329 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v8/i3/326.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v8.i3.326>

INTRODUCTION

Primary cardiac liposarcoma is exceedingly rare and its metastatic potential varies based on the actual tumor subclass. Intestinal intussusception is also an uncommon cause of abdominal pain and bowel obstruction in adults and it usually generates at a malignant lead point in this age group. We report a case of a primary cardiac dedifferentiated liposarcoma in a pregnant woman causing small bowel seeding leading to bowel intussusception.

CASE REPORT

A 29-year-old female with recent history of surgically resected intra-cardiac liposarcoma presents to the hospital at 27 wk of gestational age with complains of sudden onset of abdominal pain associated with intractable nausea and vomiting.

Three months prior to this presentation, the patient was admitted with severe shortness of breath and dyspnea on exertion. She was found to have a left intra-atrial mass on a transthoracic echocardiogram involving a large portion of the left atrium, anterior leaflet of the mitral valve and pulmonary venous system causing complete occlusion of the right inferior pulmonary vein. Cardiac magnetic resonance imaging confirmed the lesion (Figure 1). The patient underwent resection of the mass and porcine mitral valve replacement. In addition, she also had pulmonary venous endarterectomy. Due to the size of the mass with significant extension into the pulmonary venous vasculature, a complete en-block resection could not be performed. Postoperative course was complicated by a transient 3rd degree atrio-ventricular block. She was discharged on post-operative day 14. Outpatient tumor staging included non-contrast MRIs of the brain, abdomen and pelvis that were negative for any metastatic disease.

On this presentation to the hospital, an magnetic resonance imaging (MRI) of the abdomen and pelvis was performed for severe abdominal pain, which revealed an entero-enteric left upper quadrant intestinal intussusception with proximal small bowel dilation suggestive of a mechanical small bowel obstruction (Figures 2 and 3). After an unsuccessful trial of conservative management with nil per os and naso-gastric tube decompression, she developed acute cardiopulmonary decline associated with significant fetal cardiac distress. A multi-disciplinary meeting was held and she had an emergency caesarian section, exploratory laparotomy and placement of a transcutaneous cardiac pacemaker prior to the surgical interventions. A healthy, viable 950 g female infant was delivered. At exploratory laparotomy, a 10 cm long intussuscepted small bowel segment without evidence of ischemia, beginning at 50 cm from the ligament of Treitz was identified. After several and unsuccessful manual attempts at reducing this bowel segment, partial small bowel resection of the affected area with end-to-end anastomosis was performed.

The surgically resected specimen showed two polypoid

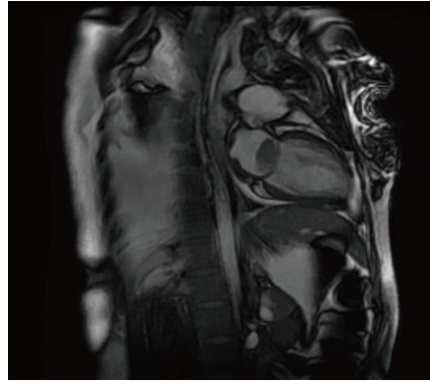


Figure 1 Cardiac magnetic resonance imaging showing a large mass in the left atrium.

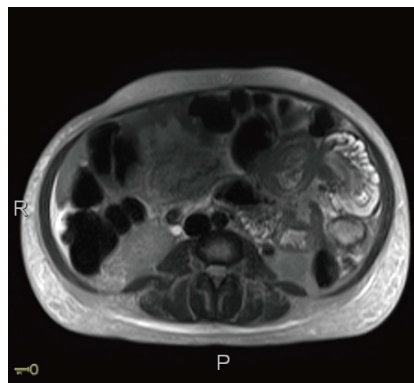


Figure 2 Magnetic resonance imaging of abdomen showing entero-enteric intussusception in axial cut.

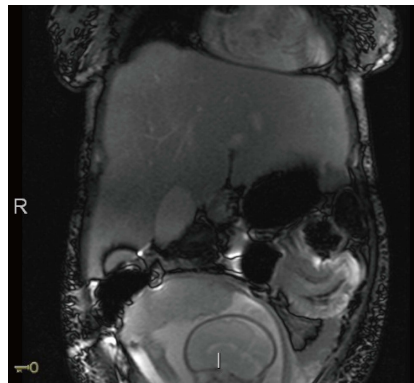


Figure 3 Magnetic resonance imaging of the abdomen showing an entero-enteric intussusception in the left upper quadrant.

lesions, which were 5 cm and 1 cm in their greatest dimension. Pathology was consistent with pleomorphic neoplasm with spindle cells, giant cells, vesicular nuclei with macro nuclei and occasional intra nuclear inclusions (Figures 4 and 5). Immunohistochemical staining was positive for Murine Double Minute (MDM) 2 and CDK 4. The histological features were identical to the original neoplasm seen in the heart 3 mo earlier (Figure 6). Post operatively the patient developed respiratory failure and was not able to be weaned off the ventilator. On post-operative day 8, the family decided to withdraw care.

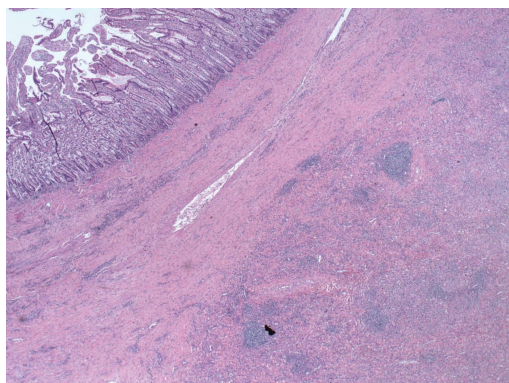


Figure 4 Normal small bowel mucosa (left hand corner) in contrast to infiltrative area of increased cellularity (right lower hand corner).

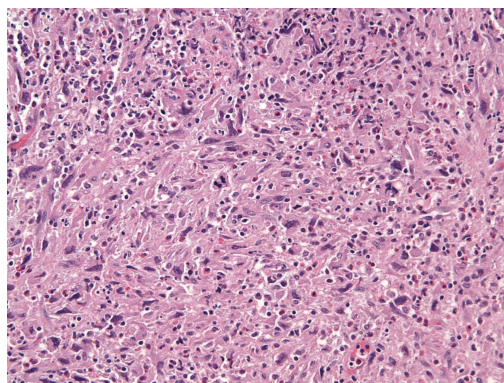


Figure 6 Poorly differentiated malignant neoplasm composed of variably spindled polygonal or histiocytoid cells and irregular vesicular nuclei consistent with a dedifferentiated liposarcoma.

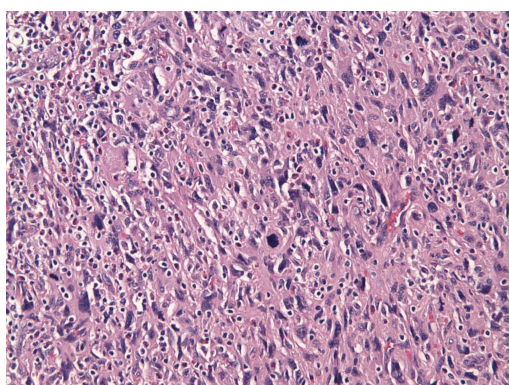


Figure 5 High power: Population of poorly differentiated malignant cells that are high grade (pleomorphic, hyperchromatic and contain increased mitotic activity) and are similar to the intracardiac dedifferentiated liposarcoma.

Unfortunately, the patient passed away the same day.

DISCUSSION

Primary cardiac neoplasms are exceedingly rare. Secondary (metastatic) cardiac lesions are 20-40 times more common than primary tumors^[1,2]. Malignant cardiac neoplasms constitute between 15%-25% of these primary lesions and sarcomas are found to be the most common malignancy^[3,4]. Liposarcoma accounts for about 13% of primary malignant tumors of the heart^[5]. There are five histological subtypes of these adipocyte precursor tumors including well differentiated, dedifferentiated, myxoid or round cell type, pleomorphic and mixed variant. Dedifferentiated liposarcoma commonly arises from the retroperitoneum and extremities and carries a lower incidence of distal seeding compared to the pleomorphic variant^[6-8]. Most common sites of metastasis include the lung, liver, bone, brain and soft tissues. Metastasis to the small bowel is rare with only few previously reported cases in the medical literature.

Surgical management is the preferred approach for cardiac liposarcoma, given its metastatic potential and the significant associated cardiorespiratory morbidity.

Intestinal intussusception is a rare cause of intestinal

obstruction in adults accounting for 1%-5% of the cases^[9]. It is defined as telescoping or prolapsing of a proximal intestinal segment into the adjacent more distal bowel segment secondary to peristaltic movements. Depending on the location of intestinal segment involved, intussusceptions are classified as enteroenteric, ileocolic, ileocecal or colocolonic. Majority of cases in adults are related to pathologic conditions that serve as a lead point for initiation of the prolapsing process. Intestinal diverticulum, luminal strictures and benign or malignant neoplasm are some of the more common causes^[9,10]. Enteroenteric intussusceptions due to malignant conditions accounts for up to 30% of all cases^[11]. Metastatic disease, adenocarcinoma and small bowel lymphomas are some of the leading metastatic causes^[12].

Clinical presentation varies depending of the degree of obstruction and ranges from chronic abdominal pain to intractable nausea, vomit and frank hematochezia in cases of complete obstruction associated with intestinal ischemia. Computer tomography scan of the abdomen and pelvis is the preferred imaging modality, with reported accuracy rates from 30%-100%^[12]. Abdominal Ultrasound and abdominal MRI are two other alternatives and can be used in pregnancy to minimize radiation exposure to the fetus.

Surgical resection of the intussuscepted area is the treatment of choice in the adult population^[9]. Although surgical resection was performed in our patient, with appropriate treatment of intestinal obstruction, her simultaneous pregnancy, heart block and respiratory condition increased her overall risks for mortality.

There have been few reported cases of liposarcoma causing metastasis to the small bowel. To the best of our knowledge, this is the first reported case of a primary cardiac dedifferentiated liposarcoma causing metastasis to the small bowel with associated intussusception.

COMMENTS

Background

Primary cardiac liposarcoma is exceedingly rare and its metastatic potential

varies based on the actual tumor subclass.

Research frontiers

Intestinal intussusception is also an uncommon cause of abdominal pain and bowel obstruction in adults and it usually generates at a malignant lead point in this age group.

Innovations and breakthroughs

The authors report a case of a primary cardiac dedifferentiated liposarcoma in a pregnant woman causing small bowel seeding leading to bowel intussusception.

Applications

On this presentation to the hospital, a magnetic resonance imaging of the abdomen and pelvis was performed for severe abdominal pain, which revealed an entero-enteric left upper quadrant intestinal intussusception with proximal small bowel dilation suggestive of a mechanical small bowel obstruction.

Peer-review

The case is interesting, well written and easy to read. References are new and adequate.

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Zuo-Feng Zhang, *South Los Angeles*
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**TOPIC HIGHLIGHT**

- 330** MicroRNAs as diagnostic and prognostic biomarkers in colorectal cancer
Yi R, Li Y, Wang FL, Miao G, Qi RM, Zhao YY
- 341** *Helicobacter pylori* associated Asian enigma: Does diet deserve distinction?
Zaidi SF
- 351** Status of colitis-associated cancer in ulcerative colitis
Kinugasa T, Akagi Y

REVIEW

- 358** Chromodomain-helicase-DNA binding protein 5, 7 and proneurotic mixed lineage kinase domain-like protein serve as potential prognostic biomarkers in patients with resected pancreatic adenocarcinomas
Seldon CS, Colbert LE, Hall WA, Fisher SB, Yu DS, Landry JC
- 366** Molecular therapeutics in pancreas cancer
Narayanan V, Weekes CD
- 380** Targeting inflammation in pancreatic cancer: Clinical translation
Steele CW, Kaur Gill NA, Jamieson NB, Carter CR
- 389** Medical treatment for gastro-entero-pancreatic neuroendocrine tumours
Berardi R, Morgese F, Torniai M, Savini A, Partelli S, Rinaldi S, Caramanti M, Ferrini C, Falconi M, Cascinu S

MINIREVIEWS

- 402** Colorectal cancers and chlorinated water
El-Tawil AM

ORIGINAL ARTICLE**Retrospective Study**

- 410** Clinicopathological features of patients with middle third gastric carcinoma
Kim JH, Joo JK, Ryu SY, Kim HG, Lee JH, Kim DY

Contents

World Journal of Gastrointestinal Oncology
Volume 8 Number 4 April 15, 2016

ABOUT COVER

Editorial Board Member of *World Journal of Gastrointestinal Oncology*, Karl-Friedrich Becker, PhD, Professor, Institute of Pathology, Technical University of Munich, D-81675 Munich, Germany

AIM AND SCOPE

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World Journal of Gastrointestinal Oncology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-85381891
Fax: +86-10-85381893
E-mail: editorialoffice@wjnet.com
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PUBLISHER

Baishideng Publishing Group Inc
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Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjnet.com
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2016 Colorectal Cancer: Global view

MicroRNAs as diagnostic and prognostic biomarkers in colorectal cancer

Rui Yi, Yao Li, Fei-Liang Wang, Gang Miao, Ruo-Mei Qi, Yan-Yang Zhao

Rui Yi, Ruo-Mei Qi, Yan-Yang Zhao, the Key Laboratory of Geriatrics, Beijing Institute of Geriatrics, Beijing Hospital, Ministry of Health, Beijing 100730, China

Yao Li, Fei-Liang Wang, Gang Miao, Department of Surgery, Beijing Hospital, Ministry of Health, Beijing 100730, China

Author contributions: Yi R, Li Y, Wang FL and Zhao YY wrote the paper; Miao G, Qi RM and Zhao YY fully discussed the paper.

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Correspondence to: Yan-Yang Zhao, MD, PhD, the Key Laboratory of Geriatrics, Beijing Institute of Geriatrics, Beijing Hospital, Ministry of Health, No.1, DaHua Road, Dong Dan, Beijing 100730, China. yanyangzhaobj@gmail.com
Telephone: +86-10-58115045
Fax: +86-10-65132969

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Abstract

MicroRNAs (miRNAs) are key regulators involved in various tumors. They regulate cell cycle, apoptosis and cancer stemness, metastasis and chemoresistance by controlling their target gene expressions. Here, we mainly discuss the potential uses of miRNAs in colorectal cancer (CRC) diagnosis. We also shed light on the important corresponding miRNA targets and on the major regulators of miRNAs. Furthermore, we discuss miRNA activity in assessing the prognosis and recurrence of CRC as well as in modulating responsiveness to chemotherapy. Based on the various pro-oncogenic/anti-oncogenic roles of miRNAs, the advantages of a therapeutic strategy based on the delivery of miRNA mimics are also mentioned. Together, miRNA seems to be an excellent tool for effectively monitoring and targeting CRC.

Key words: MicroRNA; Diagnosis; Prognosis; Colorectal cancer; Biomarkers

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Core tip: MicroRNAs (miRNAs) regulate oncogenesis, metastasis and chemotherapy by controlling corresponding target gene expressions. Here, we shed light on the diagnostic and prognostic value of some miRNAs in colorectal cancer and potential miRNA-based therapy was also discussed. We hope that this review will offer useful information for researchers who work in a related field.

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INTRODUCTION

MicroRNAs (miRNAs) are endogenous short non-coding RNAs that downregulate target gene expressions by binding to their 3'-UTR^[1]. MiRNAs function as regulators in a broad biological process. Dysregulation of miRNAs exerts a strong influence in disease progression by changing target gene expressions in various tumors^[2,3]. More and more data have suggested that miRNAs could be potential biomarkers of early-stage colorectal cancer (CRC) since they are unable to be degraded easily and their expression levels in colorectal polyps, blood and stool may give a hint of the occurrence of the disease. CRC accounts for 10% of new cancer cases and is one of the leading causes of death worldwide (over 1.23 million deaths per year)^[4]. To date, the common methods for CRC early diagnosis are computed tomography (CT), colonoscopy and fecal occult blood test (FOBT)^[5]. However, CT has a low sensitivity for the diagnosis of early CRC and could result in a large radiation exposure^[6]. Colonoscopy is also expensive and increases the risk of morbidity or mortality due to perforation of the gut^[7], although it can effectively detect neoplastic occurrence and allow for the removal of polyps when found. FOBT is commonly used but other digestive diseases such as ulcerative colitis and hemorrhoids may also cause blood in stool^[8] so the detection of FOBT is not a sensitive test for an early diagnosis. Taken together, current methodologies for early detection are neither sensitive nor specific. Differential miRNA expression in CRC individuals vs normal individuals is a common event and may be pivotal for tumor onset and progression. What's more, some dysregulated miRNAs are associated with progression and grade malignancy of CRC. Multiple studies have identified the values of miRNAs in CRC diagnosis. Some reports indicated that changed expression levels of miRNAs correlate closely with cancer progression and prognosis^[9-11]. Here, we review the literature to summarize the association of some important miRNAs with early-stage diagnosis, prognosis and recurrence of CRC and to discuss some miRNAs that might give a hint to guide treatment decisions (Table 1).

MiR-21

MiR-21 is one of the most extensively investigated oncogenic miRNAs whose expression is frequently upregulated in CRC^[12-14]. Overexpression of miR-21 is closely correlated with CRC cell proliferation, invasion, lymph node metastases and advanced clinical stage, all of which are the main prognostic factors for CRC. MiR-21 can downregulate several tumor suppressor genes, including PTEN and RECK. The tumor suppressor protein PTEN acts as a lipid phosphatase to dephosphorylate phosphatidylinositol 3,4,5-trisphosphate (PIP3), antagonizing the PI3K/Akt pathway^[15]. This pathway has an important effect on numerous biological functions, such as cell proliferation, adhesion, angiogenesis, migration, invasion, metabolism and anti-apoptosis^[16]. Besides, the key action of RECK (reversion-inducing cysteine rich protein with kazal motifs) is to suppress the cell metastasis by

inhibiting matrix metalloproteinases (MMPs) involved in the breakdown of the extracellular matrix (ECM)^[17]. All of these suggest the oncogenic role of miR-21 in CRC.

CRC tissue has been extensively reported on, as discussed in head and neck squamous cell carcinoma (HNSCC), non-small-cell lung cancer (NSCLC) and carcinomas of the digestion system^[18,19]. Moreover, some but not all polyps end up as malignant tumors through a string of increased genetic events. Therefore, it is important for early diagnosis to determine which polyp has the potential to become invasive carcinoma and then to remove it early. Accumulating studies show that miR-21 is correlated with the malignant progression of polyps and is highly expressed in CRC^[20-22]. Yamamichi *et al.*^[23] evaluated the expression of miR-21 during CRC progress in 39 surgically excised colorectal tumors and 34 CRC endoscopically resected colorectal polyps using nucleic acid *in situ* hybridization and found that miR-21 keeps increasing from precancerous polyps to early cancer, further increasing from the early to advanced stage of CRC. MiR-21 from CRC tissues may also be used to identify prognosis. Oue *et al.*^[12] used formalin-fixed, paraffin-embedded (FFPE) tumor tissues from 301 CRC patients at different TNM stages to discuss the relationship between miR-21 and prognosis and found that high miR-21 expression is significantly associated with poor survival. Furthermore, a meta-analysis of miR-21 expression level in 1174 CRC tissues suggested that increased miR-21 can be predictive of poor survival; however, CEA level shows no correlation with miR-21 expression^[24]. The relationship between Pdc4 (programmed cell death 4) and miR-21 is of great interest as well because miR-21 posttranscriptionally regulates Pdc4 and Pdc4 suppresses invasion and intravasation by suppressing the expression of the invasion-related urokinase receptor (*u-PAR*) gene *via* the transcription factors Sp1/Sp3^[25]. More importantly, Pdc4 was revealed by Asangani *et al.*^[26] to be a novel and independent prognostic factor in CRC. All of these suggested that miR-21 may be a poor prognostic biomarker. Consistently, the expression of miR-21 in a Japanese cohort ($n = 156$) and a German cohort ($n = 145$) was measured and analyzed by Harris's group. Elevated expression of miR-21 is correlated with poor prognosis in both stage II/III Japanese and stage II German cohorts^[12]. Similar results were also observed in another Japanese cohort^[11], American^[13], Hong Kong^[13], Czech^[27] and Danish^[28,29] cohorts of CRC patients.

In addition, the prognostic significance of miR-21 level was investigated in 306 patients with CRC at each Dukes' stage. However, a significant prognostic impact is not demonstrated in the analysis of Dukes' stage A patients, which may be due to the low rate of recurrence and death. This study showed that high miR-21 expression indicates poor overall survival (OS) and disease-free survival (DFS) in patients of Dukes' stage B, C and D^[30]. The prognostic value of miR-21 was also assessed for TNM stage. The TNM system describes a degree to which the tumor has invaded the

Table 1 Overview of functions of microRNAs in colorectal cancer

MiRNA	Disease progression	Biomarker	Treatment
MiR-21	Increased miR-21 correlated with CRC cell proliferation, invasion, lymph node metastases and advanced clinical stage ^[11,23,24] MiR-21 keeps increasing during the process from precancerous polyps to early cancer ^[20] High expression of miR-21 associated with poor progress in the stage II / III Japanese ^[11,21] and stage II German cohorts ^[21] , Hong Kong ^[25] , Czech ^[26] and Danish ^[27,28] cohorts of CRC patients	Increased miR-21 as an indicator for poor OS and DFS in patients of Duke stage ^[29] Elevated miR-21 as a marker for lymph metastasis in patients of TNM stage ^[30] MiR-21 in serum ^[33] as a noninvasive marker to detect early stage of CRC	Decreased miR-21 sensitizes CRC cells to 5-FU-treatment ^[21]
MiR-29	Elevated miR-29a is significantly correlated with metastasis, especially liver metastasis ^[38,39,42] Upregulation of miR-29a associated with a better outcome at 12 mo ^[43,44] High miR-29b expression associated with higher 5-yr DFS and OS ^[48]	MiR-29a as a biomarker for early detection of CRC and prediction of survival ^[38,43,44] MiR-29b as a biomarker for 5-yr DFS and OS (stage III CRC) ^[48]	
MiR-34a	Downregulation of miR-34a associated with CRC development ^[57] MiR-34a predicates recurrence of CRC patients ^[59] Increased miR-34b/c observed in more advanced tumors and associated with poor prognosis ^[60]	MiR-34a as a biomarker to predict recurrence of stage II and stage III CRC patients ^[59]	Increased miR-34a sensitizes CRC cells to 5-FU-treatment ^[58]
MiR-124a	High frequency of methylation of miR-124a in chronic inflammation and CRC ^[65,66] Methylation of miR-124a is emerging during oncogenesis in UC patients and could be used to estimate individual risk for cancer ^[71]	The methylation level of miR-124a as a factor for evaluating the risk of carcinogenesis in UC patients ^[71]	
MiR-130b	High level of miR-130b in advanced tumor stages (III-IV), miR-130b-PPAR γ axis plays a novel role in progressing towards more invasive CRC ^[77] MiR-130b inhibits CRC cells migration ^[75]	Increased miR-130b in advanced tumor stages ^[77]	
MiR-139-3p	Decreased miR-139-3p in CRC tissues ^[82,83] Downregulation of miR-139-3p associated with poor survival, especially in patients with TNM stages I and II ^[82]	MiR-139-3p as a marker for poor survival ^[82] MiR-139-3p in plasma used for predicting occurrence of CRC ^[84]	
MiR-155	High expression correlated with an advanced TNM stage and metastasis ^[85] Increased expression of postoperative miR-155 correlated with recurrence and metastasis of CRC ^[87]	MiR-155 as a prognostic maker for OS and DFS of CRC patients ^[11,86]	
MiR-224	Increased expression of miR-224 associate with tumor growth and metastasis of CRC ^[90] MiR-224 inhibits CRC cells migration ^[95]	MiR-224 as a predictor for the short-time relapse and shorter metastasis-free survival ^[90-93]	Suppression of miR-224 sensitizes CRC to chemoradiotherapy ^[94]
MiR-378	MiR-378 is up-regulated in CRC samples ^[96,97] and promotes cell survival, invasion, and angiogenesis ^[98,99] Expression of miR-378 is increased in plasma of CRC patient, and rapidly goes down within 4-6 mo after surgery ^[103] Decreased miR-378 in CRC tissues and cell lines is associated with increased the size of tumor, metastasis and short OS ^[104,105]	MiR-378 in plasma used to predict the occurrence of CRC ^[103] Reduction of miR-378 in CRC tissues as a predictor for short OS ^[104]	

UC: Ulcerative colitis; CRC: Colorectal cancer; OS: Overall survival; DFS: Disease-free survival.

intestinal wall and spread to the regional lymph nodes as well as distant organs. Compared to T1, T2 and T3 cases, Harris's group found that miR-21 expression level is significantly elevated in T4 cases. A similar result was also observed in the comparison of N1 to N0^[12]. Besides, patients with a high level of miR-21 expression are insensitive to 5-FU therapy, while decreasing miR-21 enables patients to achieve a better response to 5-FU. Recently, anti-miRNA based therapies have achieved primary progress in treatment of patients with chronic hepatitis C infection^[31]. Hence, anti-miR-21 based therapies seem to be promising for the future.

More importantly, the prognostic value of miR-21 in serum and stool of CRC patients has also been extensively investigated. Due to the ineffectiveness of a

direct amplification method, the importance of circulating miR-21 was vague^[32]. Recently, TaqMan assays as an effective approach was used by Kanaan *et al.*^[33] and they found dramatically upregulated plasma miR-21 in patients with CRC. Toiyama *et al.*^[14] systematically investigated the expression of miR-21 in medium collected from 2 CRC cell lines and serum from 12 CRC patients and 12 healthy volunteers and concluded that miR-21 is a secretory miRNA. They further expanded verification of circulating miR-21 expression in 246 CRC cases, 53 controls and 43 patients with polyps. They also tested whether serum miR-21 reflects that of CRC tissues in 166 matched CRC specimens. MiR-21 significantly increases in serum from patients with precancerous polyps and CRCs. Notably, its expression decreases in patients' serum after surgery.

Moreover, serum miR-21 expression shows an apparent difference between patients with precancerous polyps and controls. Accumulated miR-21 level is correlated with tumor size, metastasis and poor survival. Thus, miR-21 in serum could be an ideal noninvasive biomarker to detect CRC early and evaluate the prognosis. Additionally, the expression of miR-21 in stool samples is different in healthy individuals compared to CRC individuals. Link *et al.*^[34] found higher expression of miR-21 in stool from 29 patients with CRC compared to 8 healthy individuals. A similar result was also revealed by Wu *et al.*^[35] from 88 patients with CRC and 101 healthy controls. Furthermore, miR-21 in later TNM carcinoma stages was reported to exhibit a more pronounced expression^[36]. To sum up, miR-21 expression could be a promising biomarker to predict the outcome of CRC patients.

MiR-29 family

MiR-29 family consists of three members: MiR-29a, miR-29b and miR-29c. Members of this family have been shown to be dysregulated in many different types of cancers. MiR-29 family members exert function by targeting genes involved in cell proliferation, senescence and metastasis at genetic and epigenetic levels, which makes them effective regulators of tumorigenesis and cancer progression^[37].

MiR-29a: Huang *et al.*^[38] analyzed 100 CRC samples and 59 controls and found that the expression levels of miR-29a in plasma are significantly upregulated. The authors further investigated the diagnostic significance of plasma miR-29a in 37 early lesions of CRC and found an obvious increase in miR-29a expression compared to that of the control, suggesting the plasma miR-29a appears to be a novel biomarker for early detection of CRC^[38]. In addition to serving as a noninvasive tool to detect the CRC earlier, the prognostic value of miR-29a can also be applied to the early detection of CRC metastasis. Tang *et al.*^[39] analyzed the expression levels of miR-29a and KLF4 mRNA in 85 cases using quantitative real-time polymerase chain reaction (qRT-PCR). Because KLF4 has been identified as a novel target of miR-29a, KLF4 inhibits metastasis through inhibition of MMP2 and upregulation of E-cadherin^[40,41]. Tang *et al.*^[39] found that low KLF4 mRNA expression is correlated with metastasis. More importantly, a correlation between miR-29a expression and metastasis was observed in this study, with elevated miR-29a indicating metastasis and worse survival of CRC patients. Wang *et al.*^[42] recruited a total of 114 participants, including 58 liver metastatic patients and 56 non-metastatic CRC patients, into their study since colorectal liver metastasis is most common. They discovered that the expression of miR-29a in the serum is significantly elevated in colorectal liver metastatic patients. Besides, the significantly elevated expression of miR-29a was found in CRC patients with advanced tumor T stage, while miR-29a shows a non-significant elevation in patients with advanced tumor N stage. Consequently,

they discovered that miR-29a can serve as a promising non-invasive, economic screening tool for early detection of colorectal liver metastasis^[42]. In conclusion, high expression of miR-29a is associated with poor prognosis and metastasis.

With regards to the prediction of CRC early recurrence, Kuo *et al.*^[43] found that both miR-29a and miR-29c show significantly decreased expression levels in 43 patients with early recurrence compared to 35 patients of non-early recurrence. Increased miR-29a or increased miR-29c suggests a better outcome at 12 mo. However, only miR-29a can be used as a predictor of the early recurrence. That miR-29c fails to predict the early recurrence may be due to a short follow-up or a small sample size^[43]. Furthermore, the prognostic value of miR-29a was also found in stage II CRC. Weissmann-Brenner *et al.*^[44] examined the microRNA array expression profile in 51 stage I and 59 stage II CRC samples and then 903 miRNA expressions were verified by qRT-PCR. The authors defined poor prognosis as recurrence within 3 years after surgery. Their data revealed that in stage II, miR-29a solely shows an obvious difference between patients with good prognosis and those with poor prognosis. However, in stage I, there are no miRNA with different expressions between the two groups. The result showed the prognostic value of miR-29a on the recurrence in patients with stage II. They also concluded that higher expression of miR-29a is associated with a longer DFS^[44].

MiR-29b: As a member of the miR-29 family, miR-29b is the most highly expressed in the miR-29 family and is found at two genomic loci^[45]. MiR-29b can inhibit proliferation and induce apoptosis in CRC cells and mediate the inhibition of epithelial-mesenchymal transition (EMT), which is closely related to the prognosis of CRC. Moreover, Yuan *et al.*^[46] performed qRT-PCR to test miR-29b in 41 matched-paired CRC samples and reported that miR-29b is significantly decreased in CRC, suggesting that miR-29b is associated with tumor size, advanced clinical stage and lymph node metastasis of CRC^[46,47]. Furthermore, Inoue *et al.*^[48] analyzed miR-29b expression in 245 patients with CRC. The patients were divided into two groups: Those under the median (low) and those above the median (high) of miR-29b expression. Their analysis revealed that high miR-29b expression is significantly associated with higher 5-year DFS and OS. In sub-analyses by each stage, they found that miR-29b expression has a prognostic impact on 5-year DFS solely in patients with stage III CRC, which showed that low miR-29b expression is an independent predictor of a reduced 5-year DFS. In addition, low miR-29b expression is predictive of lymph node metastasis and a pathological T classification, indicating the prognostic value of miR-29b in stage III CRC^[48]. Hence, the dysregulation of miR-29a and miR-29b can serve as a biomarker to predict early recurrence and shorter DFS in CRC.

MiR-34

The miRNA-34 family (miR-34a, miR-34b and miR-34c) has been reported to be a tumor suppressor regulated by the TP53 and DNA hypermethylation. The miR-34 family influences a series of cancer cell activities, such as stemness, metastasis and chemoresistance^[49]. It is well known that miR-34 is directly regulated by p53 at transcriptional level and miR-34 exerts p53 downstream effects through targeting c-MET, CDK6 and c-MYC to regulate proliferation arrest and to induce apoptosis^[50]. MiR-34a treatment results in downregulation of NOTCH1 and induction of apoptosis^[51]. The expression level of miR-34a is crucial for CRC cells to self-renew or divide. In addition, the NOTCH signaling regulates asymmetric division of stem cells. High miR-34a levels downregulate NOTCH signaling and suppress symmetric division, thus reducing the production of colon cancer stem cells (CCSC)^[52,53].

Proteinase activated receptor 2 (PAR2) is positively correlated with tumor progression in CRC. Ma *et al.*^[54] described that miR-34a is inhibited by PAR2, resulting in the upregulation of Cyclin D1 and TGF- β in CRC cells. MiR-34a promoter methylation in CRC tissues is associated with metastasis. Consistently, in 101 of 111 CRC tissues and in 9 of 9 cell lines, the miR-34 family is epigenetically silenced; hence, the authors pointed out that the methylation of miR-34a promotes motility and metastasis^[55,56]. In another study, the downregulation of miR-34a was noticed in some CRC patients, indicating the role of miR-34a in CRC development^[57].

The potential treatment value of miR-34a was also tested. In 5-FU-resistant CRC DLD-1 cells, low levels of miR-34a were observed. The restoration of miR-34a significantly sensitizes cells to 5-FU treatment and inhibits cell growth^[58]. Furthermore, miR-34a as a recurrence biomarker has been investigated. In two independent cohorts of 268 CRC patients, miR-34a expression is positively associated with DFS survival and can serve as a prognostic factor for recurrence of CRC. In addition, compared to patients with p53-negative expression, miR-34a is much higher in those with p53-positive expression. The authors concluded that miR-34a inhibits cell growth and invasion of CRC in a p53-dependent manner and predicts recurrence in stage II and III CRC patients^[59].

Most recently, the effects of the miR-34 family on prognosis was also systematically surveyed in CRC and an increased miR-34b/c predominantly expressed in stromal tissues was revealed to be associated with poor prognosis in CRC^[60]. The relationship between miR-34b/c and the development of disease was investigated in CRC samples from 159 American and 113 Chinese by qRT-PCR. They found that miR-34b/c was accumulated in advanced tumors and associated with poor cancer-specific mortality in two independent cohorts. TP53 regulates the expression of miR-34b/c at transcriptional level. Moreover, compared with epithelial tissue, miR-34b/c is enhanced greatly in cancer stroma. Collectively, miR-34 b/c may contribute to cancer-stromal interaction associated with CRC progression. All data presented

above revealed that the miR-34 family might be an ideal tool to predict the prognosis and recurrence in CRC.

MiR-124a

MiR-124a is known as a tumor suppressor gene, which has been shown to downregulate oncogenic cyclin-dependent kinase 6 (CDK6) involved in carcinogenesis, resulting in cell-cycle arrest at the G1-S checkpoint^[61,62]. MiR-124a was reported to be expressed at low levels in CRC due to the methylation^[63]. On one hand, miR-124a is the most frequently methylated in CRC compared to other tumor types, indicating the methylation status of miR-124a may be a specific marker for CRC. As mentioned by Deng *et al.*^[64], the lower expression of miR-124a is associated with the methylation of it and the treatment with 5-aza-2'-deoxycytidine can induce the expression of miR-124a. Among CRC, cancer of the pancreas, stomach, liver, lung, breast, kidney and prostate and melanoma, they found that CRC shows the highest frequency of methylation of miR-124a and a high frequency of methylation of miR-124a was also observed in polyps^[65,66]. Together, the methylation of miR-124a may be an early event in all pathways of colorectal carcinogenesis. On the other hand, the transcription of miR-124a is controlled by DNA methylation of the promoter regions of three miR-124a isoforms (miR-124a-1, 2 and 3) in CRC^[61,62,67,68]. It was known that aberrant methylation of miR-124a is induced in chronic inflammation^[69]. In the colorectal mucosa of pediatric patients with ulcerative colitis (UC), miR-124a was reported to positively regulate the expression of signal transducer and activator of transcription 3 (STAT3), which is a major factor in inflammatory response^[70], indicating that silencing of miR-124a is related to the promotion of inflammation in colorectal mucosa through the STAT3 signaling pathway. Ueda *et al.*^[71] tested the miR-124a level in 40 UC patients without CRC, 4 patients with CRC or dysplasia, 8 sporadic CRC patients and 12 normal controls. They found that miR-124a-1, 2 and 3 genes are all methylated and cyclin-dependent CDK6, as the target of miR-124a, is elevated in neoplastic samples. The methylation level of miR-124a-3 is significantly higher in pancolitis than in HV and methylation levels in long-standing UC are higher than in short-term UC. Moreover, in contrast to patients without long-standing UC and pancolitis, the methylation level of patients with these risk factors shows a 7.4-fold increase. Collectively, methylation of miR-124a-3 is emerging during oncogenesis in UC patients and could be used to estimate individual risk for cancer.

MiR-130b

Previous studies demonstrated that miR-130b is significantly dysregulated in some tumors, such as CRC, clear cell renal cell cancer (ccRCC), liver cancer, osteosarcomas and pancreatic cancer, and contribute to the tumorigenesis^[72-76]. In 52 pairs of pancreatic cancer tissues, Zhao *et al.*^[72] showed that miR-130b is significantly downregulated, which is correlated with a worse prognosis, increased tumor size, late TNM stage,

lymphatic invasion and distant metastasis of pancreatic cancer. Wu *et al.*^[74] used the microarray technology to profile miRNA expression of 28 localized and metastatic ccRCC specimens and 78 benign tissues and samples from ccRCC patients who had at least 5 years follow-up if no metastasis developed. They speculated that miR-130b is associated with ccRCC metastasis and prognosis. However, in CRC, Colangelo *et al.*^[77] identified that miR-130b directly targets peroxisome proliferator-activated receptor γ (PPAR γ). Furthermore, they provided data that miR-130b exerts biological functions mostly through suppression of PPAR γ , leading to deregulation of E-cadherin, Snail, PTEN and VEGF. As the enhanced miR-130b was found in III-IV tumor stages of CRC, they proposed that the miR-130b-PPAR γ axis plays a novel role in progressing towards more invasive tumors. Thus, miR-130b-PPAR γ may be a promising biomarker to predict prognosis. On the other hand, we previously reported the inhibitory function of miR-130b in migration and invasion of CRC because it downregulates the expression of integrin β 1^[75], which mediates cell migration in a wide variety of human cancers. Blocking integrin β 1 can inhibit the transformation of human breast cancer cells^[78,79]. All of the above gave us a hint that miR-130b may have potential value in the prognosis of CRC.

MiR-139-3p

Previous studies reported the abnormal expression of miR-139-3p in some types of human cancers, such as adrenocortical cancer, clusters in bladder carcinoma and CRC^[80-82]. Accumulating evidence suggested the potent role of miR-139-3p as a molecular biomarker for CRC. Chen *et al.*^[83] screened the difference of miR-139-3p expression among CRC tissues, matched normal samples and celecoxib-treated HT-29 CRC cells through miRNA microarray, then verified the readout by qRT-PCR. They found that miR-139-3p is downregulated in CRC tissues and that the expression of miR-139-3p is different from the early to advanced stage. Similar data were also reported by Kanaan *et al.*^[84] in that miR-139-3p in plasma may be used to distinguish CRC patients and normal individuals by examining 12 healthy controls and 20 CRC patients. Therefore, miR-139-3p may have a potential role in CRC early diagnosis. In addition, miR-139-3p may be related to poor prognosis of CRC. Liu *et al.*^[82] examined the expression level of miR-139-3p in 63 pairs of CRC and adjacent tissues. Compared with the adjacent normal controls, miR-139-3p expression levels in CRC tissues are notably decreased and decreased miR-139-3p is significantly associated with poor OS, especially in patients with TNM stages I and II. In conclusion, miR-139-3p has potential as an early diagnostic and prognostic biomarker for CRC.

MiR-155

MiR-155 is encoded by the non-protein-coding transcript of the B-cell integration cluster gene (*BIC* gene). Altered expression of miR-155 has been described in multiple

tumors, reflecting staging, progress and treatment outcomes. Zhang *et al.*^[85] found a significant increase in miR-155 in cancer tissues compared to the matched normal samples after analyzing 76 clinical samples of patients with CRC. MiR-155 can suppress E-cadherin and upregulate ZEB-1 through promoting expression of claudin-1, leading to increased cell migration and metastasis. Furthermore, there is a correlation between miR-155 expression and lymph node metastasis, advanced TNM stage and distant metastasis. Together, all of these results indicated that miR-155 plays an important role in CRC development and metastasis^[85]. In another study, Shibuya *et al.*^[11] revealed that patients with increased miR-155 show poorer OS and DFS than those with decreased miR-155, suggesting that miR-155 has independent prognostic values for OS and DFS of CRC patients. The link of miR-155 with the poor prognosis in CRC was also proved by Lv *et al.*^[86] by multivariate analysis. Consistent with the data above, Hongliang *et al.*^[87] assessed the levels of serum carcinoembryonic antigen (CEA) and miR-155 in 84 matched-pairs specimens from patients with CRC before and after surgery. It is well known that the CEA is mainly used for prognosis, observation of curative effect and monitoring recurrence and metastasis in CRC. The authors found that miR-155 expression is significantly increased in CRC tissues and is notably associated with tumor relapse and metastasis. Before surgery, miR-155 expression in patients correlates positively with the serum CEA levels and the high postoperative miR-155 expression level is associated with the short duration before the serum CEA level increases again^[87]. Moreover, Lv *et al.*^[86] discovered that there is no change in serum miR-155 expression level between controls and stage I CRC patients after measuring the serum of 146 CRC patients and 60 healthy controls. However, there is a great elevation of miR-155 expression in stages II-IV patients. Thus, miR-155 in serum cannot be used as a biomarker for early diagnosis^[86]. Taken together, miR-155 in tissues combined with serum CEA could provide concrete clues for the diagnosis of CRC and prediction of recurrence.

MiR-224

MiR-224 is consistently reported to be upregulated in CRC and it can potentially affect many cellular processes associated with cancer, including cell proliferation, growth, differentiation and cell death^[88]. High expression of miR-224 was observed in CRC tissues. The clinicopathological significance of miR-224 in CRC was recently evaluated in 110 CRC patients by Liao *et al.*^[89] who found that increased expression of miR-224 is significantly associated with an aggressive phenotype and poor prognosis of CRC. They also pointed out that miR-224 accelerates the G₁/S-phase transition through activation of Akt/FOXO3a signaling by targeting PHLPP1 and PHLPP2, antagonists of PI3K/Akt. MiR-224 also downregulates p21Cip1 and p27Kip1 and upregulates

cyclin-D1. Therefore, miR-224 promotes CRC tumor growth^[89]. The study by Nicoloso's group^[90] supported that miR-224 is an activator for CRC metastasis *via* targeting SMAD4 and miR-224, alone or combination with SMAD4, may be an independent prognostic marker for survival of patients with CRC. Clinical outcomes correlated with miR-224 status were analyzed in 6 sets of 449 CRC cases in order to assess the difference in survival between patients with low or high levels of miR-224 expression. Their data indicated that miR-224 expression increases consistently with tumor burden and microsatellite stability status and enhances CRC metastasis through targeting SMAD4. Patients with high miR-224 levels display shorter OS in multiple CRC cohorts and shorter metastasis-free survival^[90,91]. Consistent with the data from Nicoloso's lab, Zhang *et al.*^[92] also pointed out that SMAD4 is regulated by miR-224, suggesting miR-224 as a new biomarker for recurrence of CRC. They collected a total of 108 stage I - II colorectal patients who received radical resection and evaluated clinicopathological information of 40 patients with tumor relapse and 68 without relapse within 3 years after surgery. Their data suggested that miR-224 is notably increased in CRC tissues and this upregulation is associated with recurrence and poor DFS. By analyzing precancerous polyps, a similar conclusion was also achieved by Adamopoulos *et al.*^[93]. In addition, miR-224 also has some links with the treatment of CRC. Preoperative chemoradiotherapy (CRT) is the standard treatment for locally advanced rectal cancer and miR-224 was found to result in an increased resistance to CRT in CRC cell lines^[94]. In another study, when the expression of miR-224 was investigated in 79 specimens from CRC patients and 18 healthy controls, miR-224 in CRC tissues was greatly downregulated. Moreover, miR-224 suppresses the migratory ability of the CRC cell line through targeting Cdc42. In a word, this research indicated the vital role of miR-224 in suppressing cell migration of CRC. They concluded that miR-224 might be used as a promising biomarker to predict CRC development. The high expression of miR-224 predicts the short-time relapse and shorter metastasis-free survival and besides, miR-224 can increase the resistance to CRT^[95]. Collectively, miR-224 may be a prognostic biomarker predicting the patients' survival outlook.

MiR-378

MiR-378 is expected to participate in the process of multiple tumorigenesis and play an important role in CRC. Previous studies demonstrated that miR-378 is upregulated in CRC samples^[96,97], targets the tumor suppressor genes Sufu and Fus-1 and regulates cancer progression by promoting cell survival, invasion and angiogenesis^[98,99]. The difference of miR-378 expression level between cancer individuals and healthy individuals in blood and tumor tissues was reported. For example, Hauser *et al.*^[100] reported that miR-378 in serum is significantly increased in 25 cCRC patients compared with 25 healthy individuals. Liu *et al.*^[101] showed that

serum miR-378 could serve as a novel noninvasive biomarker in gastric cancer detection. All of the above suggested that miR-378 might be a possible tumor biomarker. Moreover, miR-378 in plasma may have the highest predictive capability in CRC^[102]. The authors further investigated miRNA expression in the plasma of 65 CRC patients and 70 healthy individuals and found that miR-378 significantly increases in plasma from CRC patients once the level of miR-378 goes down notably after surgery. In addition, they also found that miR-378 expression decreases in patients who have no relapse within 4-6 mo after surgery, which further explained that plasma levels of miR-378 may be used to discriminate CRC patients from normal individuals^[102].

However, in another study, Zhang *et al.*^[103] pointed out the downregulation of miR-378 in 84 matched pairs CRC samples and cell lines. MiR-378 was considered a tumor inhibitor since it can suppress tumor cell growth in a nude mice model^[103,104]. In addition, there is a strong association between the reduction of miR-378 and increased tumor volume, metastasis and short OS of CRC patients. Consistently, miR-378 as a tumor suppressor was also observed by Wang *et al.*^[105] after analyzing the expression of miR-378 in 47 pairs of CRC samples. All of the above suggested that miR-378 plays a vital role in carcinogenesis and could serve as a biomarker to predict the outcome of CRC.

In a word, miR-378 may predict the presence of CRC and serve as a potential and reasonable biomarker for the early diagnosis of CRC.

CONCLUSION

MiRNA might be a powerful tool in diagnosis and treatment of CRC through modulating various crosstalks of oncogenic signaling pathways. MiRNAs we discussed here are, to date, the most extensively investigated tumor suppressor/enhancer miRNAs in CRC. Through *in vivo* and *in vitro* experiments, related miRNAs have been proved to be dysregulated in CRC and to be possible ideal diagnostic, prognostic and therapeutic tools (Table 1). However, some obstacles in miRNA-based therapies need to be overcome, such as degradation by nucleases, inefficient delivery to cells, fast blood clearance, renal toxicity and hemodynamic toxicity. Currently, much more efficient intracellular delivery systems are being developed to benefit miRNA-based clinical treatments, such as nanoparticle and the combination of miRNA in combination with other anticancer agents. In our review, contradictory findings regarding some miRNAs are introduced since we consider that the phenomenon could be explained by less informative clinical data, especially the lack of the definition of "healthy" control subjects, which will very likely affect the miRNA quality. Secondly, population ethnicity may be a potential confounding variable. Lastly, we hope that further studies concerning diagnosis and therapy will be done by more groups in the future in order to finally optimize the uses of miRNAs for

subsequent translation in the clinical setting.

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2016 *Helicobacter pylori*: Global view

Helicobacter pylori associated Asian enigma: Does diet deserve distinction?

Syed Faisal Zaidi

Syed Faisal Zaidi, Department of Basic Medical Sciences, College of Medicine, King Saud bin Abdulaziz University of Health Sciences, Jeddah 21423, Saudi Arabia

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Correspondence to: Dr. Syed Faisal Zaidi, PhD, Assistant Professor, Department of Basic Medical Sciences, College of Medicine, King Saud bin Abdulaziz University of Health Sciences, Jeddah 21423, Saudi Arabia. sfaisalhaz@gmail.com
Telephone: +966-9-22245778
Fax: +966-9-20008668

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Abstract

Helicobacter pylori (*H. pylori*) is one of the most widespread infections in humans worldwide that chronically infects up to 50% of the world's population. The infection is involved in the pathogenesis of chronic active gastritis, peptic ulcer, mucosa-associated lymphoid tissue lymphoma and gastric cancer, therefore, it has been

classified as class I definite carcinogen by the World Health Organization. Despite the established etiological role of *H. pylori*, its actual distribution and association with related diseases is controversial and there is a large intercountry variation especially among Asian countries. *H. pylori* infection is more frequent in developing countries like India, Pakistan, and Bangladesh as compared to developed Asian countries like Japan, China and South Korea. However, the frequency of gastric cancer is comparatively lower in India, Pakistan, and Bangladesh with that of Japan, China and South Korea. Such phenomenon of clinical diversity, defined as enigma, is judged by genetic variability of the infecting *H. pylori* strains, differences in the host genetic background in various ethnic groups, and environmental factors such as dietary habits. Most of the studies have so far focused on the bacterial factor while environmental issues, including dietary components, were not given due attention as one of the factors related with *H. pylori* associated gastric carcinogenesis. The dietary factor has been suggested to play an important role in *H. pylori* related carcinogenesis, and in this respect several studies have corroborated the intake of various dietary components as modulatory factors for gastric cancer risk. In this review, such studies, from *in vitro* experiments to clinical trials, are being focused in detail with respect to enigma associated with *H. pylori*. It may be conceivably concluded from the available evidence that dietary factor can be a game changer in the scenario of Asian enigma, particularly in high risk population infected with virulent *H. pylori* strains, however further affirmation studies are desperately needed to achieve conclusive outcomes.

Key words: *Helicobacter pylori*; Asian enigma; Gastric cancer; Dietary factor; Salt; Fermented food; Spices

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Core tip: Despite the established etiological role of *Helicobacter pylori* (*H. pylori*), its actual distribution and association with related diseases is controversial,

especially among Asian countries, a phenomenon termed as Asian enigma. This is judged by genetic variability of the infecting *H. pylori* strains, diversity in the host genetic background, and environmental factors such as diet. Amongst these, the dietary factor was not given much attention. In this review, dietary components are focused in detail with respect to *H. pylori*-associated enigma with a specific emphasis and comparison of dietary ingredients between Asian countries in order to critically evaluate its role in Asian enigma.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) have been found in the stomachs of humans in all parts of the world. It is one of the most common bacterial infections worldwide, infecting more than half of the world's population^[1]. *H. pylori* infection in the stomach induces mucosal inflammatory response and oxidative stress that leads to diverse clinical outcomes in humans such as gastritis, peptic ulcer and gastric cancer^[2]. There is a strong correlation between prevalence of infection and socioeconomic status^[3]. In some developing countries 70%-90% of the population is infected with *H. pylori*, whereas in high-income countries the prevalence is 25%-50%. Most infections are acquired in childhood^[4,5]. However, the incidence of *H. pylori* infection is declining, and today only 10% of children in high income countries are infected^[6,7]. Regardless of the established etiological role of *H. pylori*, its actual distribution and association with related diseases is controversial especially in Asian countries. *H. pylori* infection is more frequent in developing Asian countries like Thailand, Malaysia, India, Pakistan, and Bangladesh, whereas, the occurrence of gastric cancer is comparatively lower than in developed Asian countries like Japan, China, and South Korea^[8-10]. Such phenomenon of clinical diversity, defined as Enigma, is judged by genetic variability of the infecting *H. pylori* strains with respect to virulence factors, difference in the host genetic makeup, and environmental factors such as dietary habits (Figure 1)^[11].

PREVALENCE OF *H. PYLORI* INFECTION IN ASIAN COUNTRIES

Epidemiological studies indicate that Asian countries have a high prevalence of *H. pylori* infection as compared to Western countries, with a correspondingly high incidence of severe gastroduodenal (GD) diseases, especially gastric neoplasia^[8]. However, the frequency of *H. pylori* infection differs markedly between and within populations

of different Asian countries. In developing countries like India, Pakistan, Bangladesh and Thailand, infection with *H. pylori* is more frequent among the general population^[9]. In contrast, in more industrialized and developed regions of Asia like Japan, China, and South Korea, frequency of *H. pylori* infection has been reported to be somewhat lower^[8]. Singapore is also in the same category and the prevalence of *H. pylori* infection is quite low compared to developing countries^[10].

FREQUENCY OF GASTRIC CANCER IN ASIAN COUNTRIES

Although the overall incidence of gastric cancer is declining, it is still the world's second most widespread malignancy, having been overtaken by lung cancer^[12]. There is a marked variation internationally in gastric cancer incidence with highest rates reported from Japan^[8,13]. It is noteworthy that despite Japan being a developed country with a lower prevalence of *H. pylori* infection, it has the highest frequency of gastric cancer. The annual incidence of gastric cancer in Japan is around 100 times higher than those in India. Similarly, the frequency of gastric cancer is quite high in China despite a lower frequency of *H. pylori* infection^[8]. Contrary, people living in underdeveloped countries of Asia with high frequency of *H. pylori* infection have a lower incidence of gastric cancer^[8,14]. Some studies from India revealed no correlation between *H. pylori* infection and gastric cancer, while studies from China and Japan are consistent with previous findings, affirming association between *H. pylori* infection and gastric cancer^[15-17]. It has also been observed that frequency of gastric cancer varies within regions of a particular country; for example, in Japan and India^[16,18]. These corroborations support a potential role of other factors in the diverse and contradictory presentation of gastric diseases in different regions and populations.

The above mentioned evidences point out the fact that only *H. pylori* infection is solely not enough to cause life threatening conditions like gastric cancer, highlighting the importance of factors behind enigma, like genetic variability of the infecting strain and infected individual, along with life style habits.

As much has been focused on the genetic differences of host and agent, therefore, in this review the basics of these factors will be covered, while more emphasis will be on environmental factors including life style and dietary components, to critically evaluate their plausible role in *H. pylori* associated enigma.

REASONS BEHIND ENIGMA

Bacterial factors

H. pylori is a micro-aerophil gram-negative bacterium with several flagella required for bacterial motility^[19]. Numerous biochemical factors are produced by *H. pylori* that are

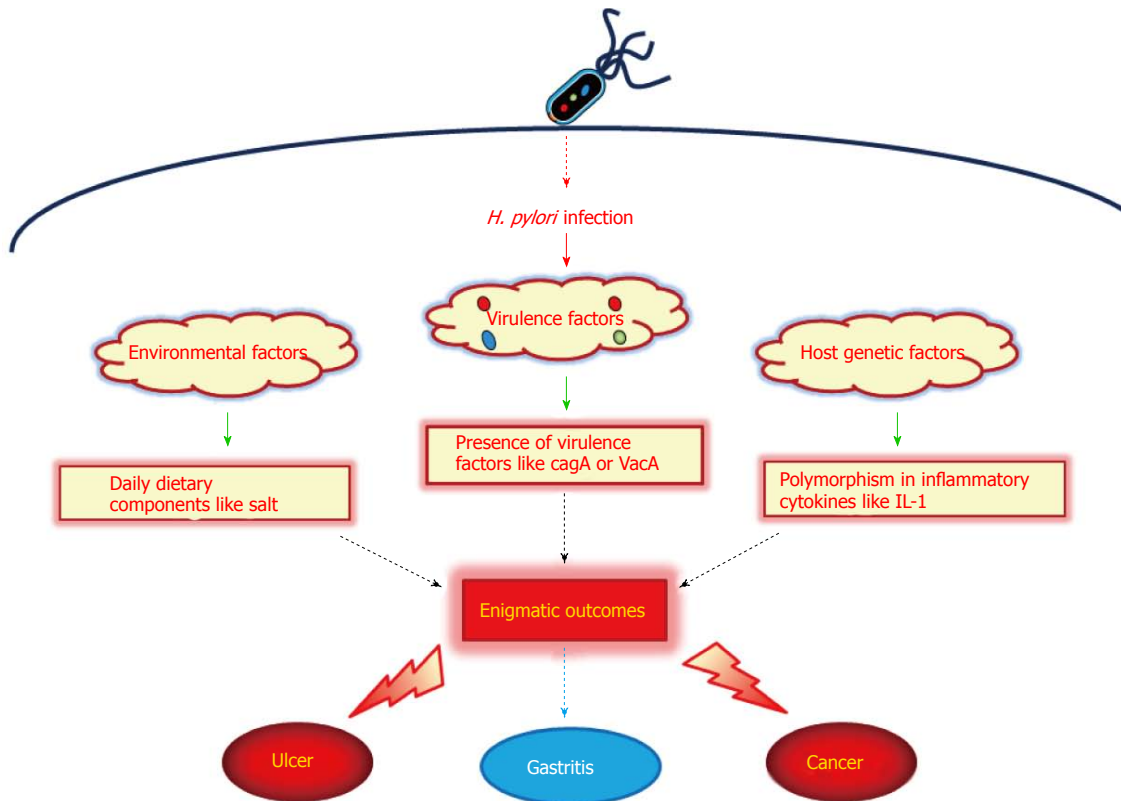


Figure 1 Three main factors behind *Helicobacter pylori* associated enigma namely: (1) Bacterial virulence factors; (2) Host genetic factors; and (3) Environmental factors. *H. pylori*: *Helicobacter pylori*; cagA: Cytotoxin associated gene-A; VacA: Vacuolating cytotoxin A; IL-1: Interleukin-1.

important to the organism's survival, virulence and initiation of pathophysiological effects in the infected host. Multiple pathways are modulated after *H. pylori* infection either through direct interaction or *via* virulent factors injected in the host cell which leads to the pathological outcome. Some of the important virulence factors are mentioned below that are responsible for bacterial colonization and pathogenesis, which are postulated to play some role in enigmatic diversity of clinical outcomes.

It is postulated that all strains of *H. pylori* are not pathogenic and the genetic diversity of *H. pylori* virulence factors has been linked with clinical outcome. The most pathogenic and well-established cluster of virulence in *H. pylori* is the *cytotoxin associated pathogenicity island (cag PAI)*; this being a 27-gene locus that is present in a majority of the clinical strains found in Europe, United States and Japan. *H. pylori* strains with the cag PAI have been shown to be more virulent, with an increased risk of development of GD disorders like duodenal ulcers and gastric adenocarcinoma, than strains lacking the gene complex^[20]. The *cytotoxin associated gene A (cagA)* is located in the cag PAI, and encodes for the cagA protein. Inside the host cell the cagA interferes with cell signaling pathways and induces cytoskeletal rearrangements^[21,22]. It has been suggested that cag PAI positive strains are involved in the activation of transcription factor nuclear factor-kappa B (NF- κ B), resulting in production of inflammatory or carcinogenic molecules such as interleukin-8 (IL-8) or activation-induced cytidine

deaminase (AID)^[21-23]. The *in vitro* observation of large vacuoles in the cytoplasm of cells incubated with *H. pylori* lead to the discovery of the *Vacuolating cytotoxin A (VacA)*^[24]. VacA induces apoptosis in epithelial cells, but it is still not clarified why vacuolation is required for this type of apoptosis. The VacA protein inserts itself into the epithelial cell membrane and forms a channel through which bicarbonate and organic anions can be released^[25]. Several studies have documented the genetic polymorphism in VacA gene and have concluded that the presence of both cagA and VacA genotype s1/m1 is more associated with severe disease outcomes^[26-28]. The gene *babA2* encodes for the protein BabA, which is an outer-membrane protein. *H. pylori* strains that possess the *bab2* gene are associated with an increased incidence of gastric adenocarcinoma. BabA-expressing strains adhere more tightly to epithelial cells, which might promote pathogenesis^[29]. Another virulence factor, *iceA*, has been linked with peptic ulceration and increased mucosal IL-8 secretion^[30,31]. Although the above mentioned studies provide evidence for the linkage of genetic variation in *H. pylori* with the disease outcomes, it is still not always conclusive due to controversial results. Genotyping analysis of *H. pylori* from an Indian population showed high prevalence of pathogenic strains in both adults and children with GD diseases as well as in control subjects^[9,32]. However, the incidence of gastric cancer is quite low in India as discussed above. Hence other factors behind enigma play simultaneous role in producing the

final outcome of *H. pylori* infection.

HOSTS GENETIC FACTORS

As discussed above, the basis for the diverse clinical outcomes of enigmatic scenario particularly in Asian countries cannot be entirely explained on the strain genetic diversity, as most patients are infected by more virulent strains than in Western countries^[8]. Another reason behind enigma is the host genetic makeup or ethnicity. Infection with any microorganism results in immune response leading to expression of inflammatory cytokines. Polymorphism in the genes of these cytokines may affect the dynamics of response to any infection, including that of *H. pylori*. Few studies have reported such polymorphism in the gene cluster of IL-1, a proinflammatory cytokine with a potent acid inhibitory effect, and its association with gastric cancer risk^[33,34]. Polymorphisms in other candidate cytokine genes, such as tumor necrosis factor- α and IL-10, may enhance or suppress inflammation of the gastrointestinal (GI) mucosa resulting in different disease outcomes^[35,36]. Besides inflammatory cytokines, gastric atrophy associated with reduced acid secretion is also documented to be linked with the ethnic background. For example, the Japanese population has much lower acid secretion as compared to the western population^[37]. As reported, gastric atrophy occurs more readily in subjects with lower acid secretion than in those with high acid secretion^[38]. It was further confirmed by Kuipers *et al*^[39] that the use of proton pump inhibitors, drugs that reduce gastric acid secretion, in the patients of reflux esophagitis aggravated the severity of *H. pylori*-associated gastritis. This may be one of the reasons that the Japanese have high incidence of gastric cancer, as gastric atrophy is a precancerous lesion. In short, the above findings support the role of genetic makeup or ethnicity in *H. pylori*-associated enigmatic disease outcome but still further studies are required from other Asian countries like Malaysia, India or Pakistan to conclude the precise role of polymorphisms in specific genes or combinations of genes for disturbances in acid secretory output and gastric cancer risks in Asian countries.

DIETARY AND ENVIRONMENTAL FACTORS

Stomach and intestines are among the parts in our body that are exposed at maximum to our daily diet and of course environmental factors like personal hygiene can equally affect them. Hence, it is postulated that diet may play a critical role in GD disorders like peptic ulcer or gastric carcinoma. When talking about enigma associated with *H. pylori* under the light of recent scientific and molecular studies, dietary components can modulate the pathogenic processes by simple anti-oxidant to complex anti-carcinogenic activities. The paradoxical clinical outcomes after *H. pylori* infection might be crucially regulated by dietary habits of the

population, especially when talking about the Asian population under Asian enigma. It has been known since ancient times that healthy diet prevents digestive problems and avoid chronic malaise, but it was not long ago when the dietary factor was brought into focus with respect to *H. pylori* associated pathogenesis. It was first reported by Holcombe^[40] back in 1992 in his leading article on African enigma that environmental factors including dietary habits can influence the outcome of *H. pylori* infection. Several studies from various countries thereafter followed Holcombe's hypothesis and documented the plausible role of dietary components in disease outcome.

East Asian population and dietary habits

So as to discuss about Asian enigma, two sets of populations can be broadly classified as mentioned above; one with low incidence of gastric cancer and high prevalence of *H. pylori* such as the South Asian population including India, Pakistan, and Bangladesh and the second with high incidence of gastric cancer and low prevalence of *H. pylori* such as the East Asian population including Japan, China and Korea. Among the latter, Japanese researchers initiated the focus on the dietary factor in their population to uncover the plausible role of diet in Asian enigma not only by comparing it with western dietary habit but also within different regions of Japan^[8]. Both aggravating (negative) and alleviating (positive) effects of diet have been documented with respect to *H. pylori* associated pathogenesis but majority of the studies from Japan linked dietary components as a negative regulator. This may be due to the fact that the Japanese population is well recognized for high prevalence and mortality due to gastric cancer and the diet therein might be a negative regulator. However, it is interesting to note that different regions of Japan have a variable incidence of gastric cancer. For example, northern regions in Japan such as Akita prefecture have a higher prevalence of gastric cancer than the Southern region of Okinawa prefecture^[41]. While looking at the dietary pattern in Akita, it was found that salt has been highly consumed in this region almost double to that of Okinawa^[42]. This may further provide the convincing evidence that in a population of similar host genetic makeup and *H. pylori* strains, it may be due to different dietary habits, like high salt intake in that region that can lead to enigmatic outcomes.

High salt consumption is one of the most extensively investigated dietary component which is well known to increase the risk of gastric cancer^[43,44]. Furthermore, interesting data was published in a prospective study of a Japanese population which demonstrated the aggravating effect of high-salt diet in *H. pylori* infected subjects with an increase in gastric cancer risk, when compared with infected subjects with lower consumption of salt^[45]. This may be one of the leading clues for the potential role of diet in *H. pylori* associated enigma. *In vivo* studies have also shown that high salt not only

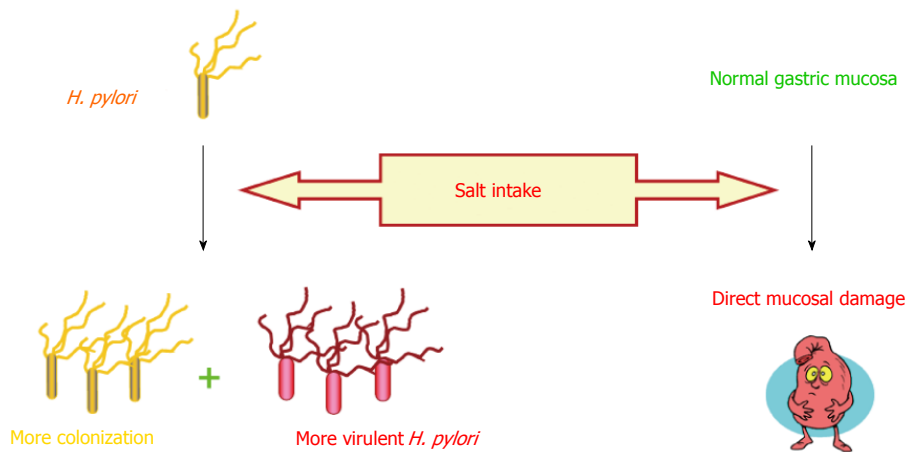


Figure 2 Multiple roles of salt in augmenting *Helicobacter pylori*-associated diseases. Salt can not only cause direct mucosal damage but also increase the colonization as well as virulent *Helicobacter pylori* strains. *H. pylori*: *Helicobacter pylori*.

directly damages the gastric mucosa but also increases the colonization of *H. pylori* in stomach resulting in loss of parietal cells, atrophy, and intestinal metaplasia^[46-48]. A recent study conducted in Mongolian gerbils provided the evidence that both *cagA*⁺ *H. pylori* strains and a high salt diet are synergistically significant risk factors for gastric adenocarcinoma compared with mutant *cagA* strain and a regular diet^[49]. An interesting finding was revealed by Loh *et al*^[49] that changes in salt concentration altered the gene expression in *H. pylori* especially *cagA* which was later observed in *H. pylori* infected gerbils as well^[50]. These findings provide evidence that salt can aggravate gastric mucosal damage in multiple ways, shown in Figure 2, which might form the basis of different disease outcomes in a genetically similar population like in Japan. It may be intriguing to evaluate the effect of high salt intake in different populations infected with *H. pylori* in a single study along with the genetic status of *H. pylori*. This may help in identifying the role of host genetic makeup in the presence of high salt intake and *H. pylori* infection in the process of gastric carcinogenesis.

Other than salt, high consumption of pickled, preserved, and smoked food in the Japanese population has also been postulated as a risk factor for gastric cancer^[43]. Pickled vegetables are also very much common in the East Asian region such as Japan, China, and Korea and are almost a part of daily cuisine. Nitrosamines are dietary carcinogens found in pickled and smoked food and their intake increased the risk of gastric cancer^[51,52]. It has been documented that nitrosamine can augment *H. pylori* associated gastric carcinogenesis not only in gerbils but in rhesus monkeys as well^[53,54]. A recent study in *H. pylori*-infected Chinese population documented the similar finding; that high intake of sodium, heme iron, and red meat increase the risk of gastric cancer while abundant consumption of fresh fruits decrease the risk^[55]. Another important source of carcinogens in the East Asian population is fermented products such as soybean pastes and kimchi, a traditional Korean dish made of fermented cabbage^[51,56]. Nan *et al*^[57] documented

the increase in gastric cancer risk in Korean subjects consuming kimchi and soybean pastes. This may again be due to the presence of nitrosated compounds in high quantity in such fermented products which have been reported to augment *H. pylori* associated carcinogenesis^[51,58]. In short, it can be retrieved from the above mentioned studies that the continuous intake of these salty, pickled, and fermented foods in the East Asian population might be a crucial factor in *H. pylori*-associated enigma of gastric cancer.

South Asian population and dietary habits

When it comes to the South Asian region, including India, Pakistan, and Bangladesh, dietary habits are quite different from those in East Asian population. It is due to this difference in dietary pattern that many researchers have postulated dietary habits as one of the important factors behind *H. pylori*-associated disease outcome and Asian enigma^[8,9,59]. One of the reasons behind this speculation is the low incidence of gastric cancer despite high prevalence of virulent *H. pylori* strains in South Asia^[60]. Although host genetic factors may also interplay in pathogenesis, diet can be quite crucial when comparing East with South Asian countries. As discussed above, some ingredients in the East Asian population diet are negative (aggravating) regulator in *H. pylori* associated pathogenesis, while in the case of the South Asian region, one can hypothesize that diet might be functioning as a positive regulator in alleviating *H. pylori*-linked diseases. This seems to be true to some extent under the light of available evidence. Consumption of salty, pickled, and fermented food is comparatively low in the South Asian region than in East Asian countries. Furthermore, the use of spices in daily cuisine and frequent over the counter use of herbal or traditional medicines can be a hallmark in different disease outcomes^[61]. It is interesting to note that the daily cuisine and dietary patterns in South Asian countries like India, Pakistan, and Bangladesh are quite alike, which to some extent, is a true copy of the

similarity in dietary habits between East Asian nations such as Japan, China, and South Korea.

Several studies from India have documented the plausible role of diet on the incidence of gastric cancer in different regions^[8,9]. Although the overall prevalence of gastric cancer in India is quite low when compared with Japan, it is worth to note that the frequency of gastric cancer differs in different regions of India, most probably due to differences in dietary habits of these regions; a pattern similar to that in Japan, as discussed above. Southern and eastern parts of India have high frequency of gastric cancer compared to the northern region. This is attributed to be due to frequent consumption of non-vegetarian food, like fish with excess spices and high salt. Contrarily, northern regions have a wheat-based vegetarian diet^[9]. A similar pattern is seen in the Kashmir region of India where esophageal and gastric cancer frequency is higher than other districts^[62]. This is again supposed to be due to high intake of dietary amines and nitrate in the Kashmir district^[63]. Another study by Mathew *et al*^[64] from south India documented the increase risk of gastric cancer with a high intake of rice, spicy, and high-temperature food. One of the limitations in these studies is the lack of consideration of *H. pylori* co-infection in the study subjects. However, one study by Phukan *et al*^[65] from South Indian region of Mizoram demonstrated the association of dietary components (fermented and smoked meat) with *H. pylori* infection with an increased risk of gastric cancer. These facts further ascertain the role of diet in the diverse prevalence of gastric cancer in the Indian population, thus highlighting its importance in enigma.

Several studies have shown protective effects of spices and medicinal plants against numerous diseases including *H. pylori* and GI disorders^[66-70]. It is worth mentioning here that in South Asian countries like India, Pakistan, Nepal, and Bangladesh, spices are also used as medicinal herbs as they possess medicinal values in the traditional system of medicine of these countries. In addition, many of these dietary spices are prescribed for the treatment of GI diseases^[67,71]. O'Mahony *et al*^[70] first reported the bactericidal and anti-adhesive activities of culinary herbs from Malaysia against *H. pylori*. Later, we reported in a series of studies the potential effect of commonly used spices and medicinal plants from Pakistan on *H. pylori* and its associated pathogenesis^[71-76]. All of the medicinal plants/spices used in these studies are also prescribed for the treatment of GI disorders. The results of these studies are very convincing, which reveals not only bactericidal (anti-*H. pylori*) activities of these plants but also anti-inflammatory effects against *H. pylori*-initiated pathological events. Spices like turmeric, nutmeg, mace, cardamom, black caraway, cumin, *etc.* which are part of daily cuisine in South Asian countries exhibited promising anti-*H. pylori* activities^[71]. We further documented that the plants, not having anti-*H. pylori* activity, can inhibit *H. pylori*-induced IL-8 secretion or reactive oxygen species (ROS) generation in gastric

epithelial cells. Both IL-8 and ROS are reported to play an important role in *H. pylori*-linked pathological sequel^[74]. Among the spices that inhibit IL-8 secretion, cinnamon showed most strong activity, and cinnamaldehyde was found to be the major reason for this effect^[76]. The major limitation with these studies was the use of only *in vitro* assays, which points out the dire need of *in vivo* or clinical trials with these herbs.

Some of the dietary components and spices have not only been examined against *H. pylori* in *in vitro/in vivo* assays but also in clinical trials. The most promising effects were demonstrated by the spice named turmeric or curcuma and its active ingredient curcumin. In *in vitro* assays, both turmeric and curcumin showed significant anti-*H. pylori* activity^[71,77]. Curcumin has also been documented to eradicate *H. pylori* in C57BL/6 infected mice and also reduced the level of gastric damage^[78]. Anti-inflammatory activity of curcumin was also documented by Foryst-Ludwig *et al*^[79] by decreasing *H. pylori*-induced NF- κ B activation and the subsequent release of IL-8. We also demonstrated that curcumin not only blocked NF- κ B activation but also suppressed the anomalous expression of AID, an enzyme highly linked with *H. pylori*-induced gastric carcinogenesis^[23]. Sintara *et al*^[80] reported the role of curcumin in Sprague-Dawley rats by reducing the gastric inflammation by inhibiting NF- κ B. Later, a clinical study demonstrated that curcumin based triple therapy significantly improved dyspeptic symptoms and reduced serologic signs of gastric inflammation even after 2 mo of the therapy^[81]. Hence, it can be postulated that the use of spices like turmeric/ cinnamon can, not only suppress *H. pylori* colonization, but also halt the inflammatory cascade initiated by *H. pylori*, ultimately preventing carcinogenesis.

Another commonly consumed cuisine ingredient in South Asian countries is garlic. Garlic or allium has revealed promising activities against *H. pylori*-induced gastritis model of Mongolian gerbils by decreasing the degree of gastritis^[82]. However, it has been described in the same study that *H. pylori* was not eradicated by the garlic extract treatment. A clinical trial was also conducted using garlic in *H. pylori* infected subjects revealing no effect on eradication of the bacteria^[83]. This suggests that garlic might only be helpful in attenuating *H. pylori*-induced pathological pathways while not killing the bacteria itself in *in vivo* and clinical settings. Overall these studies signify the potential role of dietary ingredients including spices in South Asian countries in modulating *H. pylori* associated pathological processes. However, it cannot be overlooked that the excessive and continued use of a spicy diet may act as an aggravating factor in *H. pylori*-infected individuals, a question still needed to be affirmed in the region of South Asia. Last but not the least, it is important to note that high amount of spices are always accompanied with high amount of salt to give proper taste. So it might be the salt as the main offender in these spicy diets rather than spices itself. It will be interesting to see the effect of a high intake of spices with or without excessive

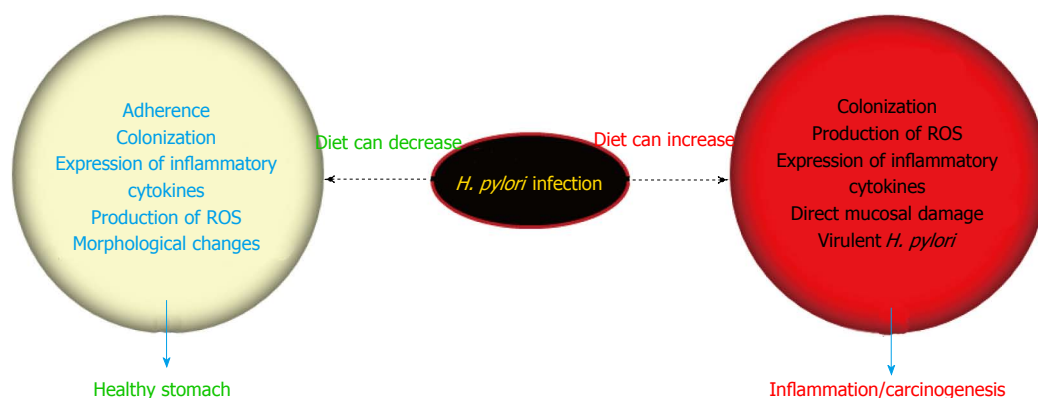


Figure 3 Dual impact of diet in either aggravating or alleviating *Helicobacter pylori* linked diverse disease outcome. Dietary ingredients can equally increase or decrease the inflammatory cascade by modulating the expression of various molecules ultimately modifying the outcome of *H. pylori* infection. *H. pylori*: *Helicobacter pylori*; ROS: Reactive oxygen species.

salt either by *in vivo* experiments or clinical trials in *H. pylori*-infected subjects.

CONCLUSION

H. pylori associated pathological outcome is a result of chronic inflammation from years to decades, and as *H. pylori* alone is not enough to cause associated diseases, several factors can play an important role in the final outcome. Each of the factors discussed above behind enigma is plausibly exerting some influence in the variable incidence of gastric cancer in Asian countries. Along with genetic status of both *H. pylori* and host, diet can also modulate the clinical outcome by aggravating or alleviating *H. pylori*-linked pathogenic processes (Figure 3). In this review, plausible mechanisms by which diet can manipulate the enigmatic consequences of *H. pylori* infection are discussed. As evident from the high prevalence of *H. pylori* infection among South Asian countries in the studies mentioned earlier, these dietary ingredients can barely eradicate *H. pylori* in the doses that are consumed on a daily basis. However, it is quite evident from the discussion that the dietary factor can modulate *H. pylori*-linked pathogenesis either by decreasing the colonization of *H. pylori* or *via* hampering the production of inflammatory/carcinogenic mediators released after *H. pylori* infection. This can end up in perplexing clinical outcomes leading to enigma. Besides the negative or aggravating role of salt among daily dietary components, it may be too earlier to firmly pose any other ingredient as a positive or negative regulator in the scenario of Asian enigma. There is no doubt concerning the role of *H. pylori* as a significant and intriguing factor in causing chronic gastritis that may later predispose the infected host to develop gastric cancer, but the nature of our daily diet can undeniably define the future course of disease outcome.

Further large scale comparative studies are required on a national and international level, not only to identify perpetrators in our dietary habits but also to develop effective ways for preventing this cancer in high risk

populations, as chemoprevention seems to be the most practical way of controlling this deadly disease. It is needless to mention that while designing clinical trials or *in vivo* models, the triad of causing factors, *i.e.*, virulence of *H. pylori*, host genetic make up, and dietary habits should be considered, otherwise exhaustive efforts will again end up with no conclusive results.

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2016 Inflammatory Bowel Disease: Global view

Status of colitis-associated cancer in ulcerative colitis

Tetsushi Kinugasa, Yoshito Akagi

Tetsushi Kinugasa, Yoshito Akagi, Department of Surgery, Kurume University School of Medicine, Fukuoka 830-0011, Japan

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Correspondence to: Dr. Tetsushi Kinugasa, Department of Surgery, Kurume University School of Medicine, 67 Asahimachi, Kurume, Fukuoka 830-0011, Japan. kinugasa_tetsushi@med.kurume-u.ac.jp
Telephone: +81-942-353311
Fax: +81-942-340709

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Abstract

Surgical therapy for ulcerative colitis (UC) depends on the medical therapy administered for the patient's condition. UC is a benign disease. However, it has been reported that the rare cases of cancer in UC patients are increasing, and such cases have a worse prognosis. Recently, surgical therapy has greatly changed, there has been quite an increase in the number of UC patients with high-grade

dysplasia and/or cancer. These lesions are known as colitis-associated cancer (CAC). The relationship between inflammation and tumorigenesis is well-established, and in the last decade, a great deal of supporting evidence has been obtained from genetic, pharmacological, and epidemiological studies. Inflammatory bowel disease, especially UC, is an important risk factor for the development of colon cancer. We should determine the risk factors for UC patients with cancer based on a large body of data, and we should attempt to prevent the increase in the number of such patients using these newly identified risk factors in the near future. Actively introducing the surgical treatment in addition to medical treatment should be considered. Several physicians should analyze UC from their unique perspectives in order to establish new clinically relevant diagnostic and treatment methods in the future. This article discusses CAC, including its etiology, mechanism, diagnosis, and treatment in UC patients.

Key words: Inflammatory bowel disease; Ulcerative colitis; Colitis-associated cancer; Surgical therapy; Colorectal cancer surveillance

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Core tip: Inflammatory bowel disease, especially ulcerative colitis, is an important risk factor for development of colon cancer. There has been quite increased in the number of patients who had high-grade dysplasia and/or cancer. The relationship between inflammation and tumorigenesis is well-established and in the last decade has received a great deal of supporting evidence from genetic, pharmacological, and epidemiological data. To avoid such a problem, there is a need for appropriate diagnosis and treatment. It should be considered that actively introduce the surgical treatment in addition to medical treatment.

Kinugasa T, Akagi Y. Status of colitis-associated cancer in ulcerative colitis. *World J Gastrointest Oncol* 2016; 8(4): 351-357 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v8/i4/351.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v8.i4.351>

INTRODUCTION

Ulcerative colitis (UC) is of unknown cause inflammatory bowel disease (IBD) associated with inflammation of the large intestine mucosa. UC number of the patients that have been recorded in Japan in 2014 at 166110 people, this number is increasing every year. As a recent problem, in patients with UC, it has been reported that it is easy to develop colorectal cancer (CRC). The longer the period of UC, the higher is the risk of developing UC-associated CRC. The mechanism of IBD-related colon cancer is different from sporadic CRC. The former is an inflammation-dysplasia-carcinoma sequence^[1] and the latter is from the adenoma-carcinoma sequence in sporadic CRC (Figure 1).

Therefore the UC patient becomes the indication of the operation when the pathological findings are demonstrated high-grade or multifocal low-grade dysplasia in colonic mucosal which means that the entire mucosal lining of the colon exposed to chronic inflammation is at increased risk of cancer^[1,2].

IBD

Crohn's disease (CD) and UC, which include IBD, is a chronic, relapsing inflammatory condition of the gastrointestinal tract. IBD has a clear pathological and clinical characteristic. Though many studies were performed about this cause of IBD for the past several decades, the cause is not yet clear. The only consensus was obtained about IBD which was deregulated the mucosal immune response in the host. Some various components, which including environmental intestinal epithelial cells, and microbial factors, genetic susceptibility, and components of the innate and adaptive immune system, are implicated in the pathogenesis of IBD.

Mechanism of colitis-associated cancer in UC patients

The connection between inflammation and tumorigenesis is well established, and in the last decade, a great deal of supporting evidence has been obtained through genetic, pharmacological, and epidemiological studies. IBD is an important risk factor for the development of colon cancer.

As a result has high mortality, it is difficult to treat colitis-associated cancer (CAC), which is the CRC subtype that is associated with IBD^[3]. When the patients with IBD are the higher the probability within 30 years disease duration, and > 50% of these patients will die from CAC^[4]. Some of the essential stages of cancer development, including the formation of aberrant crypt foci, polyps, adenomas, and carcinomas, are similar in noninflammatory CRC and CAC. However, several different pathogenic sequences CAC, has been reported including a well-defined inflammation and damage dysplasia cancer that occurs without the formation of the gland. Nevertheless, both of in the CRC and CAC had changed same genetic and signaling pathways which involving Wnt, β -catenin, K-ras, p53, and transforming growth factor (TGF)- β . However, the difference activation timing between

CRC and CAC were reported about p53, adenomatous polyposis coli (APC) and K-ras^[4,5]. A > 2-fold higher risk for colon cancer development with a family history of CRC have than without in IBD patients, suggesting an overlap in the mechanisms driving CRC and CAC^[6]. Kinugasa *et al.*^[7] also showed that high-grade dysplasia (HGD) and CAC in patients with UC results in an increase in β -catenin transcriptional activity that may contribute to increased claudin (CL)-1 expression. According to above things, the expression of CL-1 was increased in CAC and dysplasia than normal mucosa is likely to be involved in neoplastic progression in UC patients. Thus, I propose that there is a possibility that increased CL-1 expression may contribute to carcinogenesis in UC. CL-1 is a tight junction specific protein that was first described by Furuse *et al.*^[8] in 1998. The signaling pathways, including TGF- β /SMAD and β -catenin^[9,10] were interacted with the CL protein that is separate from their effects on the barrier function. We reported that CL-1 is regulated by β -catenin/TCF/lymphocyte-enhancer factor signaling^[11,12] suggests that the expression of CL-1 is concerned in β -catenin activation (Figure 2).

These mechanisms are the basis for the current discussion about CAC as well as for new approaches to prevention and therapy.

Is the frequency of colorectal cancer in UC patients higher than in the normal population?

Patients with IBD are at an increased risk of developing colorectal advanced neoplasia, including colorectal HGD and CRC^[13-16]. Chronic intestinal inflammation is considered to be a promoter of carcinogenesis^[17]. The two major drivers of the excess risk of CRC in IBD are disease extension^[18] and duration^[13]. Concomitant primary sclerosing cholangitis^[19], family history of CRC^[6], and active intestinal inflammation^[20-23] are the other established risk factors.

The risk of CRC in patients with IBD was recognized as far back as 1925 for UC and 1948 for CD^[24]. There were many reports of risk for colon cancer development about UC patients mainly, and which ratio was indicated from 16% to 43%^[25-28]. In 2001 Eaden *et al.*^[13] estimated CRC risk as 2% at 10 years, 8% at 20 years and 18% at 30 years using a widely cited meta-analysis of 116 studies with age stratified data. Interestingly, recent studies we could get two different conclusions about IBD with CRC. Jess *et al.*^[29] suggested that the risk of CAC is almost same between UC patients and the general population in a population-based study from Denmark. In contrast, the risk of CRC is 60% higher with IBD than without IBD matched cohorts of people from California, and the risk remained the same throughout the study period of 14.5 years by Herrinton *et al.*^[15]. There are few reports from Asia on CRC in UC which reports that the pressure ranges from 0.87% to 1.8% in the general population, and it can be as high as 13.5% in patients with extensive type of UC. Thus, the risk of CRC is increased in IBD, though there is variation due to various

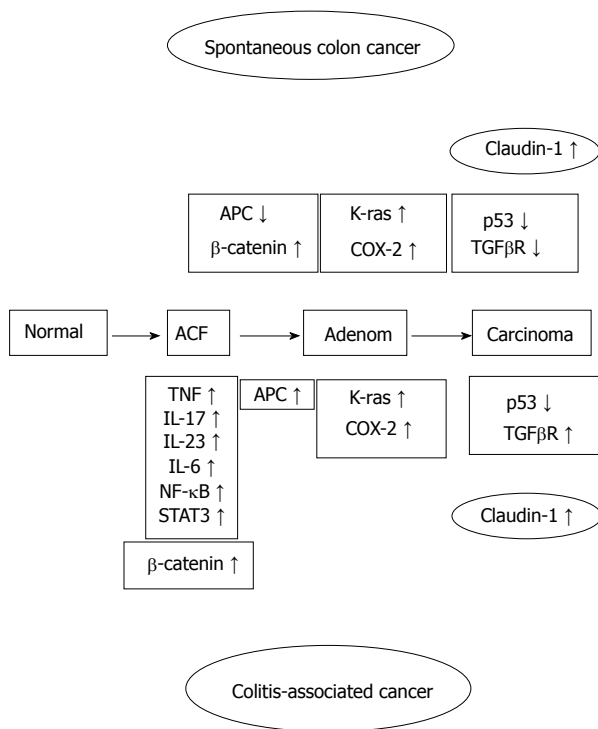


Figure 1 Mechanisms of colorectal cancer and colitis-associated cancer development. APC: Adenomatous polyposis coli; TGFβ: Transforming growth factor beta; NF-κB: Nuclear factor-κB; TNF: Tumor necrosis factor; IL: Interleukin; STAT3: Signal transducer and active of transcription 3.

factors, such as hospital- or population-based data, referral center bias, and small numbers of patients.

IBD is a lifelong diseases that primarily affects young patients. Sustained mucosal healing is becoming the standard objective of the long-term treatment of IBD^[30]. This objective can be achieved currently by maintenance treatment with immunosuppressants, including thiopurines, methotrexate, and anti-tumor necrosis factor (TNF)^[31,32]. Unfortunately, there is no major trend towards the spontaneous extinction of IBD activity with time^[33,34], and disease activity often recurs after the withdrawal of immunosuppressants^[35,36]. Similarly, another report showed that there is an unclear risk of colonic HGD and CRC among patients with IBD treated with immunosuppressants. Beaugerie *et al.*^[37] analyzed data on CRC development among patients with IBD and found that patients with IBD and long-standing extensive colitis are at increased of CRC, although the risk is lower among patients receiving thiopurine therapy. Patients without long-standing extensive colitis have a risk for CRC similar to that of the general population, but they can develop IBD-related lesions within 10 years after their diagnosis of IBD. Table 1 compares CAC with CRC.

DOSE THE SURVEILLANCE STRATEGY NEED TO TAKE INTO ACCOUNT THIS DECREASING RISK OF CAC IN UC PATIENTS?

We have to debate about the surveillance techniques for

Table 1 A comparison colitis-associated cancer and sporadic colorectal cancer

CAC: Up to 20% of UC patients develop CAC within 30 yr of disease onset
CAC: High overall mortality rate
NSAID use reduces the risk of CRC, suggesting a potential role for anticytokine therapy
CRC: Classic adenoma to carcinoma sequence
CAC: Chronic inflammation, injury, dysplasia, and CRC
The common genetic and signaling pathways are different between CRC and CAC including β-catenin, p53, k-ras, B-raf
Both CRC and CAC are associated with transcription factors, such as NF-κB and/or STAT3 which mediate the immune response and oncogenesis
Both CRC and CAC depend on the quality and quantity of intestinal microflora

CRC: Colorectal cancer; CAC: Colitis-associated cancer; UC: Ulcerative colitis; NSAID: Nonsteroidal anti-inflammatory drug; NF-κB: Nuclear factor-κB; STAT3: Signal transducer and active of transcription 3.

CAC and high grade dysplasia whether adequate or not under currently way^[38]. Presently, surveillance of using colonoscopy for UC patients is recommended every 2 years over 8 years after the onset of UC diagnosis. However, sometimes it is very hard to determinate the CRC findings under the UC mucosa, and do not match between the biopsy results and excisional tissue results in pathological diagnosis, this is that colonoscopy is often burdensome for the patient with UC patients^[39-41]. Surprisingly, 20% to 50% of UC patients with CRC were diagnosed with only dysplasia in preoperative pathological diagnosis. This indicates that it is difficult surveillance for UC patients using colonoscopy.

Furthermore, endoscopic findings of IBD-related cancers have been found to easily overlook by colonoscopy because it is not a mass-like lesions to compared with sporadic CRC. As a result, it is very difficult to appropriate for diagnosis of adenocarcinoma for UC patients using biopsied tissue taken from the lesion. Meanwhile, we could find unexpectedly in a specimen resected for medically refractive IBD without previous diagnosis of dysplasia or adenocarcinoma^[42,43].

It is clear that colonoscopic surveillance in the present form is neither ideal nor practical. We should reconsider the guidelines about the colonoscopy surveillance based on the other new reliable date and method. The ideal frequency of surveillance is not clear. Further studies are necessary to optimize the frequency of surveillance, including cost-effectiveness, and to make guidelines considering emerging methods and technologies.

Despite the proven usefulness of colonoscopy surveillance protocols and increased risk of CRC with UC, we could not determinant a useful in clinical diagnosing such as genetic or serological marker. After analyzing the association of the whole genome, the single nucleotide polymorphism (SNP) 300 or more and the genetic loci 160 or more were found to be associated with IBD^[44]. There was another genetic instability report^[45] that sought to identify IBD-associated SNPs that are potential

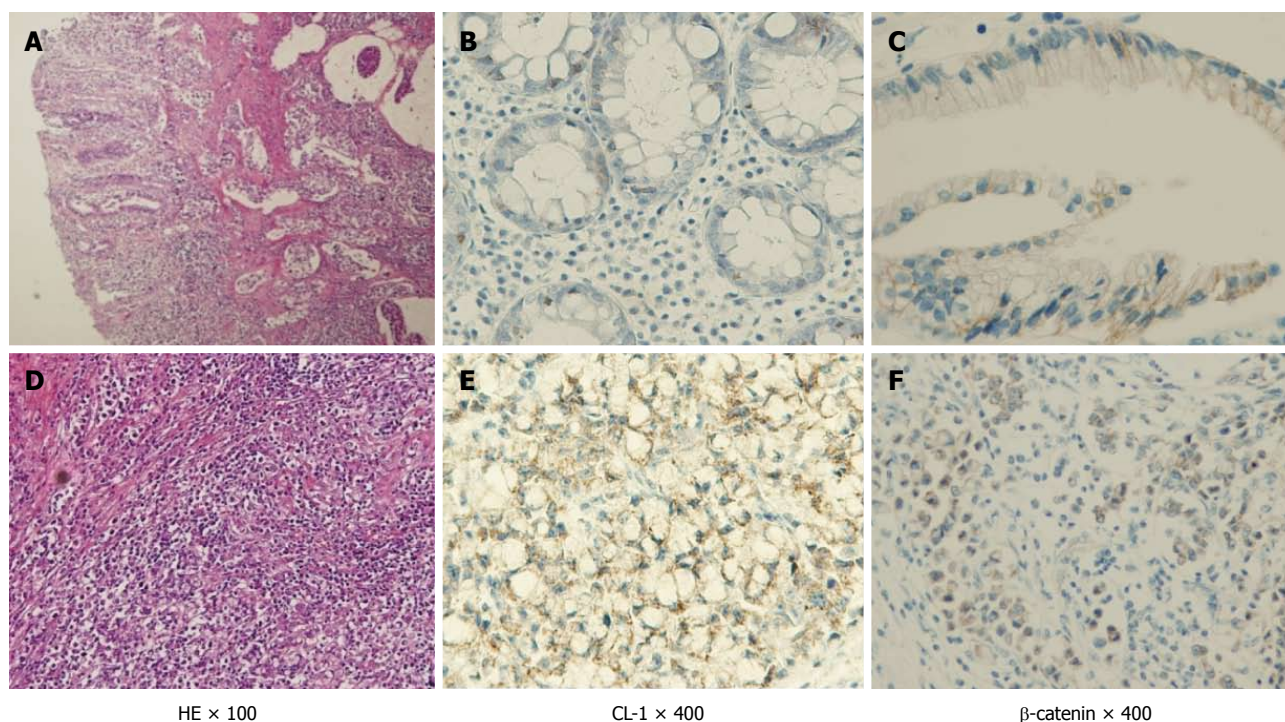


Figure 2 Hematoxylin-eosin (A, D) and immunostaining of claudin-1 (B, E) and β -catenin (C, F) in ulcerative colitis mucosa (A-C) and ulcerative colitis-associated colorectal cancer tissue (D-F) of a patient with ulcerative colitis-associated colorectal cancer.

markers for CAC by comparing groups of UC patients with and without neoplasia matched for sex, disease duration and age at diagnosis. Their conclusion was that none of the 314 studied IBD-associated SNPs were strongly associated with UC-neoplasia which may be the result of genetic mutations in molecular pathways other than those that predispose to inflammation.

On the other hand, there was a unique report that applied the tree models by considering the response variable as the CAC or UC group and the explanatory variable as the criteria studied by univariate analysis^[46].

We proposed the useful method which analysis is a tree model permits the automatic execution of the process of determining the factors, setting the threshold value and by differentiating the patients successively into two groups during the process automatically. Therefore, it might be another possibility to help identify the indication and timing for surgery in UC patients, because the ideal surveillance methods have not yet been established.

Treatment for CAC including surgery

The overall prevalence of CRC in patients with UC has been estimated as 3.7% in a meta-analysis^[13]. Chronic UC for > 10 years and pancolitis are known risk factors for CAC^[47]. On the other hand, sporadic adenoma and adenocarcinoma can arise coincidentally in patients with UC. From the perspective of clinical considerations, accurate pathological diagnosis is very important for distinguishing between different pathological entities, given their different therapeutic consequences such as sporadic adenocarcinoma and CAC.

Anal function and quality of life differ substantially

between total proctocolectomy with ileal pouch anal anastomosis (IPAA) and low anterior resection (LAR). A key point is that further proctocolectomy and IPAA may be suitable for sporadic cancers in the lower rectum. In patients with UC, irrespective of the degree of colitis, LAR should not be selected for sporadic cancer in the lower rectum except in older patients, based on considerations of quality of life and risk of further colitis. In elderly patients with poor anal function, surgical procedures should obviously be considered based on overall considerations including prognosis of the cancer, degree of inflammation with colitis, and potential requirements for further treatment. In view of the risk of recurrent colitis and cancer, partial resection might be less advantageous than proctocolectomy. Proctocolectomy with IPAA may be safe for advanced CRC regardless of the origin as colitic or sporadic cancer, because of the difficulty of differentiation, ready invasive behavior against an inflamed background and worsened potential for further progressive colitis in younger patients. Decisions on surgical procedures should be made based on full consideration of background factors including age, degree of colitis and cancer prognosis.

There was an interesting report on the risk of ileoanal pouch neoplasia in patients with IBD^[48]. Although restorative proctocolectomy with IPAA substantially reduces the risk of CRC in patients with IBD, subsequent pouch neoplasia can develop. The purpose of their study was to determine the cumulative incidence of pouch neoplasia in patients with IBD and to identify risk factors for developing pouch neoplasia. The incidence and prevalence of pouch neoplasia in patients with IBD

are probably low. According to the latest review, only 42 pouch adenocarcinomas have been described in the literature^[49]. A previous study reported a cumulative incidence of pouch neoplasia of 1.9% after 15 years and 5.1% after 25 years^[50]. However, these data were collected in a single tertiary pouch referral center and may not be representative of the general IBD population with IPAA. Furthermore, the relatively low incidence makes it difficult to assess risk factors for the development of pouch neoplasia. The result of the study indicated that the incidence of pouch neoplasia in patients with IBD without a history of colorectal neoplasia is relatively low. Prior dysplasia or colon cancer is associated with an approximately 4- and 25-fold increase in risk, respectively, of developing pouch neoplasia.

CONCLUSION

UC is a benign disease. However, according to the recent reports that the rate of CAC is increasing in UC patients, and such cases have a worse prognosis. As such, there is a need for appropriate diagnosis and treatment. Actively introducing the surgical treatment in addition to medical treatment should be considered. Several physicians should analyze UC from their unique perspectives in order to establish new clinically relevant diagnostic and treatment methods in the future. We should determine the risk factors for UC patients with cancer based on a large body of data, and we should attempt to prevent the increase in the number of such patients using these newly identified risk factors in the near future.

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Chromodomain-helicase-DNA binding protein 5, 7 and pronecrotic mixed lineage kinase domain-like protein serve as potential prognostic biomarkers in patients with resected pancreatic adenocarcinomas

Crystal S Seldon, Lauren E Colbert, William A Hall, Sarah B Fisher, David S Yu, Jerome C Landry

Crystal S Seldon, David S Yu, Jerome C Landry, Department of Radiation Oncology, Winship Cancer Institute, Emory University School of Medicine, Atlanta, GA 30322, United States

Lauren E Colbert, Department of Radiation Oncology, Memorial Sloan Kettering Cancer Center, New York, NY 10065, United States

William A Hall, Department of Radiation Oncology, the Medical College of Wisconsin, Milwaukee, WI 53226, United States

Sarah B Fisher, Division of Surgical Oncology, Department of Surgery, Emory University School of Medicine, Atlanta, GA 30322, United States

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Correspondence to: Jerome C Landry, MD, MBA, Assistant Professor, Department of Radiation Oncology, Winship Cancer Institute, Emory University School of Medicine, 1365 Clifton Road, NE, Suite C3008, Atlanta, GA 30322, United States. jland01@emory.edu
Telephone: +1-404-6166349
Fax: +1-404-6166380

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Abstract

Pancreatic cancer is one of the deadliest cancers with a very poor prognosis. Recently, there has been a significant increase in research directed towards identifying potential biomarkers that can be used to diagnose and provide prognostic information for pancreatic cancer. These markers can be used clinically to optimize and personalize therapy for individual patients. In this review, we focused on 3 biomarkers involved in the DNA damage response pathway and the necroptosis pathway: Chromodomain-helicase-DNA binding protein 5, chromodomain-helicase-DNA binding protein 7, and mixed lineage kinase domain-like protein. The aim of this article is to review present literature provided for these biomarkers and current studies in which their effectiveness as prognostic biomarkers are analyzed in order to determine their future use as biomarkers in clinical medicine. Based on the data presented, these biomarkers warrant further investigation,

and should be validated in future studies.

Key words: Chromodomain-helicase-DNA binding protein 5; Chromodomain-helicase-DNA binding protein 7; Mixed lineage kinase domain-like protein; Pancreatic adenocarcinoma; Biomarker

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Core tip: Pancreatic cancer is one of the deadliest cancers with a very poor prognosis. Recently, there has been a significant increase in studies and research directed towards identifying potential biomarkers that can be used to diagnose and provide prognostic information for pancreatic cancer. We focused on 3 biomarkers involved in the DNA damage response pathway and the necroptosis pathway: Chromodomain-helicase-DNA binding protein 5, chromodomain-helicase-DNA binding protein 7, and mixed lineage kinase domain-like protein. Based on the data presented, these biomarkers warrant further investigation.

Seldon CS, Colbert LE, Hall WA, Fisher SB, Yu DS, Landry JC. Chromodomain-helicase-DNA binding protein 5, 7 and proneurotic mixed lineage kinase domain-like protein serve as potential prognostic biomarkers in patients with resected pancreatic adenocarcinomas. *World J Gastrointest Oncol* 2016; 8(4): 358-365 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v8/i4/358.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v8.i4.358>

INTRODUCTION

With an estimated 39590 deaths in 2014, pancreatic cancer is the fourth leading cause of death from cancer in the United States^[1]. Pancreatic adenocarcinoma (PAC), the most common type of pancreatic cancer, has a very poor prognosis with a five-year survival rate of 5% for patients with all stages of disease^[2]. Patients with early-stage resected PAC have the best prognosis when followed by treatment with adjuvant therapy^[3,4], with a median overall survival (OS) of approximately 3 years^[5]. Potential predictive and prognostic biomarkers could play an important role in determining the most effective and productive treatment for individual patients. PAC is genetically heterogeneous and several well-known and some newly defined core signaling pathways likely play a role in development and behavior of PAC, including necroptosis, a form of cell death, and the DNA damage response pathway^[6]. In this review, we will explore those pathways and putative biomarkers associated with them^[7].

BIOMARKERS AND PAC

The Food and Drug Administration (FDA) defines a biomarker as "any measureable diagnostic indicator that is used to assess the risk or presence of disease"^[8]. In recent years, there has been a tremendous increase in research

directed towards identifying biomarkers in specific cancers. There are many biomarkers being used in other cancers that aid in the diagnosis and establishment of personalized treatment for patients. Though the use of biomarkers in the treatment of cancer is expanding, the role of biomarkers in the treatment of patients with PAC trails behind. To date, CA 19-9, discovered in 1981, remains as the only FDA approved biomarker in diagnosing PAC. Other cancers are also associated with elevated CA 19-9 levels including the following: Colorectal^[9], esophageal^[10], lung^[11], ovarian^[12], and breast^[10], making CA 19-9 a nonspecific marker. Patients with pancreatitis, elevated bilirubin levels, and cirrhosis can also present with elevated CA 19-9 levels^[13]. This makes it difficult to determine whether these levels are high due to tumor involvement or non-cancerous events. CA 19-9 is also viewed as a poor prognostic tool due to the fact that it is not expressed in 10% of Caucasians and 40% of Africans^[14]. This is due to a deficiency in fucosyltransferase enzyme which is involved in the production of CA 19-9 and Lewis antigen. Currently, CA 19-9 is most useful as a diagnostic tool when measured after resection for disease recurrence^[15].

Prognostic biomarkers that hold promise are SMAD4 and glypican-1 (GPC1). GPC1 is a cell surface proteoglycan located on cancer-cell-derived exosomes. Melo *et al*^[16] were able to distinguish between healthy subjects and patients with a benign pancreatic disease from patients with early- and late-stage pancreatic cancer by measuring serum levels of GPC1⁺ circulating exosomes (crExos). Levels of GPC1⁺ crExos also were found to connect with tumor burden and the survival of pre- and post-surgical patients^[16].

Mutations that inactivate SMAD Family Member 4 (SMAD4) occur most commonly in pancreatic cancers vs other cancer types^[17]. SMAD4 is silenced in 53% of pancreatic cancer cases^[18]. SMAD4 expression is lost through loss of heterozygosity and intragenetic mutations along with other alterations such as KRAS mutations^[19]. KRAS mutations, located in 95% of pancreatic cancers^[20], are usually followed by loss of SMAD4 in late development of PAC^[21]. Loss of SMAD4 promotes the progression of preneoplastic lesions and is associated with worse prognosis in patients with PAC. Numerous studies support this claim^[22-27]. Blackford *et al*^[22] determined that patients whose cancers lacked SMAD4 expression had significantly worse survival outcomes than patients with normal SMAD4 expression. Tascilar *et al*^[23] built on this observation by showing that the loss of expression of the SMAD4 protein by immunolabeling is associated with poor prognosis in patients with resected PAC, and patients with intact SMAD4 expression survived significantly longer than patients whose cancers lacked SMAD4 (median survival, 19.2 vs 14.7 mo; $P = 0.03$). Biankin *et al*^[24] concluded that SMAD4 expression predicted increased survival and improved response to surgery. Reduced survival in colon cancer was associated with decreased SMAD4 expression in a study conducted by Isaksson-Mettävainio *et al*^[25]. Reduced SMAD4 expression is also present in head- and - neck squamous cell carcinomas and esophageal squamous cell carcinoma^[19]. SMAD4 expression is lost

in 40%-50% of colon cancers^[25] and 25% of prostate cancers^[26]. In 45% of cholangiocarcinomas, loss of SMAD4 expression is present and associated with more aggressive tumor behavior^[27].

Identifying biomarkers

Identification and validation of predictive biomarkers for responsiveness to adjuvant therapy is extremely important for patients with PAC. These markers can be used clinically to optimize and personalize therapy for individual patients. At this point, no biomarkers have been identified to reliably predict patient outcome, and more knowledge of potential biomarkers may aid in tailoring and directing patient therapy. Our group has previously identified several potential prognostic markers involved in either the necroptotic or DDR pathway including chromodomain-helicase-DNA binding protein 5 (CHD5), CHD7, and mixed lineage kinase domain-like protein (MLKL) (Table 1).

DDR serves as cancer barrier

As defined by Curtin^[28] the DDR is a series of pathways that “coordinates the repair of DNA and the activation of cell cycle checkpoints to arrest the cell to allow time for repair”. The DDR has evolved in order to maintain the genomic integrity of the cell. It constantly protects the cell from endogenous and environmental damage that could disrupt DNA by causing single stranded breaks or double stranded breaks (DSBs). The DDR acts as a cancer barrier by activating DNA repair mechanisms and apoptosis so that unstable cells will not replicate and result in DDR related diseases and precancerous lesions.

One pathway of the DDR is homologous recombination repair (HRR). Occurring during the S and G₂ phases of the cell cycle^[29], HRR is associated with familial forms of pancreatic cancer associated with the following genes: *BRCA1*, *BRCA2*, *PALB2*, *ATM*, *RAD51D*, and *RAD51C*^[30]. HRR repairs DSBs. γ H2AX foci are markers for DSBs in precancerous lesions. These markers are produced during a phosphorylation reaction following chromatin engulfing the DSB^[31,32].

Data has shown that the DDR may promote the survival of PAC that outgrows the selection pressure of DDR activation^[33]. Many *DDR* genes are somatically mutated in PAC, including *ATM*, *BRCA2*, *CDKN2A*, *FANCI*, *HELB*, and *RAD9*^[34]. Dysregulated expression of tumor suppressor genes that induce DDR activation can function as biomarkers for poor outcome.

CHD5 functions as a tumor suppressor gene

CHD5 is a member of a family of chromodomain enzymes that belong to the ATP-dependent chromatin remodeling protein superfamily. It has been suggested that CHD5 is the master regulator of a tumor-suppressive network^[35]. CHD5 is regulated by DNA methylation of its promotor and histone modifications. The ability of CHD5 to bind unmodified histone 3 is essential for tumor suppression^[36]. CHD5 is epigenetically silenced in

neuroblastoma^[37], colorectal cancer^[38], breast cancer^[39], cervical cancer^[39], hepatocarcinoma^[39], gastric cancer^[40] and lung cancer^[41]. Mutations in CHD5 have been found in head and neck squamous cell carcinoma^[42], prostate cancer^[43], ovarian cancer^[44], ovarian clear cell carcinoma^[45], cutaneous melanoma^[46], hepatocellular carcinoma^[47], neuroblastoma^[48], breast and colorectal cancer^[49]. In a study conducted by Bagchi *et al*^[50] loss of CHD5 enhanced tumor proliferation whereas restoration of CHD5 inhibited proliferation. The function of CHD5 has mainly been studied in neural tissues where it was determined to control cell death and replication *via* the p19(Arf)/p53 pathway^[50]. CHD5 is also a putative substrate of the ATM/ATR checkpoint kinases, suggesting that it may have a role in the DDR^[51].

Silencing of CHD5 activates the DDR

Expression of CHD5 corresponds with a cell's capability of locating and repairing DNA damage in cells. In a study conducted by Hall *et al*^[33] preclinical data showed increased levels of γ H2AX foci markers suggesting increased levels of DSBs in pancreatic cancer cells. This was correlated with low CHD5 expression in those cells. As a result, activation of the DDR presumes due to the presence of collapsed replication forks^[33].

Low CHD5 expression is associated with worse clinical outcomes

In the same study by Hall *et al*^[33] the relationship between CHD5 levels in pancreatic cells and DDR activation was evaluated in a clinical population. The OS of 80 patients with resected PAC was analyzed in conjunction with CHD5 expression. Low CHD5 expression was associated with decreased recurrence free survival (RFS) and decreased OS in patients with PAC (5.3 vs 15.4 mo, $P = 0.03$)^[33]. The association between low CHD5 expression and poor survival has also been documented in other cancers, including gallbladder carcinoma^[52], neuroblastoma^[53], ovarian cancer^[54] and breast cancer^[55].

CHD5 as a prognostic biomarker

Available data seems to reflect that low CHD5 expression suggests a poor prognosis. If validated in an independent cohort, low CHD5 expression could be used to select patients with particularly aggressive disease for further adjuvant therapy. Due to its clinical relevance as both a tumor suppressor and a prognostic factor in numerous cancers, study of CHD5 function in the DDR warrants further review.

CHD7 as a potential DDR substrate

CHD7 is a member of a family of chromodomain enzymes that encode an ATP-dependent chromatin remodeler. Mutations in CHD7 causes CHARGE syndrome, a multiple anomaly disorder that presents with a variety of phenotypes, including ocular coloboma, heart defects, choanal atresia, retarded growth and development, genitourinary hypoplasia, and ear abnormalities^[56]. Mutations in CHD7

Table 1 Summary of chromodomain-helicase-DNA binding protein 5, 7 and mixed lineage kinase domain-like protein biomarkers in pancreatic adenocarcinoma

Biomarker	Pathway affected	Biomarker type for pancreatic cancer from literature and studies? (prognostic, predictive, diagnostic)	Mechanism of action	Other cancers	Comments
CHD5 ^[33]	DDR	Prognostic	Tumor suppressor gene. Binds to histone 3	Epigenetically silenced in neuroblastoma ^[37] , colorectal cancer ^[38] , breast cancer ^[39] , cervical cancer ^[39] , hepatocarcinoma ^[39] , gastric cancer ^[40] and lung cancer ^[41] . Mutations found in head and neck squamous cell carcinoma ^[42] , prostate cancer ^[43] , ovarian cancer ^[44] , ovarian clear cell carcinoma ^[45] , cutaneous melanoma ^[46] , hepatocellular carcinoma ^[47] , neuroblastoma ^[48] , breast and colorectal cancer ^[49]	Low expression correlates with worse clinical outcomes
CHD7 ^[66]	DDR	Prognostic	Interacts with SOX2 to regulate gene expression	-	Decreased expression is associated with improved clinical outcomes
MLKL ^[74]	Necroptosis	Prognostic	Forms necrosis-inducing complex called a "necrosome" along with RIPK1 and RIPK3	Ovarian ^[75]	Low expression is associated with worse clinical outcomes

DDR: DNA damage response pathway; SOX2: (Sex Determining Region Y)-Box 2; RIPK1: Receptor-interacting serine/threonine-protein kinase 1; RIPK3: Receptor-interacting serine/threonine-protein kinase 3.

also cause Kallman Syndrome, a genetic disorder marked by hypogonadotropic hypogonadism and anosmia^[57], and associated with colorectal carcinomas^[58]. CHD7 helps to regulate neural crest gene expression^[59], regulates ribosomal RNA biogenesis^[60], and interacts with SOX2 to regulate gene expression^[61]. CHD7 is also a potential substrate of the ATM/ATR checkpoint kinases, suggesting a role in the DDR^[51,62]. CHD7 is also dysregulated in 13% to 35% of cases of pancreatic adenocarcinoma, with aberrant expression, copy-number variation, and somatic mutations^[63-65].

Low CHD7 expression associated with better prognosis

Colbert *et al.*^[66] suggested that CHD7 deficiency may play a role in gemcitabine sensitization in pancreatic adenocarcinoma cells and delayed pancreatic tumor xenograft growth in mice treated with gemcitabine. Additionally, they showed that CHD7 knockdown impaired ATR-dependent phosphorylation of CHK1 and increased gemcitabine-induced DNA damage *in vitro*, revealing a novel function for CHD7 as a DDR protein: The maintenance of genome integrity in response to gemcitabine^[66]. Low CHD7 expression was also associated with improved RFS and OS in a retrospective analysis of patients with early-stage resected pancreatic adenocarcinoma treated with adjuvant gemcitabine^[65].

CHD7 as a prognostic biomarker

The study conducted by Colbert *et al.*^[66] suggests that CHD7 expression could potentially be explored as a prognostic biomarker to personalize adjuvant therapy for these patients by determining which patients will receive greater benefit from gemcitabine therapy and

allowing clinicians a way to better select patients for specific adjuvant therapy regimens in the future.

The necroptotic pathway and MLKL

Cell death is mediated through two processes, necrosis and apoptosis. Apoptosis is characterized by chromatin condensation, cell shrinkage, plasma membrane blebbing, and formation of apoptotic bodies^[67]. Necrosis is characterized by oncosis, organelle swelling, and plasma membrane rupture^[68]. Many cancer treatments, including chemotherapy and radiation, induce necrotic cell death^[68-70]. Necrosis has been deemed a passive and unregulated process in contrast to apoptosis, however, emerging evidence has shown that necrosis can occur in a regulated and controlled manner^[71]. Tumor necrosis factors (TNF)-induced necrotic death is called necroptosis^[72]. Necroptosis is dependent on the activities of receptor-interacting protein kinase 1 (RIPK1) and 3 (RIPK3)^[68].

Along with RIPK1 and RIPK3, MLKL forms the necrosis-inducing complex called a "necrosome"^[73]. MLKL is considered a dead kinase due to its lack of phosphate-binding glycine-rich P loop and the absence of a key amino acid, aspartate, required for kinase activity. The necrosome induces cell death through the phosphorylation of MLKL by RIPK3 through the kinase-like domain^[73]. The activity between MLKL and RIPK3 is amplified by TNF- α -mediated RIPK1 activation^[73].

Low MLKL associated with worse prognosis

Colbert *et al.*^[74] explored MLKL expression as a potential prognostic biomarker in patients undergoing resection for

early-stage PAC. Low expression of MLKL was associated with decreased OS regardless of whether adjuvant therapy was used^[74]. The HR for death associated with low MLKL expression became stronger in the group of patients treated with adjuvant therapy than in all patients, and was strongest in those patients receiving gemcitabine chemotherapy^[74]. In a study conducted by He *et al.*^[75] low expression of MLKL was significantly associated with decreased DFS and OS in patients with primary ovarian cancer. The finding low MLKL expression is associated with worse outcomes in patients with primary ovarian cancer and early-stage PAC may be a result of decreased necroptosis signaling. This suggests that necroptosis is an important determinant of cancer cell death and outcome of patients with these cancers^[75]. Study of this gene warrants further analysis as patients with low MLKL expression may benefit from more aggressive chemotherapy regimens or participation in clinical trials due to the low probability that they will benefit from traditional adjuvant therapy. Although MLKL expression may be a useful prognostic marker, further studies should be performed in other patient populations and in larger studies for validation. Also, future studies should also examine the role of MLKL in predicting response to gemcitabine therapy.

CONCLUSION

In the biomarker studies conducted for CHD5, CHD7, and MLKL, each individual gene might serve as an independent prognostic biomarker for patients with early-stage resected PAC. The findings presented provide hypothesis generating momentum to study the expression of these genes in prospective cohorts undergoing adjuvant therapy for PAC. In future studies, using larger patient cohorts, it can be determined whether multiple gene expression provides a more accurate prognostic value than single gene expression alone. The potential exists for clinicians to use biomarkers such as CHD5, CHD7, and MLKL to select the most beneficial therapy regimens and tailor them for individual patients in the future.

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Molecular therapeutics in pancreas cancer

Vignesh Narayanan, Colin D Weekes

Vignesh Narayanan, Colin D Weekes, Division of Medical Oncology, Department of Medicine, Developmental Therapeutics Program, University of Colorado Cancer Center, University of Colorado School of Medicine, Aurora, CO 80045, United States

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Correspondence to: Colin D Weekes, MD, PhD, Division of Medical Oncology, Department of Medicine, Developmental Therapeutics Program, University of Colorado Cancer Center, University of Colorado School of Medicine, 12801 E. 17th Avenue, Aurora, CO 80045, United States. colin.weekes@ucdenver.edu
Telephone: +1-303-7249238
Fax: +1-303-7243889

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Abstract

The emergence of the "precision-medicine" paradigm in oncology has ushered in tremendous improvements in patient outcomes in a wide variety of malignancies. However, pancreas ductal adenocarcinoma (PDAC) has

remained an obstinate challenge to the oncology community and continues to be associated with a dismal prognosis with 5-year survival rates consistently less than 5%. Cytotoxic chemotherapy with gemcitabine-based regimens has been the cornerstone of treatment in PDAC especially because most patients present with inoperable disease. But in recent years remarkable basic science research has improved our understanding of the molecular and genetic basis of PDAC. Whole genomic analysis has exemplified the genetic heterogeneity of pancreas cancer and has led to ingenious efforts to target oncogenes and their downstream signaling cascades. Novel stromal depletion strategies have been devised based on our enhanced recognition of the complex architecture of the tumor stroma and the various mechanisms in the tumor microenvironment that sustain tumorigenesis. Immunotherapy using vaccines and immune checkpoint inhibitors has also risen to the forefront of therapeutic strategies against PDAC. Furthermore, adoptive T cell transfer and strategies to target epigenetic regulators are being explored with enthusiasm. This review will focus on the recent advances in molecularly targeted therapies in PDAC and offer future perspectives to tackle this lethal disease.

Key words: Pancreas neoplasm; Vaccines; Targeted therapy; Immunotherapy; Kirsten rat sarcoma oncogene

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Core tip: The treatment of pancreas ductal adenocarcinoma is in an exciting phase due to a tremendous surge in knowledge regarding the molecular mechanisms that underlie pancreas cancer that has fueled interest in devising novel strategies to target signal transduction factors downstream to kirsten rat sarcoma oncogene, desmoplastic tumor stroma and cancer stem cells. Furthermore, immunotherapy by utilizing vaccines and immune checkpoint inhibitors is gaining momentum. Alluring results from studies evaluating molecularly targeted therapies have not only proven the feasibility of this approach but are also indicative of a paradigm shift in management of pancreatic cancer in the near future.

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INTRODUCTION

Over the past few decades, pancreas ductal adenocarcinoma (PDAC) has claimed notoriety by proving to be one of the most recalcitrant solid-organ malignancies. As a telltale sign of its lethality, PDAC accounts for less than 3% of new cancers diagnosed annually in developed nations and in the United States, yet it is the fourth leading cause of cancer related mortality^[1]. Ominously, PDAC is also poised to surpass breast, prostate and colon cancers to become the second leading cancer related cause of death by 2030^[2]. Owing to the late stage at presentation, most patients with PDAC are not candidates for surgical resection. Even patients with early-stage disease who undergo surgical resection and adjuvant therapy eventually relapse and succumb to it^[3,4].

Patients with advanced disease have a dismal prognosis with 5-year survival rates of less than 5%^[5]. Following the initial success of gemcitabine in the metastatic setting^[6], oncologists have traditionally relied upon cytotoxic chemotherapy to tackle locally advanced and metastatic disease but with limited success. After nearly two decades of research to identify optimal regimens for metastatic PDAC, the PRODIGE 4/ACCORD 11 and MPACT trials have proven the efficacy of combination chemotherapy with meaningful increase in overall survival (OS) although accompanied by the risk of increased toxicity^[7,8]. The median survival for patients with metastatic disease still remains less than 1 year^[7,8].

MOLECULAR THERAPEUTICS IN PDAC

The dawn of the era of precision-medicine in oncology has led to tremendous gains in understanding various molecular mechanisms of PDAC oncogenesis, but translating this knowledge to the bedside with targeted therapy has been a daunting task. The complex biology of PDAC has posed a formidable challenge against successful targeted interventions (summarized in Table 1). However, in recent years several innovative approaches have achieved early success to pave the way for impactful molecular therapeutic strategies.

GENETIC HETEROGENEITY OF PDAC

Similar to the adenoma-carcinoma sequence in colon cancer, the development of PDAC represents the culmination of progressive increments in dysplasia in precursor lesions collectively termed pancreatic intra-epithelial neoplasia (PanIN)^[9]. Molecular profiling studies in genetically engineered mouse models (GEMM) have demonstrated that histological progression of PanINs

from low to high-grade occurs in tandem with successive accumulation of gene mutations such as activation of the *KRAS* oncogene, inactivation of the tumor suppressor cyclin dependent kinase-N2A (*CDKN2A*) gene and the eventual inactivation of TP53 and deleted in pancreatic cancer 4 (*DPC4/SMAD4*) genes^[10]. All of the same genetic alterations also occur in established PDAC but at a higher frequency (Table 2). Patients with familial forms of PDAC also harbor germ-line mutations in *BRCA2* and partner and localizer of *BRCA2* (*PALB2*) genes^[11,12]. In a sentinel genomic analysis of 24 pancreatic tumors, Jones *et al.*^[13] classified the genetic alterations in PDAC into a core set of 12 cellular signaling pathways that encompass an incredibly high 63 gene mutations within an individual tumor. A recent study of 109 micro-dissected pancreatic tumors by whole-exome sequencing corroborated the high mutational burden and also identified other novel genetic mutations that confer adverse prognosis such as *MYC* amplification^[14]. Abnormalities in Wnt and Hedgehog signaling, chromatin remodeling and DNA repair mechanisms occur at a high frequency in PDAC^[14,15]. In addition to remarkable variations in genetic abnormalities in individual tumors, the realization that PDAC genes function through a relatively small number of pathways confers a level of genetic heterogeneity that makes molecular targeting exceptionally difficult.

Targeting *KRAS* and downstream signal transduction

The four human *RAS* genes encode for small guanosine triphosphatases (GTPases) and under normal circumstances cycle between an active GTP-bound and an inactive guanosine diphosphate (GDP) bound state^[16]. Upwards of 95% of PDACs possess activating mutations of the *KRAS* gene, most commonly at the G12 residue^[17]. Mutant *KRAS* remains persistently active in the GTP-bound state and results in uninterrupted downstream signal transduction of growth signals such as rapidly activated fibrosarcoma homolog B (*BRAF*), mitogen activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (*PI3K*)/mammalian target of rapamycin (*mTOR*)^[16].

Despite intensive efforts, direct pharmacologic inhibition of *KRAS* has been unsuccessful because of the high binding affinity of the oncoprotein to GTP and inability to identify an easily accessible active site within *KRAS* that is susceptible to competitive allosteric inhibition^[18]. To overcome these difficulties, alternate approaches have been attempted but with limited success. Van Cutsem *et al.*^[19] attempted inhibition of farnesylation, a crucial step in post-translational modification of *KRAS* proteins that is essential for membrane anchorage of *RAS*, using the farnesyl-transferase inhibitor tipifarnib in combination with gemcitabine. But no improvement in OS was observed when compared to gemcitabine plus placebo in patients with advanced PDAC. Likewise, other strategies such as dislodging *KRAS* from the plasma membrane and preventing interactions with *KRAS* activating proteins have been effective in pre-clinical models but are yet to be translated to the clinical setting^[20,21].

Substantial efforts have also been devoted to inhibition

Table 1 Barriers to effective molecularly targeted therapy in pancreatic ductal adenocarcinoma

PDAC biology	Barrier
Genetic heterogeneity	Inability to directly inhibit <i>KRAS</i>
	Convergence of signal transduction pathways downstream from <i>KRAS</i> with feedback inhibitory loops
Overexpression of EGFR, IGF-1R	Escape from growth factor dependence in later stages of tumorigenesis
Desmoplastic stroma	Hypoxic tumor milieu impairs effective drug delivery
Overexpression of angiogenic factors	Secretion of angiostatic factors in tumor microenvironment
PDAC stem cells	Difficult to eradicate subpopulation of cells capable of self-renewal
	Resistance to chemotherapy, radiation
Low immunogenicity	Evasion of host immunity
	Abundance of immunosuppressive cells in tumor milieu

PDAC: Pancreatic ductal adenocarcinoma; *KRAS*: Kirsten rat sarcoma oncogene; EGFR: Epidermal growth factor receptor; IGF-1R: Insulin like growth factor-1 receptor.

of downstream signal transduction, especially the PI3K and MAPK (RAF/Mek/ERK) pathways, as they are more amenable to pharmacological inhibition^[22-24]. Disconcertingly, this strategy has proven unsuccessful because inhibition of the MEK pathway resulted in feedback activation of the PI3K pathway mediated by the epidermal growth factor receptor (EGFR)^[25]. To counter this Ko *et al*^[26] investigated the effect of dual inhibition of EGFR and MEK with erlotinib and selumetinib respectively. In this phase II non-randomized trial of 41 patients with chemotherapy refractory PDAC, 26% had stable disease for 12 wk or more and 38% of patients had a greater than 50% decline in CA 19-9 levels^[26]. Though this combination approach showed promise, it needs to be validated in larger studies.

Targeting *BRCA2* and *PALB2*

The main role of the tumor suppressor genes *BRCA2* and *PALB2* is to repair double stranded DNA breaks by homologous recombination. In patients with germ-line mutations of these genes, DNA repair occurs by alternate means, predominantly by base-excision repair mediated by the enzyme poly(ADP-ribose) polymerase (PARP). Inhibition of PARP in patients with BRCA mutations renders tumor cells incapable of repairing DNA damage and cell death ensues. This concept is known as “synthetic lethality” and represents a great example of targeted therapy in PDAC^[27].

In a preclinical study, the PARP inhibitor 3-amino-benzamide in combination with gemcitabine showed strong anti-tumor activity by inducing apoptosis in PDAC cell lines^[28]. Remarkably, neoadjuvant iniparib plus gemcitabine induced a complete pathological response in a patient

with recurrent PDAC harboring the *BRCA2* mutation^[29]. In a phase I study of olaparib plus gemcitabine in patients with advanced solid tumors that also included 15 patients with PDAC, no differences in efficacy endpoints were noted with the combination^[30]. However, these patients were genetically unselected and were unable to receive full-dose gemcitabine due to myelotoxicity attributed to olaparib. The role of PARP inhibitors in PDAC might thus be limited to patients with *BRCA2* mutations. The combination of PARP inhibitors with cytotoxic chemotherapy is also being investigated (ClinicalTrials.gov identifiers: NCT01585805 and NCT01296763, Table 3).

OVEREXPRESSION OF GROWTH-FACTOR RECEPTORS ON TUMOR CELLS

PDAC cells overexpress the EGFR and its ligand transforming growth factor- α (TGF- α)^[31]. In transgenic mouse models, EGFR signaling mediated by TGF- α has been shown to be essential for the onset of ductal metaplasia, a precursor lesion that progresses to PanIN and eventually to PDAC^[32]. EGFR signaling is perceived to be a vital cog in mediating the oncogenic effects of *KRAS* as evidenced in mouse models wherein genetic or pharmacological inhibition of EGFR signaling eliminates tumorigenesis^[33,34]. The exact mechanism by which EGFR overexpression contributes to the development of PDAC is unclear but could be related to induction of the Notch pathway^[35]. EGFR overexpression is also associated with increased propensity for liver metastasis and poor prognosis^[36]. Paradoxically, despite compelling pre-clinical evidence, the EGFR inhibitor erlotinib showed only a marginal clinical benefit for patients with advanced PDAC by prolonging OS by a mere 2 wk^[37]. It is hypothesized that EGFR signaling might be essential earlier in tumorigenesis while advanced PDAC cells escape EGFR dependence^[38].

Targeting insulin-like growth factor-1 receptor

PDAC cells also overexpress insulin-like growth factor-1 receptor (IGF-1R)^[39]. IGF-1R signaling promotes tumorigenesis by activating PI3K, MAPK, AKT and Rac pathways^[40]. This results in uncontrolled cellular proliferation, survival and metastasis. Based on exciting and positive results from preclinical^[41] and early-phase trials^[42,43], ganitumab, a fully humanized monoclonal antibody against IGF-1R combined with gemcitabine was investigated in a phase III, double-blind, placebo-controlled randomized controlled trial (RCT) as first-line therapy for patients with metastatic PDAC^[44]. Though the ganitumab-gemcitabine combination was safe, the study was terminated early based on results of a pre-planned futility analysis which revealed no improvement in the primary objective of OS, and the reason for lack of efficacy is yet unclear^[44]. Inhibition of IGF-1R using small interfering RNA (siRNA) had an anti-proliferative effect on HPAC and Panc-1 pancreatic cancer cell lines invoking the possibility of a novel target for

Table 2 Frequency and consequences of common genetic mutations in pancreatic ductal adenocarcinoma

Mutation category	Frequency in PDAC	Effects of mutation	Consequence
Gain of function <i>KRAS</i>	> 95%	Continuous transduction of downstream growth signals (BRAF/MAPK, PI3K/mTOR)	Enhanced cell growth and survival
Loss of function <i>CDKN2A</i>	95%	Disruption of RB1 by CDK4	Uncontrolled cellular proliferation
<i>TP53</i>	75%-85%	Impaired DNA damage repair, loss of cell cycle checkpoint activation	Chromosomal instability, aneuploidy
<i>DPC4/SMAD4</i>	50%	Loss of inhibition of TGF- β	Loss of cell growth inhibition
<i>BRCA2</i>	6%-17%	Impaired DNA damage repair by homologous recombination, loss of cell-cycle checkpoint activation	Genomic instability
<i>PALB2</i>	1%-3%	Impaired <i>BRCA2</i> function	Genomic instability

KRAS: Kirsten rat sarcoma oncogene; *BRAF*: Rapidly activated fibrosarcoma homolog B; *MAPK*: Mitogen activated protein kinase; *PI3K*: Phosphatidylinositol-3 kinase; *mTOR*: Mammalian target of rapamycin; *CDK*: Cyclin dependent kinase; *DPC4*: Deleted in pancreatic cancer 4; TGF- β : Transforming growth factor- β ; *BRCA2*: Breast cancer 2; *PALB2*: Partner and localizer of *BRCA*.

Table 3 Summary of selected ongoing clinical trials evaluating molecular therapies in pancreatic ductal adenocarcinoma (according to www.clinicaltrials.gov, accessed July 2015)

Category	Clinical trial number	PDA setting	Medications studied	Phase	Status	Estimated completion
Tumor suppressor genes	NCT01585805	Locally advanced/ metastatic	Gem and Cisplatin \pm Veliparib <i>vs</i> Veliparib alone	II	Recruiting	07/2017
	NCT01296763	Advanced	Irinotecan + Cisplatin + Mitomycin C \pm Olaparib	I / II	Ongoing, not recruiting	01/2014
Recombinant hyaluronidase	NCT01959139	Metastatic	FOLFIRINOX \pm PEGPH20	I / II	Recruiting	12/2017
	NCT01839487	Metastatic	Gem + Nab-paclitaxel <i>vs</i> Gem + Nab-paclitaxel + PEGPH20	II	Recruiting	04/2016
Vaccine therapy	NCT02004262	Metastatic	Cy + GVAX + CRS-207 <i>vs</i> Chemotherapy <i>vs</i> CRS-207	II	Recruiting	12/2016
	NCT01072981	Adjuvant	Chemotherapy <i>vs</i> Chemo-radiotherapy \pm Algenpantucel-L	III	Ongoing, not recruiting	06/2016
	NCT01836432	Neoadjuvant	FOLFIRINOX \pm Algenpantucel-L	III	Recruiting	09/2015
Immune checkpoint	NCT02472977	Metastatic	Ulocuplumab (CXCR4) and nivolumab (PD1)	I B	Recruiting	7/2017
CAR-T cell therapy	NCT01897415	Metastatic	Autologous redirected RNA mesothelin specific CAR-T cells	I	Not recruiting	01/2015
	NCT01583686	Metastatic	CAR-T cell receptor	I / II	Recruiting	12/2018
Micro-RNA-21 targeted therapy	NCT01274455	Locally advanced	Gem + Plasmid DNA CYL-02	I	Not recruiting	12/2013
Signal transduction inhibitors						
Janus kinase targeted	NCT02119663	Locally advanced/ metastatic	Capecitabine + Ruxolitinib <i>vs</i> Capecitabine + Placebo	III	Recruiting	06/2017
	NCT02117479	Locally advanced/ metastatic	Capecitabine + Ruxolitinib <i>vs</i> Capecitabine + Placebo	III	Recruiting	12/2015
Wnt targeted	NCT02050178	Metastatic	OMP-54F28 + Gem-Nab-paclitaxel	I	Recruiting	12/2016
	NCT01764477	Metastatic	PRI-724 + Gem	I	Recruiting	03/2016
Notch inhibitor	NCT01647828	Locally advanced/ metastatic	OMP-59R5 + Gem-Nab-paclitaxel	I / II	Recruiting	01/2016
	NCT01373164	Locally advanced/ metastatic	LY2157299 + Gem	I / II	Not recruiting	11/2015

PDA: Pancreas ductal adenocarcinoma; Gem: Gemcitabine; FOLFIRINOX: 5-fluorouracil, leucovorin, irinotecan, oxaliplatin; CAR-T: Chimeric antigen receptor T cell; TGF- β : Transforming growth factor- β ; Cy: Cyclophosphamide.

future clinical studies^[45].

DESMOPLASTIC STROMA

The stroma of PDAC is characterized by an intense fibrotic reaction termed “desmoplasia”^[46]. This is attributed to collagen, laminin, fibronectin, hyaluronan and various other components of the extracellular matrix (ECM) secreted by activated pancreatic myofibroblasts (stellate cells) in response to stimuli from TGF- β , platelet derived growth factor (PDGF) and fibroblast growth factors (FGF) produced by the tumor microenvironment (TME)^[46]. The accumulation of ECM components renders the tumor milieu rigid, and the ensuing increase in extracellular fluid pressure results in collapse of blood vessels in the tumor stroma. The resultant hypoxic peritumoral milieu is thus a significant impediment to the effective delivery of chemotherapy to the tumor^[47]. Furthermore, matrix metalloproteinases (MMP) produced in the ECM damages the structural integrity of the ECM to self-perpetuate tumor invasion and metastasis^[48]. The desmoplastic stroma in PDAC represents an ever-changing compartment that not only functions as a mechanical barrier to drug delivery, but also favors tumorigenesis and invasion.

STROMAL TARGETING STRATEGIES

Pegylated recombinant hyaluronidase

Hyaluronan is a visco-elastic glycosaminoglycan found in abundance in normal tissues, notably in joint cartilage^[49]. It is also found in the stroma of PDAC, where it contributes to significantly elevated interstitial fluid pressure (IFP) and vascular collapse^[47]. In the KPC mouse model, enzymatic depletion of hyaluronan with pegylated recombinant hyaluronidase (PEGPH20, Halozyme, San Diego, CA) rapidly normalized the IFP and hence restored normal vascular caliber^[50]. Importantly, co-administration of PEGPH20 and gemcitabine resulted in an 83% increase in survival and a dramatic decrease in metastatic burden in mice, owing to the enhanced delivery of gemcitabine to the tumor^[50]. Based on the encouraging results from a phase I b trial that combined PEGPH20 with gemcitabine^[51], this strategy is now being investigated in phase II trials in combination with conventional chemotherapy regimens for metastatic PDAC (ClinicalTrials.gov identifiers: NCT01959139 and NCT01839487, Table 3). Initial reports demonstrate that patients with high hyaluronan expressing tumors have greater clinical benefit^[52].

Nanoparticle albumin-bound (nab)-paclitaxel with gemcitabine

Though cytotoxic agents are not considered to be within the realm of targeted therapy, nab-paclitaxel might be an exception. In a small yet novel study, the combination of nab-paclitaxel and gemcitabine was administered to 16 patients in a neo-adjuvant fashion^[53]. The effects on tumor stroma were determined by endoscopic

ultrasound (EUS) elastography and examination of surgically resected tumor specimens. Not only was there a significant decrease in tumor stiffness on EUS elastography, but also a decrease in cancer associated fibroblasts (CAF) and significant disruption of the intense collagen architecture^[53]. Similarly, stromal disruption was also noted in a patient-derived xenograft mouse model treated with the same combination^[54]. In this study, genetically engineered mice bearing tumors received nab-paclitaxel, gemcitabine or the combination of the two. The intra-tumoral concentration of gemcitabine was nearly 3-fold higher in mice treated with nab-paclitaxel plus gemcitabine than in those receiving gemcitabine alone. The exact mechanism of action of nab-paclitaxel in depleting tumor stroma has not been elucidated, but could be mediated by secreted protein acidic and rich in cysteine (SPARC) - a matrix glycoprotein and marker of activated fibroblasts^[55] proposed to be a crucial driver of PDAC invasiveness^[54,56,57].

Targeting myofibroblasts/stellate cells

Though the anti-inflammatory properties of 1,25(OH)₂D₃ have been well established^[58], the finding that activated myofibroblasts (also known as stellate cells) overexpress the vitamin D receptor (VDR) was an unexpected finding^[59]. The VDR plays an important role in the transcriptional regulation of activated myofibroblasts by converting them back to their quiescent state^[59]. This is substantiated by a preclinical study in mouse models in which calcipotriol, a VDR agonist resulted in stromal depletion, facilitated intra-tumoral delivery of gemcitabine and caused reduction in tumor volume^[59]. Unlike other therapies that focus on stromal ablation, reprogramming the stroma using vitamin D analogs might be a useful adjunct to PDAC therapy.

Hedgehog pathway inhibition

The hedgehog (Hh) signaling cascade activates the Gli family of receptors when the sonic Hh ligands bind to its receptor Patched1 that in-turn relieves the repression on Smoothened1 (Smo)^[60]. This paracrine signaling is vital for the proliferation of the desmoplastic stroma in PDAC^[60]. IPI-926 is a powerful inhibitor of Smo, which when administered in combination with gemcitabine to KPC mice resulted in increased mean vessel density in the stroma and increased intra-tumoral concentration of gemcitabine^[61]. However, when the combination of gemcitabine with IPI-926 resulted in worse progression free survival (PFS) and OS compared to gemcitabine plus placebo in a phase II trial that had to be terminated early^[62]. More recent studies have shown a possible protective effect of the stroma, which when depleted resulted in a more aggressive and hypervascular phenotype^[63,64]. The incongruity in outcomes between pre-clinical and clinical trials is a fine example to exemplify the complexity of targeting the TME in PDAC.

ANGIOGENESIS

Tumor cells can activate quiescent endothelial cells through

an “angiogenic switch” which causes overexpression of pro-angiogenic factors, chiefly vascular endothelial growth factor (VEGF)^[65]. VEGF and its two high-affinity tyrosine kinase receptors namely flk-1/KDR and flt-1 are overexpressed in PDAC and associated with disease progression^[66]. VEGF enhances MAPK phosphorylation in pancreatic cancer cell lines, and PD9805 an inhibitor of MAPK inhibits the proliferative effects of VEGF^[67]. In contrast to pancreatic cancer cell lines, actual pancreatic tumors have a much lower microvessel density compared to normal pancreatic tissue^[68]. Unsurprisingly, anti-angiogenic therapy directed against circulating VEGF using bevacizumab in combination either in combination with gemcitabine alone or with gemcitabine and erlotinib has been unsuccessful^[69,70]. Likewise, VEGF receptor targeted agents such as axitinib and aflibercept have not improved outcomes either^[71,72]. As explained previously, the desmoplastic stroma contributes significantly to altered vasculature. Additionally, the abundance of angiostatic factors such as angiostatin and endostatin that are secreted in the TME also explains the discordance between VEGF overexpression and lack of clinical benefit with VEGF inhibition^[46,73].

TUMOR STEM CELLS

Cancer stem cells (CSC) constitute a very small proportion of pancreatic tumors (< 1%), but have the potential for unlimited proliferation^[74]. They were identified in PDAC using a xenograft model of immunocompromised mice and proven to have a 100-fold higher tumorigenic potential compared to non-tumorigenic cancer cells^[74]. A distinct population of CD133⁺ PDAC stem cells also predicts propensity to metastasis^[75]. Moreover, cancer stem cells are extremely resistant to chemotherapy and radiation^[76,77], attributed to the overexpression of the early developmental sonic hedgehog (SHH) pathway^[78]. The self-renewing nature of CSCs poses a significant challenge in molecular therapeutics of PDAC.

Targeting CSCs in PDAC

Data emerging from preclinical studies have demonstrated that it is indeed possible to target and eliminate CSCs. Salinomycin, an antibiotic with a greater than 100-fold efficacy against CSCs compared to paclitaxel, inhibited the growth of CD133⁺ pancreatic CSCs and the effects were synergistic with gemcitabine, which curbed the growth of non-CSC cells^[79]. Pancreatic CSCs also overexpress epithelial cell adhesion molecule (EPCAM) and this feature has been the focus of immunotherapy directed against CSCs^[80]. MT110 is a bi-specific T cell engaging antibody (BiTE) that simultaneously targets EPCAM on CSCs and T cell-CD3 complexes on T cells to effectively eliminate the highly tumorigenic CSCs both *in vivo* and *in vitro* in a mouse model of PDAC^[80]. Natural agents such as isoflavones, 3,3'-diindolylmethane (DIM) and curcumin analogues have also garnered attention because of their inhibitory effects on CSCs through cell-signaling

molecules and microRNAs (miRNA)^[81]. Pancreatic CSCs also overexpress Nodal and Activin belonging to the TGF- β superfamily and pharmacological inhibition or knockdown of their receptor activin-like 4 and 7 (Alk 4/7) reversed gemcitabine resistance in an orthotopic mouse model and dramatically reduced their tumorigenicity^[82]. In addition to newer agents, the anti-neoplastic effects of the timeworn drug metformin are also attributed to its activity against pancreatic CSCs^[83]. Results from these preclinical studies await clinical translation.

IMMUNE BIOLOGY OF PDAC

The immune system serves as an innate defense against tumorigenesis and metastasis. To counteract immune-surveillance, tumors develop adaptive mechanisms and PDAC is adept at immune evasion because of its inherently low immunogenicity^[84,85]. The lack of anti-tumor effector T lymphocytes in preclinical mouse models of PDAC compared to a very high proportion of immunosuppressive cells such as regulatory T cells (Tregs), tumor-associated macrophages and myeloid derived suppressor cells tips the balance in favor of tumorigenesis^[85]. Tumor and stromal cells also secrete several inflammatory mediators, notably TGF- β and interleukin 10 (IL-10) which down-regulate T cell and antigen presenting cell (APC) proliferation in the PDAC microenvironment^[86,87]. Despite the purported low immunogenicity of PDAC, the presence of CD4⁺ helper T cells and CD8⁺ cytotoxic T cells (CTL) in resected pancreatic tumors was associated with longer OS, suggestive of a definite immune response against PDAC^[88]. Though it has been a challenging endeavor to devise effective strategies to harness the host's immune system against PDAC, results of recent vaccine trials and immune checkpoint inhibitors in PDAC have been quite encouraging.

VACCINE THERAPY

Immune mediated anti-tumor response occurs in two steps; first, tumor associated antigens (TAA) are presented by APC, notably dendritic cells to effector/CTL, which in turn recognize antigenic epitopes bound to major histocompatibility (MHC) molecules. Next, concomitant binding of co-stimulatory molecules such as B7-1 on APCs and CD28 on T cells results in T cell activation. However, tumor cells lack the additional co-stimulatory molecules and immune evasion ensues^[89]. Vaccine-based therapies are designed to circumvent immune evasion by delivering TAAs to APCs and stimulate a robust cell-mediated immune response to attack and eliminate tumor cells.

Initial vaccine designs for PDAC utilized peptide antigens such as mucin-1 (MUC1), carcinoembryonic antigen and protein products of *KRAS* oncogene that are capable of binding exact MHC molecules^[89]. Because peptide vaccines contain only single antigenic epitopes, it leads to immune tolerance with minimal and transient efficacy^[90]. The expansion of proteonomics and gene expression based assays has led to the identification of

several TAAs that are selectively expressed by pancreatic cancer cells and has widened the scope for development of whole-cell vaccines that utilize these antigens to trigger tumor-specific immunity. Mesothelin is one such example of a TAA that is overexpressed in nearly all PDACs (but not in normal cells) and is implicated in cell adhesion and metastases^[91,92]. Mesothelin-specific CD8⁺ T cell responses have been associated with improved OS following vaccine therapy^[93].

Granulocyte-macrophage colony-stimulating factor vaccines

GVAX: GVAX is a whole-cell irradiated allogeneic vaccine that is composed of tumor cells from two pancreatic cell lines (Panc 10.05 and Panc 6.03) that have been genetically modified using a plasmid vector encoding for the granulocyte-macrophage colony-stimulating factor (GM-CSF) gene^[94]. When injected transdermally, high GM-CSF secretion at the vaccine site causes mobilization and differentiation of APCs, a feature that patients with PDAC typically lack. APCs subsequently migrate to regional lymph nodes and activate CD4⁺ and CD8⁺ T cells to mount an effective anti-tumor response^[95].

Initial trials demonstrated the safety and tolerability of GVAX when administered in the adjuvant setting followed by conventional chemoradiation. Delayed-type hypersensitivity (DTH) reactions and induction of mesothelin-specific CD8⁺ cells correlated with prolonged disease free survival in the phase I and phase II trials respectively^[94,96]. Based on the favorable results in the adjuvant setting, GVAX was studied in the metastatic setting in patients who had progressive disease after gemcitabine^[97]. In this open-label phase II study, the combination of GVAX with immune-modulating dose of cyclophosphamide (Cy) was compared to GVAX alone. The rationale for adding Cy was to enhance treatment related immune response by inhibiting immunosuppressive Tregs. Although OS was better in the combination arm compared to GVAX alone, the results were not statistically significant (median OS - 4.7 mo vs 2.3 mo). CD8⁺ T cell responses to mesothelin were enhanced in the combination arm and associated with a trend towards prolonged PFS^[97]. Whether metronomic Cy plus GVAX will counter immune tolerance mediated by Tregs is being evaluated in a randomized clinical trial (NCT00727441, Table 3).

GVAX-prime and CRS-207-boost

CRS-207 is a live-attenuated strain of *Listeria monocytogenes*, genetically engineered to secrete mesothelin into the cytosol of APCs. In addition to activating effector T cells by delivering TAAs directly to the APCs, the cytokine mediated inflammatory response that is triggered by CRS-207 also serves to recruit more APCs^[95]. The synergy between GVAX and CRS-207 was demonstrated in a phase I trial^[98] which led to a multi-center, randomized phase II trial among 90 patients exclusively with PDAC^[99]. Patients with previously treated metastatic PDAC were randomized 2:1 to either 2 doses of GVAX immune

priming followed by 4 doses of CRS-207 as a boost (arm A) or 6 doses of GVAX alone (arm B). All patients received Cy to inhibit Tregs. After a median duration of follow-up of 6.6 mo, the OS was 6.1 mo in arm A compared to 3.9 mo in arm B (HR for death, 0.59; 95%CI: 0.36 to 0.91, $P = 0.02$). Toxicity with the combination was minimal and included transient fevers, fatigue, lymphopenia and elevated liver enzymes. As with previous studies, detection of enhanced mesothelin specific CD8⁺ T cell responses was associated with longer OS regardless of treatment arm^[99]. A larger phase II b trial is currently underway to compare the combination of GVAX plus CRS-207 to CRS-207 alone or chemotherapy alone in the metastatic setting (NCT02004262, Table 3).

Algenpantucel-L

Algenpantucel-L (NewLink Genetics Corporation, Ames, IA) is an allogeneic vaccine that contains two PDAC cell lines (HAPa-1 and HAPa-2) that have been genetically engineered to express $\alpha(1,3)$ -galactosyl epitopes (α -Gal)^[100]. Though human cells lack the α -Gal epitopes, the gut flora stimulates antibodies against it. These antibodies are the primary mediators of hyperacute rejection characterized by rapid organ destruction through complement activation within minutes of organ transplantation^[100]. When these antibodies are coupled with tumor cells such as in algenpantucel-L, it promotes opsonization and phagocytosis of tumor cells by APCs and results in T cell activation. In a phase II study of 70 patients with resected PDAC, algenpantucel-L was added to either gemcitabine or 5-fluorouracil based chemoradiotherapy^[100]. After a median follow-up of 21 mo, the DFS and OS at 1 year were 62% and 86% respectively. Notably, the OS in this trial was better than the reported 81% in the sentinel RTOG-9704 trial using the same chemoradiotherapy regimen. Patients who received a higher dose of 300 million cells/dose fared better than those who received 100 million cells/dose with regard to both DFS (81% vs 51%) and OS (81% vs 68%) at 12 mo respectively, suggesting a strong dose-response effect. Apart from mild adverse events such as injection site pain and induration the vaccine was well tolerated. Phase III trials evaluating algenpantucel-L in the adjuvant (NCT01072981) and neoadjuvant setting (NCT01836432) are ongoing (Table 3).

Immune checkpoint inhibitors

Cytotoxic T lymphocyte antigen-4 (CTLA-4) is expressed on the surface of activated T cells and down-regulates immune activation by competitively inhibiting the binding of CD28 to B7-1 and turning off the intracellular signaling cascade of B7-1^[101]. Monotherapy with ipilimumab (Yervoy, Bristol-Myers Squibb Company), an anti-CTLA-4 mAb, was ineffective in the treatment of locally advanced or metastatic PDAC^[102]. However, the combination of GVAX with ipilimumab showed striking clinical and immunological synergy in previously treated patients with advanced PDAC^[103]. Compared to single-agent ipilimumab, patients

in the combination therapy arm had better OS at 1 year (27% vs 7%) although the study was not powered to detect differences in OS. Significantly however, combination therapy was not associated with increased adverse events, despite the higher dose of ipilimumab (10 mg/kg) used in this study. Increase in peak mesothelin-specific T cells and enhancement of the T cell repertoire was associated with longer OS^[103]. Most responders in this study required at least 12 wk of therapy, thus underscoring the need for selecting patients with early stage disease in future trials to evaluate delayed responses often seen with immunotherapy.

Programmed cell death ligand-1 (PD-L1) and its receptor PD-1 are expressed on the cell surface of tumor cells as well as activated T cells. This receptor-ligand interaction down-regulates CD4⁺ and CD8⁺ T cells and is a natural immune checkpoint to prevent excessive immune mediated tissue damage^[104]. PD-L1 expression is up regulated in PDAC cells and results in a blunted T cell response against the tumor^[104]. Blocking the interaction between PD-1 and PD-L1 successfully augmented anti-tumor immune responses *in vitro* and formed the basis for investigating the efficacy of BMS-936559, an anti-PD-L1 mAb in various solid tumors^[105]. Durable responses were noted in patients with melanoma, non-small cell lung cancer and renal-cell carcinoma but disappointingly no responses were seen in 14 patients with PDAC^[105]. The resistance to PD-L1 inhibition in PDAC is due to the high expression of fibroblast activation protein (FAP) by carcinoma-associated fibroblasts (CAF) in the tumor stroma^[106]. These FAP⁺ CAFs produce chemokine (C-X-C motif) ligand 12 (CXCL12) that binds with chemokine receptor 4 (CXCR4) to prevent T cells from infiltrating the tumor and causing local immunosuppression. Inhibition of CXCR4 by AMD3100 in a GEMM of PDAC caused cancer regression synergistically with PD-L1 inhibition^[106]. Therefore it might be feasible to overcome tumoral immunosuppression through a combined approach. A clinical trial to test this hypothesis has recently opened to enrollment as a phase I study of ulocuplumb (anti-CXCR4) and nivolumab (anti-PD1) (NCT02472977, Table 3). Since these immune-modulating agents are not cancer T cell specific and can cause activation of other quiescent T cell populations, autoimmune toxicities occur frequently^[107]. Hence future studies will also need to focus on effective management of these toxicities.

CD40 agonist therapy

CD40 belongs to the tumor necrosis factor receptor superfamily and is expressed by multiple APCs including dendritic cells, B cells and macrophages^[108]. Activated CD40 plays a crucial role in the priming and activation of tumor-specific T cells, but also mediates T cell independent antitumor immunity by activating macrophages^[108]. CP-870, 893 is a CD40 agonistic mAb that potentiates anti-tumor immunity by these aforementioned mechanisms. In the initial pre-clinical study CP-870, 893 when administered in combination with gemcitabine in the KPC mouse model caused rapid regression of tumors mediated by

T cell-independent macrophage infiltration^[109]. Notably, depletion of tumor stroma was also noted and attributed to the effect of stromal infiltrating macrophages. In a phase I trial conducted subsequently, 22 patients with previously untreated advanced PDAC were administered CP-870, 893 with gemcitabine^[110]. The radiological response rate (19% vs 9.4%) and median OS (8.6 mo vs 6.8 mo) were better than expected with single agent gemcitabine. The addition of gemcitabine is postulated to cause antigenic release akin to that of a vaccine with co-stimulation of APCs by CD40 agonist therapy^[111]. Apart from transient cytokine release syndrome and depletion of B cells, none of the auto-immune toxicities seen with the immune check-point inhibitors were noted^[110].

FUTURE STRATEGIES

Adoptive T cell transfer

Stemming from the successes in hematological malignancies notably acute lymphoblastic leukemia^[112], adoptive T cell transfer is an exciting new paradigm that holds tremendous promise in PDAC. This therapeutic strategy involves *ex vivo* genetic engineering of T cells collected from patients to produce chimeric antigen receptors (CAR) capable of recognizing mesothelin expressed on PDAC cells^[113,114]. Infusion of CAR-T cells back to the patient results in immediate recognition of tumor cells and obviates antigen processing and HLA expression. In preclinical studies, CAR-T cells exhibited potent anti-tumor activity^[115]. Beatty *et al*^[116] have also reported a marked decline in ascitic fluid malignant cell burden in a patient with metastatic PDAC, in addition to transient decline in [¹⁸F] fluorodeoxyglucose uptake on positron emission tomography (PET) scan after infusion of CAR-T cells. CAR-T cell therapy is a subject of active research in PDAC and studies are ongoing (ClinicalTrials.gov identifiers: NCT01897415 and NCT01583686, Table 3).

Targeting epigenetic regulators

Epigenetics is the study of changes in gene expression by mechanisms other than changes in the DNA code. Histone modification by acetylation or methylation, DNA methylation and miRNA expression are the main mechanisms of epigenetic regulation^[117]. Histone acetylation by histone acetyltransferase promotes transcriptional activity but histone deacetylases (HDAC) repress transcription of tumor suppressor genes and are overexpressed in PDAC^[118]. HDAC inhibitors serve to abrogate the transcriptional repression and impair tumorigenesis by playing a crucial role in differentiation, cell-cycle inhibition and apoptosis in tumor cells^[117]. Multiple HDAC inhibitors such as hydroxamic acid derivatives (vorinostat), cyclic peptides (romidepsin), short-chain fatty acids (valproic acid) and benzamides have been studied, but results in PDAC have been disappointing^[119,120]. However, the recognition that miRNAs play an important role in PDAC has resulted in increased attention towards exploiting them as potential

therapeutic targets^[121]. Acting at the post-transcriptional level, these non-coding RNAs play a crucial role in apoptosis, differentiation and proliferation. Aberrant overexpression of multiple miRNAs, particularly miRNA-21 has been demonstrated in PDAC and its inhibition by Lentiviral vectors has shown promising antitumor effects in preclinical studies^[121,122]. MiRNA targeted therapy especially in combination with chemotherapy is in its early stages and expected to gain momentum in the future (ClinicalTrials.gov identifier: NCT01274455, Table 3).

Targeting signal transduction

As described previously, targeting signaling pathways downstream from *KRAS* has been unsuccessful so far. However, there is renewed interest in targeting the effects of Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway after its importance in PDAC and associated cachexia became apparent^[123,124]. The addition of the JAK inhibitor ruxolitinib to capecitabine in patients with refractory metastatic PDAC in a phase II trial showed OS benefit for a subgroup of patients with elevated levels of C-reactive protein^[125] and has formed the rationale for phase III trials evaluating ruxolitinib in metastatic PDAC (ClinicalTrials.gov identifiers: NCT02119663 and NCT02117479, Table 3). Global genomic analysis data also revealed alterations in genes in the Wnt/Notch and TGF- β signaling pathways in all PDACs^[13]. Ongoing clinical trials to evaluate the efficacy of specific inhibitors of these pathways are currently underway (ClinicalTrials.gov identifiers: NCT02050178, NCT01764477: Wnt inhibitors, NCT01647828: mAb against Notch, NCT01373164: Oral anti TGF- β receptor type 1, Table 3).

CONCLUSION

As evinced in this review, with improved understanding of the biology, genetic basis and molecular mechanisms that initiate and propagate PDAC carcinogenesis, the focus has shifted from identifying effective cytotoxic chemotherapy regimens to molecularly targeted therapies. These efforts have been further burnished by significant strides in the field of onco-immunology that now allows for cautious optimism that effective therapeutic options for PDAC are finally within reach.

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Targeting inflammation in pancreatic cancer: Clinical translation

Colin William Steele, Nina Angharad Kaur Gill, Nigel Balfour Jamieson, Christopher Ross Carter

Colin William Steele, Nigel Balfour Jamieson, Christopher Ross Carter, Department of Pancreaticobiliary Surgery, Glasgow Royal Infirmary, Glasgow G4 0SF, United Kingdom

Nina Angharad Kaur Gill, Department of General Surgery, South Glasgow University Hospital, Glasgow G51 4TF, United Kingdom

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Correspondence to: Colin William Steele, MD, Department of Pancreaticobiliary Surgery, Glasgow Royal Infirmary, Queen Elizabeth Building, Glasgow G4 0SF, United Kingdom. cwsteele@hotmail.co.uk
Telephone: +44-0141-2114000

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Abstract

Preclinical modelling studies are beginning to aid development of therapies targeted against key regulators of

pancreatic cancer progression. Pancreatic cancer is an aggressive, stromally-rich tumor, from which few people survive. Within the tumor microenvironment cellular and extracellular components exist, shielding tumor cells from immune cell clearance, and chemotherapy, enhancing progression of the disease. The cellular component of this microenvironment consists mainly of stellate cells and inflammatory cells. New findings suggest that manipulation of the cellular component of the tumor microenvironment is possible to promote immune cell killing of tumor cells. Here we explore possible immunogenic therapeutic strategies. Additionally extracellular stromal elements play a key role in protecting tumor cells from chemotherapies targeted at the pancreas. We describe the experimental findings and the pitfalls associated with translation of stromally targeted therapies to clinical trial. Finally, we discuss the key inflammatory signal transducers activated subsequent to driver mutations in oncogenic Kras in pancreatic cancer. We present the preclinical findings that have led to successful early trials of STAT3 inhibitors in pancreatic adenocarcinoma.

Key words: Pancreatic cancer; Inflammation; Stroma; Microenvironment

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Core tip: Many advances have been made in preclinical assessment of therapies in pancreatic cancer. Here we review the successes and failures of translation to clinical trial of therapies targeting the pancreatic cancer microenvironment. Using data from preclinical trials we expose opportunities for further clinical trial within pancreatic cancer. We focus on therapies that modulate the immune response to pancreatic cancer, stromally active therapies and therapies targeting inflammatory signal transduction that are key in pancreatic cancer progression. We provide experimental results that have led to clinical trial and those findings that may be exploited in future. We attempt to rationalize the failure of certain therapies to

translate to clinical practice and provide a realistic overview of why at present tumor microenvironment targeted therapies are not licensed in pancreatic cancer.

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INTRODUCTION

Rationale for targeting inflammation in pancreatic cancer

Inflammation is a hallmark of cancer^[1]. For over 100 years scientists have been interested in the relationship between inflammation and cancer. Researchers within Glasgow Royal Infirmary have for some time been interested in the relationship between cachexia, inflammation and poor prognosis in cancers of different origins. The modified Glasgow Prognostic Score (mGPS) that assesses blood albumin in combination with inflammation, C-reactive protein (CRP), has for over a decade been used to accurately predict outcome across a range of tumor types. Raised mGPS correlates with poor patient prognosis in colorectal, renal and pancreatic cancers^[2]. Additionally, large observational studies have analyzed both cancer incidence and outcome based on daily aspirin use during previously performed randomized controlled trials. The long-term use of aspirin, a non-selective COX inhibitor, improves survival from cancer as a result of reduction in cancer incidence and metastatic burden^[3-5]. These findings demonstrate a clear link between inflammation and cancer initiation and behaviour. Thus, there is observational evidence that inflammation promotes incidence, enhances progression and impacts on prognosis in patients with cancer.

Pancreatic adenocarcinoma (PDAC) presents at a late stage of progression and is associated with very poor outcomes (www.cancerresearchuk.org/cancer-info/cancerstats/). Surgery remains the only potentially curative treatment, though as few as 15% of patients have disease amenable to surgical intervention, and despite surgery the majority of these patients will succumb to recurrent disease. Therefore, new therapies and methods of instituting these therapies are required if survival is to improve in PDAC.

In addition to standard clinicopathological features, presence of systemic inflammation, as assessed by CRP, is a poor prognostic factor in patients undergoing surgical resection for PDAC. In a cohort of 135 patients who underwent potentially curative Whipple's resection for PDAC an elevated mGPS was independently associated with lower overall survival^[6]. Furthermore a high neutrophil to lymphocyte ratio (NLR), a further index of host innate response, has been categorically shown to confer poor

prognosis in PDAC^[7]. Interestingly, in this study of 74 patients, NLR had improved utility at predicting disease recurrence than CRP. This phenomenon was not confined to resectable cases of PDAC, inoperable cases of PDAC appear to respond poorly to chemotherapy in the presence of a raised NLR^[8]. Indeed in the randomized controlled clinical trial of nab-paclitaxel in PDAC an elevated NLR conferred poor prognosis in both treatment arms^[9]. Therefore, assessment of host inflammation at the time of diagnosis of PDAC, has clinical implications for patient survival regardless of therapeutic modality.

The treating physician should consider the inflammatory insults they are subjecting patients to during their treatment course. Over the last decade minimally invasive surgery of the pancreas has increased significantly. When 65033 resections of liver and pancreas were assessed, patients who had minimally invasive pancreatic resections had reduced morbidity, mortality, and length of stay in hospital compared with those having open resections. Traditionally inflammatory insults generated by minimally invasive surgery are smaller than open procedures, however, at present, studies show no oncological benefit^[10]. Intra-operative blood transfusion is associated with loss of immune surveillance in cancer patients, with associated increases in morbidity and mortality following surgery. These data suggest transfusion should be avoided in the peri-operative period if possible^[11]. Furthermore, a profoundly elevated systemic inflammatory response in the post-operative period has been associated with increasing rates of infectious complications following a number of operations including pancreatectomy^[12]. To our knowledge no randomized controlled data exist to confirm the findings of this meta-analysis, however, the study raises the question of the benefits of use of anti-inflammatories in the post-operative setting. Such benefits would have to be offset against potential increases in the risk of anastomotic leak. Thus, when dealing with the small percentage of patients who have PDAC suitable for operative management, surgeons must consider the implications of their treatments. Limiting inflammatory insults involved seems sensible but requires clarification *via* clinical trial.

In vivo models of PDAC

Preclinical studies in PDAC have improved greatly in the past decade with the development of murine models that genetically and histologically recapitulate the human disease. Murine models use pancreas specific promoters to drive oncogenic *Kras* and tumor suppressor gene mutations including mutant *Tp53* to create experimental PDAC murine models with an active microenvironment. These models permit preclinical interrogation of targeted therapies in the hope of translation to patients *via* clinical trial.

Importantly, progression of murine models of PDAC based on initiating oncogenic *Kras* mutations are greatly accelerated in the presence of pancreatic specific inflammation^[13]. Further work by the same authors

revealed this was due to the requirement of pancreatitis to overcome oncogene-induced senescence, which can be blocked by anti-inflammatory medication^[14]. Lee *et al.*^[15] found that when Kras was mutated within the pancreas, pancreatic inflammation led to reduction in *Ink4a* expression. Low levels of Ink4a allowed tumor cells to escape senescence and progress to form tumors. In the presence of Kras mutations, pancreatic inflammation is sufficient to induce tumor formation.

Following initiation of PDAC, inflammation promotes tumor progression. Within the tumor microenvironment there are many pro and anti-tumoral interactions^[16]. Immune cells present have plasticity that permits differing both pro or anti-tumorigenic actions based on received stimulus. Ultimately, during PDAC evolution, a myriad of stromal elements, immune cells and key transducers of inflammatory signals cooperate to permit disease progression. Improvements in understanding the tumor microenvironment are permitting trial of novel therapeutics against key disease progression mediators. Ongoing research in this area will elucidate more completely the complex interactions within the tumor microenvironment and help future development and assessment of multi-target drug regimens.

DISCUSSION

Inflammatory targets identified by in vivo modeling studies in PDAC

Possible inflammatory therapeutic targets in PDAC can be classified into one of three categories: (1) immune modulation to target tumors; (2) targeting tumor stroma; (3) targeting signal transduction (Table 1).

This review will consider the progress made by preclinical studies in each of these three areas and how better understanding of the PDAC microenvironment has potential to translate to the clinical arena. Figure 1 provides a summary of potential inflammatory targets for therapy in PDAC.

Immune modulation

Tumor immunosurveillance is a term that refers to identification and clearance of tumor cells in the early stages of tumorigenesis by the adaptive immune system. It is the role of CD8⁺ T cells to provide cytotoxic protection against "foreign" tumor cells, and hence the development of tumor immunogenicity. Different facets of immunosurveillance are now being interrogated to establish how PDAC so effectively evades detection.

Dendritic cells are a good example of an immune cell capable of adopting dual roles within the PDAC microenvironment. Dendritic cells can engage both CD8⁺ and CD4⁺ T cell responses dependent on stimulus^[17]. Chemokine CXCL17 may be important for migration of dendritic cells to tumor sites while ICAM 2 upregulation was necessary for activation of a CD8⁺ cytotoxic response against tumor cells. Downregulation of CXCL17 and ICAM2 by tumor cells during evolution from precursor lesions to

PDAC allowed tumors to develop immune tolerance^[18]. In contrast Ochi *et al.*^[19] have demonstrated that blockade of TLR4 signaling promotes CD4⁺ T helper cell activity which has a positive effect on pancreatic tumorigenesis through mediation of pro-tumorigenic inflammatory responses. The plasticity of the immune system was highlighted by the findings of Beatty *et al.*^[20]. In patients with metastatic PDAC, targeting CD40 with monoclonal antibodies led to tumor regression. Authors anticipated CD40 ligation would result in enhanced anti-tumoral T cell responses, however in fact resulted in anti-tumoral effects through macrophage infiltration. This phase 1 trial holds promise for trials of similar agents to activate an anti-tumoral immune response.

Tumor-associated macrophages (TAMs) are ever present from pre-invasive Pan-Ins to established PDAC^[21]. TAMs exhibit an M2 phenotype that is pro-tumorigenic while suppressing adaptive immunity^[22]. In cancer, signals received by macrophages from tumor cells including interleukin (IL)-10 and transforming growth factor (TGF)- β lead to adoption of an M2 phenotype^[22]. Macrophages are attracted to the tumor microenvironment *via* production of chemokines by tumor cells^[23]. CSF1 and CCL2 are crucial mediators of this chemo-attraction. CCL2 overexpression mediates migration of M2 macrophages to PDAC and is thought to play a key role in recruiting pro-tumorigenic macrophages to metastatic sites in development of the metastatic niche^[24,25]. *In vivo* studies of anti-CCL2 drugs were effective in enhancing tumor immunity and impacting on metastasis in PDAC^[26]. In addition, patients with high CCL2 expression and low CD8 T cell infiltration suffer poor outcomes following tumor resection.

Direct depletion of TAMs may also be a therapeutic option. Trabectedin has recently been licenced for study in PDAC and is currently in phase 2 trials in advanced disease (NCT01339754). Trabectedin can actively target macrophages *via* caspase-8 dependent apoptosis with selectivity to TAMs achieved through differential expression of TRAIL receptors by macrophages^[27].

Promotion of anti-tumoral cell mediated responses has been successful, particularly in metastatic melanoma. These strategies focus on engaging T cell responses. CTLA4 inhibitor, ipilimumab, was the first drug shown to improve outcome in patients with metastatic melanoma. Following this development and success of Programmed cell death 1 (PD1)/Programmed cell death ligand (PDL1) T cell checkpoint inhibitors has led to great excitement in the field of oncology. These drugs have made a significant impact on survival of patients with metastatic melanoma. CTLA4 is a cell surface protein that suppresses T cell function. When drugs such as Ipilimumab bind CTLA4, T cell function is activated. Likewise, PD1 inhibits T cell function. Production of PD1s major ligand PDL1 by tumor cells and pro-tumorigenic immune cells permits tumors to escape T cell mediated adaptive immunosurveillance^[28]. Hence PD1 is an ideal target when attempting to generate anti-tumoral immune responses.

Concerns exist that PDAC may not respond to such

Table 1 Preclinical assessment of inflammatory targets in pancreatic adenocarcinoma

Target	Drug	PMID	Year	Authors summaries
Hedgehog acyltransferase (Hhat)	RU-SKI 43	24469057	2015	<i>In vivo</i> mouse study targeting Hedgehog acyltransferase (Hhat). A lentivirally delivered hairpin RNA impeded the proliferation of pancreatic cancer <i>in vitro</i> and <i>in vivo</i>
Hedgehog	GDC-0449	25679326	2015	Combination therapy with GDC-0449 or miR-let7b <i>vs</i> single agent therapy effectively inhibited tumor growth when injected to athymic nude mice bearing ectopic tumors generated using MIA PaCa-2 cells
Sonic hedgehog pathways	Ormeloxifene	25840985	2015	Ormeloxifene caused potent inhibition of the SHH signaling pathway <i>via</i> downregulation of SHH and its related important downstream targets. Ormeloxifene potentiated the antitumorigenic effect of gemcitabine by 75% in PDAC xenograft mice
Hedgehog pathway	MEDI-5304	24344235	2014	MEDI-5304 displayed robust pharmacodynamic effects in stromal cells that translated to antitumor efficacy as a single agent in an HT-29/MEF coimplantation model of paracrine hedgehog signaling. MEDI-5304 also improved responses to carboplatin in the HT-29/MEF model. The antibody, however, had no effect as a single agent or in combination with gemcitabine on the CSC frequency or growth of several primary pancreatic cancer explant models
Hedgehog	GDC-0449	25278454	2014	GDC-0449 for 3 wk leads to downmodulation of GLI1 and PTCH1, without significant changes in CSCs compared with baseline. GDC-0449 and gemcitabine were not superior to gemcitabine alone in the treatment of metastatic pancreatic cancer
Hedgehog pathway	Metformin	24692708	2014	<i>In vitro</i> , BxPC3 human pancreatic cancer cells were treated with metformin, and Sonic hedgehog (Shh) mRNA and protein levels were examined. Metformin reduces the expression of Shh in several cancer cell lines including pancreatic cancer cells
Hedgehog	Curcumin	23563640	2013	Curcumin can inhibit the proliferation of TGF- β 1-stimulated PANC-1 cells, it can induce apoptosis, and reverse the EMT. The possible underlying molecular mechanisms are through inhibition of the Shh-GLI1 signaling pathway
COX 5-lipoxygenase (5-LOX)	Dietary licofelone	25906749	2015	<i>In vivo</i> mouse study of licofelone, an agent that targets both COX-2 and 5-LOX
LOX	Zileuton	25483364	2014	Zileuton suppressed the proliferation of SW1990 cells in a concentration- and time-dependent manner. In addition, zileuton induced SW1990 cells to undergo apoptosis and significantly decreased 5-LOX expression
STAT3	Thiosemicarbazones	25561562	2015	<i>In vitro</i> and <i>in vivo</i> iron-binding ligands inhibit constitutive and interleukin 6-induced activation of STAT3 signaling DFO, Dp44mT, and DpC significantly decreased constitutive phosphorylation of the STAT3 transcription factor at Tyr705 in the pancreatic cancer cell lines and when injected <i>in vivo</i>
STAT3	Aspirin metformin	26056043	2015	Metformin combined with aspirin significantly inhibited the phosphorylation of mTOR and STAT3, and induced apoptosis as measured by caspase-3 and PARP cleavage Taken together, the combination of metformin and aspirin significantly inhibited pancreatic cancer cell growth <i>in vitro</i> and <i>in vivo</i>
JAK2 STAT3	MicroRNA (miR)-216a	25220761	2014	MiR-216a overexpression markedly inhibited the JAK2/STAT3 signaling pathway and xenograft tumor growth <i>in vivo</i>
ALK pathway including STAT3	Crizotinib	25193856	2014	Crizotinib strongly suppressed the growth and proliferation of pancreatic cancer cells in a dose-dependent manner. Crizotinib strongly inhibited the expression of activated ALK in pancreatic cancer cells, modulating its downstream mediators such as STAT3, AKT, and ERK
STAT3 NF- κ B COX-2 EP4	Nexrutine	24520096	2014	Nexrutine treatment inhibited growth of pancreatic cancer cells through induction of apoptosis Reduced levels and activity of STAT3, NF- κ B, and their crosstalk led to transcriptional suppression of COX-2 and subsequent decreased levels of prostaglandin E2 (PGE2) and PGF2. Nexrutine intervention reduced the levels of NF- κ B, STAT3, and fibrosis <i>in vivo</i> . Expression of prostaglandin receptor EP4 that is known to play a role in fibrosis was significantly elevated in human pancreatic tumors. Dual inhibition of STAT3-NF- κ B by Nexrutine may overcome problems associated with inhibition of either pathway
JAK/STAT Src/FAK	Guggulsterone	23920124	2013	<i>In vitro</i> , guggulsterone treatment decreased mucin MUC4 expression in Capan1 and CD18/HPAF cells through transcriptional regulation by inhibiting Jak/STAT pathway
Notch JAK2	GSI IX and AG-490	24293409	2014	Combinational treatment with anti-NOTCH and JAK/STAT drugs significantly attenuates tumor progression <i>in vivo</i> and suppresses conversion from acinar-ductal-metaplasia to PDAC

Outlines the significant interest shown by preclinical researchers in targeting inflammation in PDAC. Using the search criteria pancreatic cancer/pancreatic adenocarcinoma + hedgehog, JAK/STAT, LOX we identified preclinical studies that have attempted to assess therapeutics targeted against these important inflammatory mediators of PDAC progression in the past 2 years. We have included those published in journals with an impact factor > 5. PDAC: Pancreatic adenocarcinoma; PARP: Poly-ADP-ribose polymerase; JAK: Janus kinase; STAT: Signal transducer and activator of transcription.

T cell interference because they are extremely fibrotic and desmoplastic. As a result PDACs have a relative

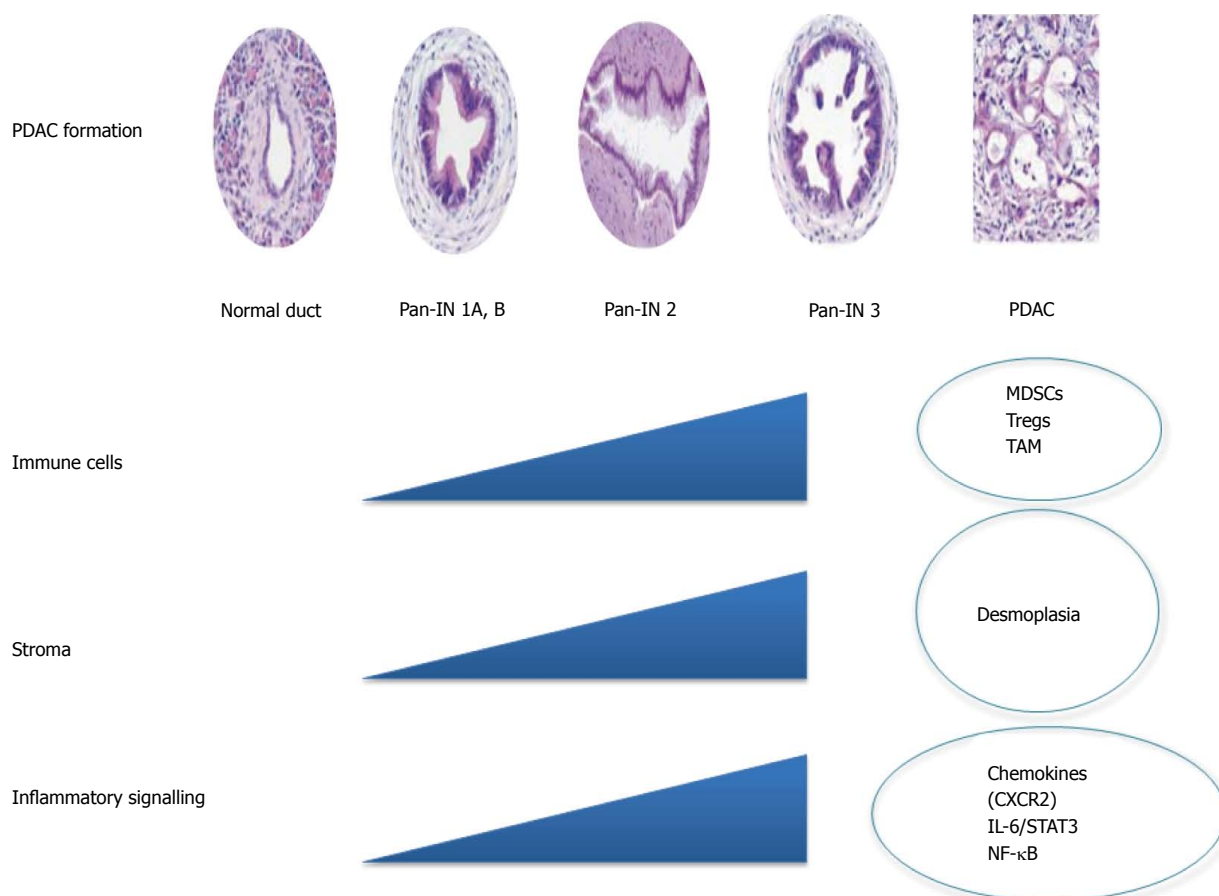


Figure 1 Changes in the pancreatic adenocarcinoma microenvironment during tumor formation. Pancreatic cancer forms from normal tissue via progression through pre-invasive pancreatic intra-epithelial neoplasia (Pan-IN) to invasive PDAC. Changes in immune cell components, stroma, and inflammatory signaling pathways all contribute to PDAC progression. Here we identify possible targets for therapy in PDAC. PDAC: Pancreatic adenocarcinoma; NF-κB: Nuclear factor kappa B; STAT: Signal transducer and activator of transcription; IL: Interleukin; MDSCs: Myeloid derived suppressor cells.

paucity of anti-tumoral T lymphocytes seen at histology compared with other epithelial tumors. Preliminary studies assessing ipilimumab have proven unsuccessful as a single agent^[29]. Feig *et al.*^[30] have recently shown that immunosurveillance can be overcome in PDAC *in vivo* by expression of fibroblast activating protein (FAP) and production of CXCL12 by cancer associated fibroblasts (CAFs). These CAFs were able to prevent T cell infiltration to the tumor microenvironment. Interestingly when these CAFs were depleted genetically, or indeed CXCL12 was inhibited, tumors were sensitised to T cell checkpoint inhibition. Furthermore, myeloid derived suppressor cells (MDSCs) are orchestrated by PDAC to suppress proliferation and induce apoptosis in activated T cells^[31]. Selective depletion of this granulocytic subset of MDSCs led to enhanced CD8⁺ T cell responses promoting immunosurveillance. These data suggest that greater understanding of the processes of evasion of tumor immunosurveillance by PDAC will open up therapeutic opportunities *via* combination with therapies that enhance the effectiveness of immunogenics.

Tumor vaccines are designed to target tumor specific antigens, activating adaptive immunity, eradicating tumor cells. Studies have assessed PDAC specific antigens including MUC-1 that is expressed by over 90% of PDAC cells. Vaccine PANVAC-VF was developed

to be active against cells expressing MUC-1, oncofetal protein carcinoembryonic antigen (CEA) and 3 co-stimulatory molecules. Unfortunately no survival benefit was seen with the addition of PANVAC-VF to standard therapy in a phase III trial in palliative PDAC patients^[32]. Ribonucleoprotein enzyme Telomerase, which maintains telomeric stability, has been assessed unsuccessfully as a potential vaccine target in the Telovac trial^[33]. 1062 palliative patients were randomised to standard chemotherapy, sequential chemotherapy with Telovac or concurrent chemotherapy and Telovac. Neither Telovac groups showed any survival advantage. Smaller trials have been established against other targets including KRAS, although none has proven successful in clinical trial as yet showing how difficult it is to raise a successful adaptive immune response against PDAC.

Targeting tumor stroma

The majority of the tumor bulk of PDAC is composed of stromal cells. This dynamic network of immune cells, stellate cells and extracellular matrix is now believed to play a crucial role in sustenance and support for invasive tumor cells^[34]. Stellate cells are key coordinators of fibrosis as a result of received signals from tumor cells in PDAC^[35]. SPARC is one such factor present in high levels in the tumor microenvironment. SPARC functions

normally to promote wound healing, however its role in PDAC is less certain. High expression of SPARC is associated with poor patient survival in resected cohorts of PDAC patients^[36]. Albumin-bound Paclitaxel (nab-paclitaxel) binds SPARC-expressing fibroblasts, allowing therapeutic targeting of this cell type. When nab-paclitaxel was combined with gemcitabine patients with metastatic PDAC survived significantly longer compared with standard gemcitabine chemotherapy^[37]. Surprisingly no appreciable histological changes to the stroma were evident in the tumors of mice treated with nab-paclitaxel raising the possibility that targeting SPARC improved outcome *via* a different biological mechanism than predicted^[38].

PDAC is desmoplastic, avascular and relatively acellular. These attributes are believed to be responsible for failure of chemotherapies to adequately access tumor cells. Recently, high tissue pressures within the PDAC stroma have been suggested to prevent chemotherapy delivery to tumor cells^[31]. Preclinical model work has suggested relieving such pressures will enhance chemotherapy delivery and subsequent tumor cell death. Unfortunately, these findings have not translated to patients with drugs including anti-MMP and VEGF inhibitors failing to have a therapeutic effect in clinical trials^[35]. Sonic hedgehog paracrine signaling through smoothened has been implicated in the coordination of stromal elements by tumor cells in PDAC. Despite this, a phase II trial based on preclinical data assessing smoothened inhibition in PDAC was stopped early as patients receiving gemcitabine alone survived longer than those receiving the smoothened inhibitor in addition to gemcitabine^[39] (clinicaltrials.gov, Infinity Pharmaceuticals, Cambridge, MA). In addition Catenacci *et al.*^[40] have recently found that Vismodegib, a Sonic hedgehog antagonist, when combined with gemcitabine provided no survival benefit in advanced PDAC patients than gemcitabine alone in a multicentre randomised controlled phase II trial. Hyaluronan is a prominent element within the stroma of PDAC and when targeted in preclinical studies experimenters have found significant improvements in tumor vasculature and lowering of tissue pressures permitting access by chemotherapeutics^[41,42]. This agent is currently the subject of phase II clinical trials in combination with best current chemotherapeutic regimens FOLFIRINOX and gemcitabine/nab-paclitaxel (clinicaltrials.gov: NCT01959139 and NCT01839487).

At present no stromal agent is licenced for therapeutic use in PDAC, with sonic hedgehog in particular representing a cautionary tale of “bench to bedside” medicine. Recent studies, contrary to the findings of Olive *et al.*^[39], have proven that deletion of Shh specifically within the pancreas of *in vivo* PDAC models led to development of more aggressive tumors^[43]. Furthermore, Özdemir *et al.*^[44] found eliminating CAFs from the tumor microenvironment led conversely to suppression of immunosurveillance, increasing numbers of T regulatory cells infiltrating the microenvironment, leading to tumor

progression. What is clear from this work is that the PDAC stroma exists in a state of flux, with an interdependent network of stromal components which when manipulated therapeutically do not always produce expected results.

Targeting inflammatory signal transduction

Mutant *KRAS* is the major oncogenic driver of PDAC in more than 90% of cases^[45]. Development of temporally controlled, inducible models of PDAC has recently permitted interrogation of signaling mechanisms required for PDAC tumorigenesis and progression. Use of inducible and reversible *Kras* alleles has demonstrated the requirement of ongoing stimulus from *Kras* for precursor lesions to progress to PDAC. Removal of *Kras* stimuli prevented progression from pan-INs to PDAC. However, when mutant *Kras* expression remained switched on, striking stimulation of the hedgehog signaling pathway was observed in addition to upregulation of inflammatory mediators IL-6, STAT3, and COX2. *Kras* inactivation resulted in decreased expression of these inflammatory mediators and resultant pan-IN regression^[46], providing clear evidence of the relationship between *Kras* mutation and coordination of the inflammatory response in PDAC. *KRAS* activates RAF phosphorylation resulting in production of chemokines including CXCL1 and CXCL8^[47]. CXCR2 a G-protein coupled receptor is crucial for MDSC migration to the tumor microenvironment and metastatic sites in breast and colon cancer and is activated by CXCL1, 2, 5, 7 and 8^[48,49]. CXCR2 inhibition in preclinical models of PDAC successfully delayed tumor progression, suggesting it merits further study^[50].

Ochi *et al.*^[51] recently reported high expression of TLR7 in PDAC. Activation of TLR7 promotes PDAC formation *via* downstream signalling through inflammatory signalling pathways including STAT3 and nuclear factor kappa B (NF- κ B). When TLR7 was knocked out of immune cells within a murine model of PDAC and exposed to the same pro-tumorigenic conditions animals were completely protected from pancreatic carcinogenesis. Pharmacological TLR7 inhibition is yet to be assessed.

Transcription factor STAT3 represents a key-signaling node in PDAC^[52,53]. When mouse models expressing endogenous mutant *Kras* combined with experimentally induced pancreatitis were assessed, STAT3 activation was significantly increased. Absence of *STAT3* from the pancreata of these mice led to a block in acinar to ductal metaplasia and pan-IN formation, while reduced immune cell infiltration and IL-6 expression was also observed. Infiltrating macrophages were identified as producing IL-6 leading to STAT3 upregulation. Corcoran *et al.*^[54] have proposed that patients could be selected to trial based on phosphoSTAT3 levels as they predict PDAC cell sensitivity to JAK/STAT inhibitors. As the Jak/STAT signaling cascade has been recognised to impact on survival following resection for PDAC, investigators are now beginning to target it in randomized controlled trials^[55].

Ruxolitinib targets the IL6/JAK/STAT signaling cascade. Assessment *via* double blind randomised controlled trial in

advanced PDAC with capecitabine vs capecitabine/placebo showed a marginal survival advantage in the ruxolitinib group^[56]. Intriguingly, those patients that benefited most were those with high mGPS scores. This work and that in preclinical studies by Corcoran *et al.*^[54] suggests trial of anti-inflammatory agents requires careful patient selection to optimise outcome in PDAC. Two ongoing phase III trials of JAK/STAT inhibition in PDAC, JANUS 1 and 2, basing patient selection on high systemic inflammatory scores, will test the hypothesis of the need for better patient selection in PDAC trials of inflammatory targeted agents.

NF- κ B is also important in PDAC progression downstream of Kras mutation, specifically IKK2/ β releases NF- κ B from inhibition leading to progression of pancreatitis, ductal metaplasia, PanIN formation and eventually PDAC formation^[57]. Genetic inactivation of IKK2/ β in preclinical PDAC models led to failure of mice to develop tumors, while IKK2/ β deficient animals showed reductions in pancreatic cell proliferation rates and reduced inflammatory cell infiltrate. These observations support a critical role for NF- κ B in PDAC tumorigenesis. Unfortunately NF- κ B is difficult to pharmacologically target effectively due to the complexity of regulation within the signaling cascade. Inhibitors of IKK2/ β have so far failed to reach clinical trial.

SUMMARY

Preclinical trials are beginning to inform us as to tumor generated and tumor associated inflammation, how these factors help progress PDAC, and how they may be countered therapeutically.

Robust murine models of human disease now exist to allow preclinical trial of therapeutic agents. From these models researchers have established MDSCs, CAFs and TAMs are key cellular mediators of immunosuppression. Targeting these cells may sensitise tumors to immunotherapies such as anti-PD1 and CTLA4 antibodies. Immunotherapies have been extremely successful in diseases such as metastatic melanoma and if tumors can be “unmasked” from immunosuppressive elements this strategy is an exciting prospect in PDAC. IL-6/STAT3 and NF- κ B represent established inflammatory signaling nodes that progress PDAC. Trial of JAK/STAT inhibition shows early promise in clinical trial. However, NF- κ B remains an extremely difficult target to develop drugs against.

Early trials with trabectedin (immune cells), targeting hyaluronan (tumor stroma), and JAK/STAT inhibitors (inflammatory signaling), are extremely promising, however, as yet no large randomised controlled phase III trials have been published.

In future, we envisage combination trials targeting all three aspects of the pro-tumoral PDAC microenvironment will lead to better results in carefully selected patients. Pre-clinical assessment of such strategies is already under trial in robust mouse models of PDAC. A number of inflammatory targets, as outlined in this commentary, have been identified for trial, therefore the next decade of randomised controlled clinical trial data will determine the

effectiveness of agents against these key inflammatory mediators in PDAC.

It is important to note the relative lack of success of translation of stromal/inflammation targeting therapies to clinical trial from the laboratory at present. Only those therapies that demonstrate the most robust preclinical data should be taken forward. Patient selection for tumor microenvironment targeted therapies is a key issue, as is identification of biomarkers of response to such therapies. While those patients presenting with high levels of inflammation are easy to identify clinically, objective monitoring of response to therapy is more difficult due to the lack of robust biomarkers and paucity of available tissue to assess response. Strategies that incorporate pre and post-treatment endoscopic ultrasound biopsies must be considered to help develop techniques required to run robust clinical trials. Immune cell profiling could be employed to stratify subgroups of resected PDACs, potentially enabling individualized targeted immunotherapeutic strategies.

In PDAC the multitude of pathways and factors that determine progression remains the main obstacle in combating this aggressive disease. It is probable that to generate durable responses, in such a plastic disease as PDAC, carefully selected combination therapies will be required. Such strategies are likely to evolve to incorporate chemotherapeutics, immunogenics and therapies targeted against tumor stroma and signal transduction. The recent steady progression made in advanced PDAC with FOLFIRINOX and nab-paclitaxel will hopefully progress further with complementary therapeutics targeted against these different components of the tumor microenvironment.

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Medical treatment for gastro-entero-pancreatic neuroendocrine tumours

Rossana Berardi, Francesca Morgese, Mariangela Torniai, Agnese Savini, Stefano Partelli, Silvia Rinaldi, Miriam Caramanti, Consuelo Ferrini, Massimo Falconi, Stefano Cascinu

Rossana Berardi, Francesca Morgese, Mariangela Torniai, Agnese Savini, Silvia Rinaldi, Miriam Caramanti, Consuelo Ferrini, Stefano Cascinu, Department of Medical Oncology, Università Politecnica delle Marche, 60126 Ancona, Italy

Stefano Partelli, Massimo Falconi, Chirurgia del Pancreas, Ospedale San Raffaele IRCCS, Università Vita e Salute, 20132 Milano, Italy

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Correspondence to: Rossana Berardi, MD, Department of Medical Oncology, Università Politecnica delle Marche, Azienda Ospedaliero-Universitaria Ospedali Riuniti Umberto I - GM Lancisi - G Salesi di Ancona, Via Conca 71, 60126 Ancona, Italy. r.berardi@univpm.it
 Telephone: +39-071-5965715
 Fax: +39-071-5965053

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Abstract

Gastro-entero-pancreatic neuroendocrine neoplasms (GEP-NENs) represents a various family of rare tumours. Surgery is the first choice in GEP-NENs patients with localized disease whilst in the metastatic setting many other treatment options are available. Somatostatin analogues are indicated for symptoms control in functioning tumours. Furthermore they may be effective to inhibit tumour progression. GEP-NENs pathogenesis has been extensively studied in the last years therefore several driver mutations pathway genes have been identified as crucial factors in their tumourigenesis. GEP-NENs can over-express vascular endothelial growth factor (VEGF), basic-fibroblastic growth factor, transforming growth factor (TGF- α and - β), platelet derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1) and their receptors PDGF receptor, IGF-1 receptor, epidermal growth factor receptor, VEGF receptor, and c-kit (stem cell factor receptor) that can be considered as potential targets. The availability of new targeted agents, such as everolimus and sunitinib that are effective in advanced and metastatic pancreatic neuroendocrine tumours, has provided new treatment opportunities. Many trials combining new drugs are ongoing.

Key words: Neuroendocrine neoplasms of the gastro-entero-pancreatic system; Chemotherapy; Targeted agents; Somatostatin analogues; Everolimus; Sunitinib

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Core tip: In this review, recent evidences in the biology and pathology of neuroendocrine neoplasms of the gastro-entero-pancreatic system were analysed, focusing on new biological perspectives of medical treatment. The

evidence-based data of new-targeted drugs and the new molecular knowledge are summarized looking at the basis for future studies.

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INTRODUCTION

Neuroendocrine neoplasms of the gastro-entero-pancreatic system (GEP-NENs) include a heterogeneous group of disease emerging from neuroendocrine cells of gastro-intestinal tract and pancreatic islets^[1]. Nevertheless, despite their morphologic, clinical and prognostic heterogeneity, GEP-NENs are often considered as a single entity^[2].

Although still considered a rare disease, SEER data showed an increasing incidence in the last three decades up to 3.65/100000 per years^[3]. This may be due to a remarkable improvement of diagnostic technique as well as a real change in population demography^[4]. GEP-NENs are more frequently detected in adult population^[5] and in about 50% of cases nodal (25%) or distant (25%) metastases are already existing from the beginning^[3,6]. On the basis of their morphologic features and proliferation index, NENs are currently stratified in two groups, according to WHO 2010 classification criteria^[7]: Neuroendocrine carcinomas, G3 tumours with ki67 proliferation index > 20%, and neuroendocrine tumours (NETs), including G1 (ki67 < 3%) and G2 (ki67 between 3% and 20%) neoplasms. Neuroendocrine carcinomas represent a separate cluster in the family of NENs, with specific biological features and a more aggressive behavior, so chemotherapy is currently considered the standard of care in this specific set^[8,9]. Conversely well and moderately-differentiated NENs do not represent a single entity and their pathogenesis has become clearer in recent years. In fact many driver mutations pathway genes have been identified as crucial factors in their tumourigenesis. Therefore altered pathways represent as a profitable therapeutic choice in neoplastic disease and also in NENs^[10-13].

Despite extensive and remarkable medical exertions, therapeutic choices are still unsatisfactory, mainly due to the lack of a broad knowledge of biological mechanisms and predictive factors. This review aims to summarize the present knowledge about chemotherapy and the pathways involved in sporadic well and moderately differentiated GEP-NETs, highlighting available evidences and new biological perspectives on biological and targeted therapies.

CHEMOTHERAPY

Although most of the studies were conducted on a heterogeneous population and the relationship between response rate (RR) and proliferation index value is often not clearly defined, GEP-NENs, therapy should include cytotoxic agents, especially in symptomatic subjects, progressive disease, moderated differentiation and more aggressive features. Chemotherapy should also be evaluated when the aim is to obtain a response in case of bulky lesions. However the best sequence for chemotherapy still remains uncertain^[14-18].

The most common used chemotherapy schemes include alkylating agents [streptozotocin (STZ), dacarbazine, temozolomide], antimetabolites [5-fluorouracil (5-FU), capecitabine] and platinum derivatives.

Temozolomide combined with 5-FU^[19] or capecitabine^[20] can represent the regimen of choice in G1 and G2 advanced P-NENs. Retrospective data showed a RR of 70% and progression-free survival (PFS) of 18 mo for temozolomide and capecitabine combination^[20].

Furthermore the association of STZ and 5-FU is frequently evaluated as a first-line therapy for advanced P-NENs with RRs between 6% to 40%, with the benefit in PFS ranging between 5 and 20 mo and with a median overall survival of 16-24 mo^[19].

Then, oxaliplatin in combination with capecitabine could also be considered for different setting of G1-G2 GEP-NETs^[15]. None of small retrospective studies or case reports conducted with other chemotherapy regimens have demonstrated sufficient efficacy in GEP-NETs.

SOMATOSTATIN

Many studies have shown the importance of somatostatin in the regulation of NENs' physiological functions. Currently, a cluster of five distinct somatostatin receptors (SSTRs) has been characterized in humans (SSTR1-SSTR5)^[21,22].

The presence of SSTRs has been demonstrated in over 80% of well-differentiated GEP-NENs, with a clear predominance of SSTR2 both in GI-NENs (90%) and P-NETs (80%)^[23,24].

Among the different SSTR subtypes, SSTR2 is usually the most prevalent in NENs, after that SSTR1 and SSTR5, whilst SSTR3 is less commonly expressed and SSTR4 almost absent^[25-27].

In general, tumour dedifferentiation is usually associated with a reduction of receptor density and changes in receptor subtype profile; thus, the presence of SSTRs might be also useful as a tumour specific predictor of prognosis.

Furthermore, the presence of SSTR5 seems to correlate with a major risk of angioinvasion and distant metastasis^[28]; instead, the loss expression of SSTR2 could be highly associated with the dysregulation of tumour proliferation, consequently promoting tumour growth^[29]. The lack of SSTR2

induces the generation of new membrane dimers, with development of different receptors, characterized by new function^[29-36]. It remains unclear if only numeric reduction of SSTRs or also their down-regulation are linked with tumour dedifferentiation^[37]. In pancreatic gastrinomas, glucagonomas and VIPomas, SSTRs are high expressed (80%-100% of patients). However, SSTRs seem to be expressed in 50%-70% of insulinomas, especially SSTR5 mRNA expression was demonstrated to be positively correlated with histopathological features of tumour aggressiveness in primary insulinomas^[38].

Therefore, in P-NENs subtypes, which express less SSTR, short synthetic analogues of somatostatin (SSAs) show a reduced activity in symptoms' control with a worsen hypoglycaemia^[39,40]. This high and heterogeneous expression does not show any relevant correlation between the subtype(s) expressed and the primary tumour origin, or a specific hormone secretion^[41-43].

The intracellular pathways activated by SSTRs appear different in several types of tumour cells and depend on the specific SSTR distribution pattern, signalling elements, as well as to receptor desensitization, internalization, and cross talk^[44,45].

The activation of G-proteins regulates the different critical enzymatic proteins such as adenylyl cyclase and protein kinase A, phospho-tyrosine phosphatases (PTPs) and mitogen activated kinases (MAPKs)^[22,46,47].

In particular SSTR1 induces MAPK pathway activations, SSTR2 improves SHP1 and epidermal growth factor receptor (EGFR) work, up-regulate p21 and Rb reducing MAPK switching on and blocking cellular proliferation. SSTR3 activates p53 and Bax inducing apoptosis, besides it blocks vascular EGFR (VEGFR). SSTR5 induce the activations of PTPs. Globally, these mechanisms leads to an inhibition of cellular proliferation and hormones secretion. Conversely, SSTR4 promotes cell mitosis up-regulating MAPK/ERK1/2 pathway^[21,48].

Since the 80s', several SSAs including octreotide, lanreotide, vapreotide, seglitide and pasireotide, were studied. In contrast to the endogenous somatostatin, these peptides have a more durable half-life (1.5-2 h vs 1-2 min) and activity, as they have a greater resistance to peptidase^[49].

Furthermore, compared to native somatostatin, they have diverse affinity for the aforementioned receptor subtypes^[25,37,50]. In particular the natural ligands of SSTR1-5 can bind all SSTRs with high affinity. Conversely different SSAs, in the same cell type, may elicit differential effects, due to the activation of different subsets of intracellular mediators^[45,51,52].

The analogues octreotide, lanreotide, vapreotide and seglitide exhibit elevated affinity for SSTR2 and lower for SSTR3 and SSTR5. Multi-SSTR-targeted analogue SOM230 (pasireotide) shows higher binding capacity towards SSTR1 and activates also SSTR 2, 3 and 5^[50,53].

The various SSTR binding show a different affinity with their own ligands, which is responsible for the distinct biological and clinical activity^[37]. Imam *et al.*^[54] and Eriksson *et al.*^[55] demonstrated a pro-apoptotic role of

SSAs. In fact they analysed tumor samples of GEP-NENs patients, who received high doses of SSAs^[54,55], finding increased apoptosis processes. The antiproliferative effect of SSAs is mediated by direct and indirect mechanisms. The inhibition of SSTRs, if expressed on tumour cells' membrane, operates directly on cell proliferation, stimulating antimitotic and apoptotic activities. SSAs induce cell growth inhibition also with indirect activities, such as angiogenesis inhibition, modulation of immune system and growth factors' block.

The indirect antiproliferative efficacy of SSAs does not require SSTR tumour expression and is shown by an antiangiogenic or immunomodulation mechanism, mediated by stimulation of the production of natural-killer cells^[56-58]. The antiproliferative activity of SSAs has been shown through various experimental models^[59-64]. The indication of using SSAs as fundamental therapy in NETs derives mainly from two studies: PROMID and CLARINET trials^[65,66]. The PROMID study showed a significant benefit with octreotide LAR (long-acting release) therapy in 85 subjects affected by advanced midgut NENs.

This study demonstrated an advantage in time to progression (TTP). In fact in patients treated with octreotide LAR a mTTP of 14.3 mo was observed, whilst patients in the control arm, receiving placebo, reported a mTTP of 6 mo. Sixty-four percent of subjects in the experimental arm showed stable disease (SD), which was observed only in 37.2% of subjects assuming placebo. Furthermore, patients treated with octreotide LAR experienced a 67% risk reduction of tumour progression compared with patients receiving placebo. The benefit of octreotide LAR was independent either of chromogranin level or hormone secretion.

The study did not show significant differences in OS, presumably due to the few deaths' percentage in both treatment arms. Furthermore the failure of the demonstration of an impact of octreotide in survival could be also done to the high rate of cross-over^[67].

Based on PROMID results, octreotide LAR has been approved as treatment of recurrent and advanced neuroendocrine tumors' patients, irrespective of the site of primary tumour, functional status and symptoms' presence. Lanreotide is another SSA with a similar *in vitro* hormone release inhibitory profile to octreotide^[68].

Recently, the CLARINET trial focused on 204 subjects suffering of nonfunctioning GEP-NENs who were randomized to receive either depot lanreotide, 120 mg every 4 wk for 96 wk, or placebo. The study demonstrated an improvement in PFS for patients treated with lanreotide (mPFS not reached in lanreotide arm; mPFS of 18 mo in placebo arm). This benefit was confirmed both in patients with P-NENs and midgut NENs.

Pasireotide, a new SSA, is characterized by an elevated binding affinity to four of the five SSTR sub-types^[69]. Hence, due to its broad binding profile, pasireotide may represent an effective therapeutic opportunity in tumours refractory to octreotide or lanreotide^[70]. However, its role in GEP-NETs still remains to be defined. In a phase III study pasireotide did not improve the control of flushing

Table 1 Ongoing phase III trials in gastro-entero-pancreatic neuroendocrine tumours

ClinicalTrials.gov Identifier	Investigated drug	Target	Type of enrolled pts
NCT00171873	Octreotide LAR 30 mg	SSTR	Locally inoperable or metastatic well differentiated NETs of the midgut Naïve pts
NCT01524783	Everolimus plus BSC <i>vs</i> PBO plus BSC	mTOR	Unresectable or metastatic G1 or G2 neuroendocrine tumours of GI or lung Treatment-naïve pts and pre-treated pts (all available treatment options are allowed) with PD
NCT00842348	Lanreotide autogel 120 mg	SSTR	Non-functioning GEP-NETs
NCT00690430	Pasireotide LAR 60 mg <i>vs</i> Octreotide LAR 40 mg	SSTR	Metastatic carcinoid tumours
NCT00774930	Somatuline depot (lanreotide) <i>vs</i> PCB	SSTR	Pts with disease-related symptoms inadequately controlled by somatostatin analogues Carcinoid tumours with liver metastasis
NCT00092287	Lanreotide autogel <i>vs</i> Sandostatin LAR	SSTR	Treatment-naïve pts and pts pre-treated with and responsive to somatostatin analogues Carcinoid tumours localized in lung, stomach or midgut
NCT00263659	Telotristat etiprate (LX1606) <i>vs</i> PBO	TPH	Treatment-naïve pts and pts pre-treated with and responsive to somatostatin analogues Well-differentiated metastatic NETs with carcinoid syndrome
NCT01677910	Telotristat etiprate (LX1606) <i>vs</i> PBO	TPH	Treatment-naïve pts Well-differentiated metastatic NETs with carcinoid syndrome Pts with disease-related symptoms inadequately controlled by somatostatin analogues

GEP-NETs: Gastro-entero-pancreatic neuroendocrine tumours; LAR: Long acting release; SSTR: Somatostatin receptor; mTOR: Mammalian target of rapamycin; BSC: Best supportive care; PBO: Placebo; PD: Programmed death; TPH: Tryptophan hydroxylase; pts: Patients.

or diarrhea in patients affected by refractory carcinoid syndrome^[71] (Table 1). The antiproliferative effects are being tested in several clinical studies^[72,73]. Telotristat etiprate (LX1606) is an oral serotonin synthesis inhibitor used in patients with diarrhoea related to carcinoid syndrome^[74].

A recent randomized prospective single-arm study has been conducted in patients with carcinoid tumour and diarrhoea (≥ 4 bowel movements/day) inadequately controlled by octreotide. Among patients treated with telotristat etiprate, 28% experienced a $\geq 30\%$ reduction in bowel movements frequency for more than 2 wk and 56% had a biochemical response. These results suggest a potential activity of telotristat etiprate in controlling carcinoid syndrome and diarrhoea. Pavel *et al.*^[75] made a prospective exploratory dose escalating 12-wk open label multicentre study of telotristat etiprate in metastatic well-differentiated NETs with ≥ 4 -bowel movements/day. Whole patients experienced reductions in bowel movements, 74.2% mean reduction in metabolites of serotonin and 75% of patients reported adequate relief of GI symptoms (Table 1).

MAMMALIAN TARGET OF RAPAMYCIN PROTEIN KINASE B, PHOSPHOINOSITIDE 3-KINASE AND PHOSPHATASE AND TENSIN HOMOLOG PATHWAY

A considerable number of intracellular pathways seem to conditionate tumorigenesis and neoplastic spread in NENs, as receptor tyrosine kinases (RTKs) and G-protein coupled receptors (GPCRs) transduction mechanisms. Their action seems to be modulated by Ras/Raf, MAPK, phosphoinositide 3-kinase (PI3K)-protein kinase B (AKT)-mammalian target of rapamycin (mTOR) and JNK

increasing cells' growth and number. The AKT family of serine/threonine kinases is an important mediator of PI3K signaling, promoting the principal cellular functions^[76]. Akt isoforms seem to be an eminent target for GEP-NENs therapy^[77]. PI3K/AKT/mTOR pathway is especially activated among P-NENs^[78] and their somatic mutations are detected among a minority of P-NETs^[79]. Although discrete mutations in the aforementioned pathway are rarely found in GEP-NENs, overexpression of mTOR and/or its downstream targets is being individuated in a high frequency of cases and it is correlated with higher proliferative activity and adverse clinical outcomes^[80,81]. mTOR is composed by two complexes working together guarantying many cells' activities^[82-91]. The importance of mTOR inhibitors results from the aforementioned considerations^[92,93]. RADIANT-1 (phase II study) represents the first trial demonstrating everolimus utility in GEP-NETs^[94]. The trial compared everolimus alone *vs* everolimus plus octreotide in 160 patients. Regarding combined therapy arm the median PFS was 16.7 mo with a quite well tolerance.

In RADIANT-2 (phase III trial) subjects affected by symptomatic well-differentiated NETs received everolimus plus octreotide *vs* octreotide alone. A lack of significant benefit in PFS was showed in the combination arm. The most common grade 3/4 side effects in the everolimus arm were stomatitis (6.5%), diarrhea (6%), infections (5.1%), and hyperglycemia (5.1%)^[95]. RADIANT-3 (phase III trial) contemplated everolimus *vs* placebo^[96]. The study recruited only G1-G2 P-NETs subjects. Everolimus arm was associated with a better PFS although a low ORR. Therefore everolimus was approved in the management of advanced P-NETs.

RADIANT-4 (ongoing phase III trial) investigates role of everolimus in gastrointestinal/pulmonary neuroendocrine tumors. It may lead to a better definition of the role of

Table 2 Ongoing phase II trials in gastro-entero-pancreatic neuroendocrine tumours

ClinicalTrials.gov Identifier	Investigated drug	Target	Type of enrolled pts
NCT01841736	Pazopanib	VEGFR PDGFR FGFR c-kit	Progressive carcinoid tumours
NCT02399215	Nintedanib	VEGFR FGFR PDGFR	Carcinoid tumour Metastatic carcinoid tumour Neuroendocrine neoplasm
NCT01994213	Famitinib	c-kit PDGFR VEGFR Flt	Gastroenteropancreatic neuroendocrine tumour
NCT01121939	Bevacizumab plus pertuzumab plus sandostatin LAR	VEGF HER2	Advanced neuroendocrine cancers
NCT02259725	Regorafenib	c-RAF BRAF VEGFR PDGFRa FGFR-1 c-kit RET Flt-3	Gastrinoma Glucagonoma Insulinoma Metastatic gastrointestinal carcinoid tumour Pancreatic polypeptide tumour Pulmonary carcinoid tumour Recurrent gastrointestinal carcinoid tumour Recurrent Islet cell carcinoma Somatostatinoma
NCT01784861	X-82 plus everolimus	mTOR	Pancreatic neuroendocrine tumours
NCT01508104	BEZ235 plus everolimus	PI3K	Advanced cancers of different types
NCT00781911	Cixutumumab	IGF-1R	Neuroendocrine tumours

VEGFR: Vascular endothelial growth factor receptor; PDGFR: Platelet derived growth factor receptor; FGFR: Fibroblast growth factor receptor; mTOR: Mammalian target of rapamycin; PI3K: Phosphoinositide 3-kinase; IGF-1R: Insulin-like growth factor-1 receptor; pts: Patients.

everolimus in patients with carcinoid tumours. Finally, other targeted therapies are being studied in NETs (Table 1). Furthermore temsirolimus, another mTOR inhibitor, was evaluated in NETs^[97]. However, the results were not considered clinically relevant and further studies with this agent in NETs won't be performed.

Another fundamental target implicated is PTEN (phosphatase and tensin homologue). Loss of PTEN is commonly individualized in a several human cancers^[98] and it is related to the presence of metastases and therapy resistance towards mTOR inhibition^[99-103]. PTEN is localized in the nucleus. Its activation through internalization leads to a reduction of Act^[104-106]. PTEN is frequently mutated in P-NETs and a low expression of PTEN correlates with high grading^[107].

PI3K pathway represents a hot point in NETs proliferation and some studies evaluating its inhibition are ongoing. BEZ235 is a PI3K inhibitor studied associated with everolimus (phase II study) (Table 2). Then a phase I study is on-going using BYL179 in combination with everolimus and exemestane in P-NETs.

INSULIN GROWTH FACTOR-1

Insulin growth factor 1 (IGF-1) represents a fundamental factor in tumour expansion, so its inhibition may reduce tumour proliferation. NETs have demonstrated to secrete

a significant quantity of IGF-1, then its receptor (IGF-1R) shows a key role in GEP-NETs tumorigenesis^[108,109].

Furthermore, many evidences have related a major IGF-1R expression with the presence of functioning and symptomatic NETs^[109-118]. Cixutumumab (CIX), a monoclonal antibody competitively binding IGF-1R and then causing its degradation, is currently being evaluated in an on-going trial in association with octreotide depot (Table 2). The usefulness of CIX has already been demonstrated in combination with many other therapeutic options^[119].

VEGF

Angiogenesis displays a crucial role for tumour expansion and distant spread and it's mediated by VEGF and its receptors (VEGFRs). Four VEGF forms were identified: VEGF-A, VEGF-B, VEGF-C and VEGF-D, with a different affinity to their three own receptors^[120-129]. Octreotide showed an inhibition of angiogenesis probably mediated by an interaction with VEGF pathway^[130]. The tyrosine kinase inhibitor (TKI) sunitinib^[131] has been demonstrated a valid targeted therapy option in NENs.

A phase II trial evaluated the efficacy of sunitinib in GEP-NETs demonstrating a significant antitumour activity in P-NETs, while among patients with carcinoid tumours OR were only 2.4%; the treatment was average well

tolerated with especially gastrointestinal toxicities^[132].

As a consequence of these results, a phase III trial evaluated sunitinib vs placebo in 171 low- and intermediate-grade advanced P-NETs^[133]. In the experimental arm was demonstrated an improvement of PFS although the RRs associated with the drug were only 9.3%. The benefit was independent of previous treatments and concomitant administration of SSAs. Considering the importance of VEGF in pathogenesis of NENs, bevacizumab, an antibody directed against VEGF^[134], has been used either alone or in combination with other drugs with favourable results^[135].

CYTOTOXIC T-LYMPHOCYTE ANTIGEN-4 AND PROGRAMMED DEATH-1

Recently, immunotherapy was demonstrated to be an important treatment option in various cancers. In fact several new immune-target drugs, directed towards specific immune checkpoints, showed an important antitumoral effect.

The first developed immune agents were directed against mediator of immunity inhibition, as cytotoxic t-lymphocyte antigen-4 (CTLA-4) and programmed death-1 (PD-1). These mediators are both membrane glycoprotein, which are mainly expressed in activated T-lymphocyte.

CTLA-4, known also as CD152, owns an elevated kinship with CD28 and plays a crucial role regulating immunity's homeostasis, through the switching-off of T-lymphocyte activation. Its expression seems to be mayor stimulated in switched-on effector T-lymphocytes (Teff cells)^[136]. CTLA-4 is constitutive and represented in regulatory T lymphocytes (Treg)^[137]. As aforementioned said, it joints CD28, thanks to theirs high affinity, to costimulatory proteins (CD80, CD86) represented in antigen-presenting cells (APC).

Several humanized monoclonal antibodies directed vs CTLA-4, were studied, such as ipilimumab and tremelimumab. The programmed cell death protein-1, PD-1, a membrane protein, acts inhibiting a large group of molecules owning to CD28 family of T-lymphocytes regulators. PD-1 is most represented on surface membrane of activated monocytes, T lymphocytes, and B lymphocytes. PD-1 have different ligands, the most known are PD-L1^[138] and PD-L2^[139].

PD-L1, a transmembrane protein notably presents in macrophages, in T-lymphocytes, B lymphocytes and dendritic cells (DCs), its concentration increases since cellular activating processes. PD-L1 may be presented also in some tissues not involved in immune system. The principal function of PD-1 seems to be reducing autoimmunity and switching off T-lymphocyte activities involved in inflammatory response to infection^[140-142].

In conclusion the linkage between PD-1, mainly expressed in activated T-lymphocytes and PD-L1, principally expressed in tissue DCs, induce a switching-off of T-lymphocytes activation and a blockage of their effector activity^[143]. Identifying a selected group of NENs' patients

that could benefit from immunotherapies is not still possible because no predictive biomarkers to immune drugs have been found. Further studies are needed to evaluate the exact expression of aforementioned target immune proteins (PD-1, PD-L1/L2) in the various NENs.

EGF AND TRANSFORMING GROWTH FACTOR ALPHA

EGF and transforming growth factor alpha (TGF- α) are polypeptides that bind the EGFRs regulating cellular responses to growth signals through activating signal transduction pathways (RAS-RAF-MAPK). From a biological point of view, EGF is a mitogen factor regulating growth, proliferation and differentiation of numerous cell types; abnormalities in EGF-signalling pathways have been related to tumour growth and progression^[144].

The EGFR belongs to the HER receptor family. Gastrointestinal (GI) and pancreatic NETs express and activate EGFRs^[145]. Papouchado *et al*^[146] demonstrated a most elevated presence of EGFR (> 91%) in GI-NENs, (especially in rectal NETs), whilst in P-NENs its expression was lower (< 25%).

Srivastava *et al*^[147] showed instead an elevated presence of EGFR and TGF- α , in P-NENs. Sixty-three per cent of neoplasms in fact showed positivity for TGF- α and 65% for EGFR. However the study did not demonstrate an association with measure, functional status, ability to secrete hormones, or biologic behaviour^[147].

TGF- α is expressed in approximately 70%-100% of NETs depending on the technique used (immunohistochemistry or northern blot analysis)^[148-150] and is commonly over-expressed in larger rectal NETs with a high Ki-67 index^[150]. TGF- α binds with high affinity to the EGFR extracellular domain. Cytoplasmic substrates phosphorylation occurs and initiates a signalling cascade (RAS/RAF/MAPK-ERK) that drives pro-proliferative gene expression, cytoskeletal rearrangement, and increased cell proliferation^[144].

Gefitinib is a targeted agent that selectively inhibits receptor tyrosine kinases, including EGFR. A phase II trial enrolling subjects affected by advanced NENs, gefitinib exhibited somewhat promising initial results. At 6 mo, 61% of patients affected by carcinoid tumours and 31% affected by P-NEN were progression-free; however, objective responses for each group were low, 5% and 9.6%, respectively^[151].

BASIC FIBROBLASTIC GROWTH FACTOR

The basic fibroblastic growth factor (bFGF) is involved in both physiological and pathological processes by interaction with determinated receptors localized in cellular membrane^[152,153].

Because overexpression of bFGF and/or its receptors is frequently detected in tumours, the development of antagonists to bFGF and its receptors has been studied as a potential strategy for cancer therapy^[154-156].

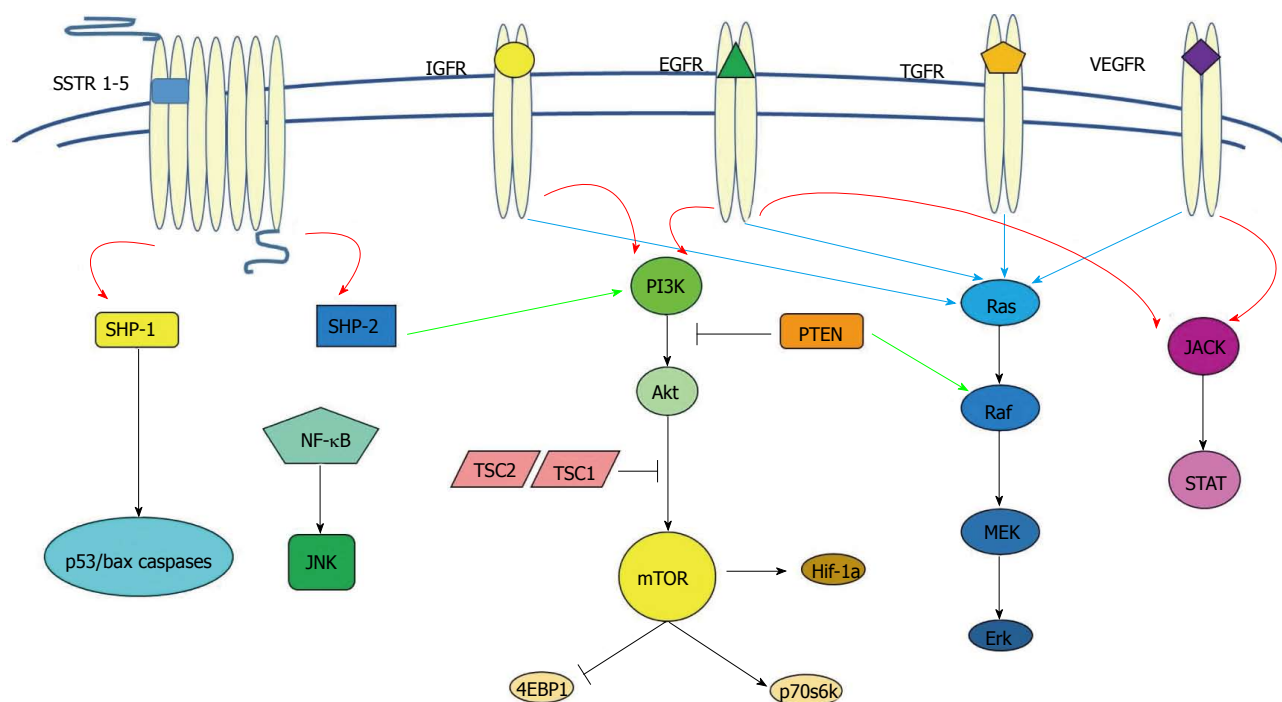


Figure 1 Illustration of principal pathways involved in cellular differentiation, proliferation, survival and apoptosis: Somatostatin receptors, mammalian target of rapamycin protein kinase B, phosphoinositide 3-kinase and phosphatase and tensin homolog, insulin-like growth factor 1 receptor, vascular endothelial growth factor receptor, epidermal growth factor receptor, transforming growth factor receptor, fibroblast growth factors. SSTRs: Somatostatin receptors; mTOR: Mammalian target of rapamycin; Akt: Protein kinase B; PI3K: Phosphoinositide 3-kinase; PTEN: Phosphatase and tensin homolog; IGFR: Insulin-like growth factor receptor; VEGFR: Vascular endothelial growth factor receptor; EGFR: Epidermal growth factor receptor; TGFR: Transforming growth factor receptor.

Almost five isoform of transmembrane FGF receptors (FGFR), able to dimerize, are well known. The first four subtypes are characterized by a tyrosine kinase activity^[157]. Chaudhry *et al.*^[158] searched for mRNA expression of 6 different transmembrane receptors (FGFR, EGFR, IGF-1R, TGF-betaR1 and betaR2), and the presence of SSTRs in determinate subtypes of GEP-NENs tissues (gastrinoma, insulinoma, tumours with carcinoid syndrome, not-functioning neoplasms) using reverse transcriptase-polymerase chain reaction. Among the four tumour subtypes, expression frequencies of the receptors aforementioned varied significantly^[158]. Taken together, these studies have accounted for high growth factor abundance in GEP-NENs. Considering these results GEP-NENs seems to have an elevated growth factors concentration.

C-KIT/ PLATELET DERIVED GROWTH FACTOR

The c-kit receptor, also referred to CD117 or platelet derived growth factor receptor (PDGFR) is a type I transmembrane glycoprotein. It is usually included in the family of tyrosine kinase receptor (RTK)^[159].

In tumor cells, PDGF promotes proliferation and neoplastic spread^[160-163]. Various subtypes of c-kit receptor have been already identified^[164] but their ligand still remains stem cell factor (SCF), a hematopoietic cytokine involved in cell survival, proliferation and differentiation^[165].

Few pre-clinical studies performed of GEP-NETs have shown a variable expression of c-kit, with ranges from 0% to 38%, and PDGFR α in carcinoids^[166], with a particularly high expression in gastrinomas (up to 100% of c-kit expression)^[167].

MULTI-TARGETED AGENTS

Famitinib is an oral tyrosine-inhibitor agent targeting at c-kit, PDGFR, VEGFR2, VEGFR3, Flt1 and Flt3. Its efficacy in GEP-NETs is currently being evaluated (Table 2).

Regorafenib is a novel multi-kinase inhibitor (c-RAF; BRAF, VEGFR-1, 2, 3; PDGFR α , FGFR-1; c-kit; RET; Flt-3) belonging to the group of biaryl urea chemicals^[168-170]. Pazopanib is an oral inhibitor of several specific cellular pathways involved in neoplastic growth and dissemination^[171]. Its efficacy in NENs was demonstrated in a phase II clinical trial combining pazopanib and SSA achieving a 17% RR in G1 P-NETs^[172]. Data related to ongoing trials with pazopanib and with regorafenib in NETs are summarized in Table 2.

CONCLUSION

In GEP-NETs tumourigenesis and progression are often involved SSTRs, mTOR/Akt/PI3K and PTEN, IGF-1, VEGF, EGF, TGF, FGF and c-kit/PDGF and its corresponding receptors^[145,148,149,173-177] (Figure 1). The recent availability

of novel drugs has provided new treatment opportunities and holds promise given the expression in GEP-NENs of this variety of targets^[33,178,179].

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Colorectal cancers and chlorinated water

Ahmed Mahmoud El-Tawil

Ahmed Mahmoud El-Tawil, Department of Surgery, University Hospital Birmingham, Birmingham B15 2GW, United Kingdom

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Correspondence to: Dr. Ahmed Mahmoud El-Tawil, Department of Surgery, University Hospital Birmingham, Mindelsohn Way, Edgbaston, Birmingham B15 2GW, United Kingdom. atawil20052003@yahoo.co.uk
Fax: +44-121-4466220

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Abstract

Published reports have revealed increased risk of colorectal cancers in people exposed to chlorinated drinking water or chemical derivatives of chlorination. Oestrogen plays a dual positive functions for diminishing the possibilities of such risk by reducing the entrance, and increasing the excretion, of these chemicals. In addition, there are supplementary measures that could be employed in order to reduce this risk further, such as boiling the drinking water, revising the standard concentrations of calcium, magnesium and iron in the public drinking water and prescribing oestrogen in susceptible individuals. Hypo-methylation of genomic DNA

could be used as a biological marker for screening for the potential development of colorectal cancers.

Key words: Chlorinated drinking water; Oestrogen; Sex hormones; Gender; Colorectal cancers; Trihalomethanes; Carcinogenesis; DNA hypo-methylation

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Core tip: Oestrogen inhibits the absorption and increases the excretion of xenobiotics and their metabolites *via* the bile. Oestrogen has anti-hypo methylation activity on the genomic DNA by reducing the plasma levels of homocysteine. Colorectal carcinomas are the third most common tumour in both sexes across the globe. The hazard to develop tumours in different specific sites including colon and rectum in association with the long-term exposure to water disinfectants in drinking water is well established. The risk to develop tumours in the large intestines is dependent on the concentrations and frequency of exposure to the trihalomethanes in the used water for drinking. The risk to develop malignant tumours due to water pollution is higher amongst user of swimming pools and is also dependent on the frequency of showering. Indeed, this risk is much higher in those who are avid consumers of fatty foods and/or their meals lacks vegetables and fruits in this susceptible group amongst those who are users of swimming pools. Yet, this risk could be reduced by adding calcium, magnesium and removing iron from the drinking water. Boiling of drinking water is another effective measure for reducing such risk. Colorectal carcinomas arising from long exposure to trihalomethanes in drinking water are characterised an aggressive courses of development and are rarely diagnosed in early stages. Accordingly, it is quite necessary to screen for their occurrence amongst the susceptible persons. Global DNA hypomethylation is most common amongst all subjects who are susceptible to develop malignant tumours and the levels of hypo methylation increase with the prognosis of the disease. Thus screening for the hypo methylation of the relevant genomic DNA and the plasma concentration of homocysteine would be useful criterion for identifying those at risk.

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INTRODUCTION

Carcinomas of the large intestines are remarkably common across the globe with around 1.4 million new cases diagnosed in 2012^[1]. The risk of developing colorectal cancer increases with age and is higher in men than in women^[2].

An association between water chlorination and the development of colorectal carcinoma is well established^[3-13].

The different frequencies of cancers in different genders, the recurring diagnosis of malignant tumors of the large intestines in women with breast cancer, the prophylactic effect of gravidity, together with the decreased risk among women on pills after menopause suggest that female hormones may play a role^[14].

In animal models, for example, male rats that were exposed to dimethyl hydrazine (a carcinogenic mediator), had twice the risk of developing colonic tumors and noticeably shorter survival times than their female peers^[14].

Estrogen mainly exerts its actions through its receptors (ERs) that exist on the target cells. ERs have been found in several malignant tumors including those of the alimentary tract^[15].

Yet, whether estrogen contributes in the carcinogenic activity of the trihalomethanes that leads to the development of colorectal carcinoma is still unknown. The objective of this review is to explore the possible mechanism played by oestrogen in the cancer development process.

METABOLISM AND EXCRETION OF XENOBIOTICS

Poisons, cancer initiators and medicines are examples of xenobiotics. Many metabolizing enzymes are involved in their detoxification. The cluster of cytochrome P450 isoenzymes is one of two known classes that are responsible for the metabolism of these compounds. It consists of many isoforms^[16] leading to the oxidation (mainly hydroxylation) of the particles^[17]. Isoforms in the clusters 1, 2 and 3 accelerate metabolism of many exogenous compounds. Many poisons and cancer promoters usually necessitate activation by cytochrome P450 isoenzymes in order to be expelled outside the body. In general, the enzymatic activity of cytochrome P450 isoenzymes has three specific and consecutive phases: Phase I metabolism, is largely for catalysing the first step of biotransformation. However, if this catalysing activity follows a variety of conjugation steps it is entitled phase II metabolism. This stage is usually accelerated by a variety of enzymatic systems, of which UDP Glucuronosyltransferases is the most important^[18]. The

purpose for conjugating the xenobiotics to the glucuronic acid is to make them soluble in water and consequently fit for excretion in bile^[19,20]. Other models of phase II systems include sulfotransferases, acetyltransferases and glutathione-s-transferase.

Later reports^[21] have added the transport steps and considered them as part of the detoxification process. The role of the export pumps is to reduce the cellular concentrations of these poisons and thereby decreasing the associated harm.

These transport proteins mainly exist in the apical membrane of epithelial cells, such as enterocytes, which lie directly opposite to these exogenous toxins. The protein transporters can limit the entrance of the harmful compounds and facilitate the excretion of their metabolites as well. The last step is entitled "phase III metabolism" in order to link it to the oxidation and conjugation steps. Yet, directing the poisons to enter into the enterocytes has been considered as first line of defence and accordingly has been entitled "phase 0 metabolism".

These protein transporters exert their activities in an active ATP dependent manner against the concentration gradients. The two most common models of these transporters are the multidrug resistance transporter 1 (MDR1) and multidrug resistance associated protein 2 (MRP2). These transporters play a remarkable role in phase 0 and phase III defence against xenobiotics. They are present in both the intestine and liver and thence can reduce the oral bioavailability by direct inhibition of gut absorption and speedy excretion of xenobiotics and their metabolites *via* bile^[19,20].

MDR1 is active against xenobiotics in both the blood-brain barrier and in the gut^[22]. MDR1 are expressed in the small and large intestines but they are mostly manifested in the large intestine^[23,24].

MRP2 was first identified in the apical membrane domain of hepatocytes^[25]. The main function of MRP2 is to facilitate the transport of a variety of organic anions, especially the conjugated formulas, into bile and thence out of the body^[26]. This means that MRP2 are capable of restricting whole body load of endogenous and exogenous toxins. MRP2 are also expressed in the kidney, in the epithelial cells of the intestine^[27], in the placenta^[28], and in the blood-brain barrier^[29]. In the rat intestine, MRP2 expression is highest in the duodenum but few are found in the colon^[30] MRP2 actively ejects the xenobiotics after hydroxylating them in the form of glucuronide, sulfate or glutathione conjugates.

REGULATION OF EXPRESSION AND ACTIVITY OF MULTIDRUG RESISTANCE PROTEINS MRP2 AND MDR1 BY ESTROGENIC COMPOUNDS IN CACO-2 CELLS

The influence of pharmacological levels of ethynylestradiol (EE) on the expression and activity of MRP2, MDR1

and breast cancer resistance proteins (BCRP) in *in vitro* models of drug transport, such as CaCo2 cells has been examined^[31].

Cells treated with either 0.5 or 5 pmol/L EE for 48 h showed an increase in MRP2 (+75% and +88%) and MDR1 (+158% and +162%) protein expression, with respect to control cells. Yet, no effects were observed when cells were treated with all other concentrations tested^[29]. In additional experiments performed to determine protein expression of MRP2 and MDR1 in total cellular membranes as well as their mRNA levels in cells treated with 5 pmol/L EE, an increase in MRP2 (+56%) and MDR1 (+128%) protein expression, with respect to control cells was noted.

When MRP2 activity was evaluated using DNP-SG as a model substrate treatment with 5 pmol/L EE increased the ratio of protein expression by 39% when compared to control cells^[28]. To the contrary, when MDR1 activity was evaluated using Rh123 as a model substrate the intracellular accumulation of Rh123 correlated inversely with MDR1 extrusion activity. Treatment with 5 pmol/L EE decreased substrate accumulation by 19% when compared to control cells^[31]. Such decreased accumulation will lead to reduction in the length and concentrations of exposure and accordingly a reduction in the risk of the associated hypomethylation of repetitive DNA elements^[32].

Further, when the protective effect of EE against CDNB and PQ cytotoxicity was evaluated through determination of the cell survival IC50 value related to CDNB, IC50 values were higher in cells treated with 5 pmol/L EE (33.3 ± 0.5) than in control cells (25.5 ± 0.5)^[31]. Likewise, the IC50 value related to PQ cytotoxicity was higher in cells treated with 5 pmol/L EE (8.8 ± 0.8) vs control cells (6.8 ± 0.5 mmol/L)^[31].

The same researchers also tried to evaluate whether ER mediates transporter modulation by EE. For achieving that goal, they measured MRP2 and MDR1 protein expression after treatment with 5 pmol/L EE for 48 h, in the presence or absence of ICI 182/780. The findings were that MRP2 and MDR1 protein up-regulation was abolished by the ER antagonist (EE vs EE + ICI)^[31].

DNA HYPO-METHYLATION IS A SIGNIFICANT INDICATOR OF THE DEVELOPMENT OF CANCERS

The first reported epigenetic changes in human cancer of losses of DNA methylation (methylated 5C component was replaced by non-methylated C component) was published in 1983^[33]. In their study, Gama-Sosa *et al*^[33] noticed this change in DNA methylation thru the genome in a variety of carcinomas against a broad diversity of ordinary tissues. Then in further published work, Feinberg *et al*^[34,35] reported hypomethylation of unrelated gene areas to cancer in colon adenocarcinomas compared with normal controls.

DNA hypo-methylation appears much more extensive in metastases. Many subsequent reports have confirmed

the recurring global genomic hypo-methylation in cancers when compared with normal tissues^[36-42]. This conclusion has been recently bolstered^[43]. In a 2014 report by Kaz *et al*^[43] genetic alterations in the methylation of genes known for their participation in the colonic carcinogenic process of the normal colon, where no colonic tumour existed suggest that these genetic alterations precede any changes in the colonic tissues and could be potentially used as predictors for the development of colorectal cancers^[43].

Phases of DNA Hypo-methylation status is a significant feature during the early stages of the development of tumours or in other abnormal growths, such as hyperplasia^[42,44-46]. This conclusion was confirmed further by the findings of DNA hypo-methylation prior to the identification of aneuploidy in gastrointestinal cancers^[47]. Hypo-methylation of DNA, in general, increases with the tumour progression or grade of malignancy^[48-51]. Yet, cancers arising from long exposure to chlorinated water are not exceptional^[52]. In a study on an animal model, Coffin *et al*^[52] examined the influence of the exposure to trihalomethanes in the used water for drinking on the tumour progression and DNA methylation of female B6 C3 F1 mouse liver cell line. The main finding of this study was that the trihalomethanes administered by gavage enhanced the multiplicity of the hepatocytes and decreased the methylation of the *c-myc* gene^[52].

ER- α GENE HYPER-METHYLATION IS A POTENTIAL INDICATOR FOR COLORECTAL CARCINOMA

Oestrogen has anti-cancer activity and plays a significant role in suppressing the development of colorectal cancer^[53]. This clearly appears from the frequently reported hyper-methylation of the *ER- α* gene in malignant tumours of the large intestines^[54,55], suggesting that *ER- α* gene hypermethylation could be used as a predictor for the development of large bowel cancers. *ER- α* is a transcription factor that, upon binding to oestrogen transfers to the nucleus to activate various genes including those involved in the inhibition of cell multiplicity^[56]. The insertion of *ER- α* gene into ER-negative colon cancer cells suppressed cell proliferation^[57]. Retrieval of an epigenetically inactivated *ER* gene resulted in suppression of large bowel cancer cells development *in vitro* and *in vivo*^[58]. Experimental work have shown that *ER- α* gene is also hypermethylated in azoxymethane (AOM)-induced carcinoma of the large intestines in rats, lending support to a pragmatic approach to cancer suppression^[59].

THE INFLUENCE OF LONG-EXPOSURE TO TRIHALOMETHANES IN THE USED WATER FOR DRINKING AND THE DEVELOPMENT OF CANCERS

Chloroform, Bromodichloromethane, Chlorodibromome-

thane, and Bromoform are common contaminants in chlorinated water.

Chloroform is considered a facilitator for the development of cancers in humans based on data from animal studies.

Oral contact to chloroform initiated tumours in two kinds of rats and at two different places. Direct gastric administration of chloroform by stomach tube caused hepatocellular carcinoma in mice of both sexes^[60] and renal epithelial tumours in male mice and rats^[61,62].

Benign hepatic adenomas were observed in female rats drank contaminated water with chloroform^[63,64] and in female mice breathed contaminated air with Chloroform^[64]. Renal tubular-cell adenomas, carcinomas, or adenocarcinoma were observed in male rats drank contaminated water with chloroform^[62,63], in male mice breathed contaminated air with chloroform^[65], and in male rats following combined exposure to chloroform *via* breathing and drinking contaminated suppliers^[66].

No cause-effect relationship has been established between human cancer and exposure specifically to chloroform. However, an association between exposure to contaminated water and development of specific kinds of cancers has been established by community-based, cohort and case control studies^[67,68], but a causal relationship could not be inferred^[69-77].

Similarly, bromodichloromethane is also considered a facilitator for the development of cancers in humans based on data from animal studies. Drinking contaminated water with bromodichloromethane caused tumours at several different places in mice and rats. Direct gut administration of bromodichloromethane by a stomach tube caused renal tubular-cell adenomas and adenocarcinomas in male mice and in rats of both sexes, hepatocellular adenomas and carcinomas in female mice, and colonic adenomatous polyps and adenocarcinomas in rats of both sexes^[71,77-79].

Drinking contaminated water with bromodichloromethane increased the frequencies of hepatocellular adenomas and carcinomas in males^[80] and caused hepatocellular adenomas in females^[81].

The data available from epidemiological studies are not conclusive to confirm on a possible relationship between the development of cancers in humans and the exposure specifically to bromodichloromethane. Several epidemiological studies indicated a possible association between drinking chlorinated water and increased risk of cancer, but these studies could not provide information on whether any observed effects were specifically related to bromodichloromethane^[78].

When the risk to develop cancers due to long-term exposure to trihalomethanes *via* drinking, breathing and dermal contact from supply water of five water suppliers were analysed chloroform was the major component that caused cancer risk through both oral and dermal routes whereas bromodichloromethane was the major component through inhalation^[82]. The main risk factors that enhance the development of cancers are the existence of Chloroform in the contaminated water, body weight and then the long-term exposure to chlorinated

water^[82,83].

Evidence exists to prove that low concentrations of calcium (Ca) and/or magnesium (Mg) in the used water for drinking increase the carcinogenic effect of TTHM and thence the development of cancers of oesophagus^[83], kidney^[84], rectum^[85] and pancreas^[86].

On the other hand, it was found that the presence of Fe³⁺ increases the carcinogenic activity of THMs in humans^[87]. It was estimated that the risk to develop cancer from long-term skin exposure to trihalomethanes while swimming is as high as 94%^[88].

THE EFFECT OF OESTROGEN ADMINISTRATION ON THE DNA METHYLATION

In a randomized double-blind, placebo-controlled, cross-over study consisting of two different stages, placebo and conjugated horse oestrogen, Friso *et al*^[89] investigated the effect of administration of oestrogen in thirteen volunteer postmenopausal women on the genomic and promoter DNA methylation in peripheral mononuclear cells and on the plasma concentrations of homocysteine, folate, vitamins B6 and B12. In this study, oestrogen was prescribed as oral pills containing 0.625 mg CEE while placebo consisted of twin pills but lacking the active constituent. Each course lasted 8 wk and these two courses were separated by a 4-wk period^[90]. At week 8 of each stage, blood samples were taken for measuring plasma homocysteine, plasma pyridoxal-50-phosphate, serum folate and vitamin B12 levels. DNA was extracted from peripheral blood mononuclear cells in order to estimate genomic and promoter DNA methylation status.

The findings of this study were that: (1) plasma homocysteine levels were markedly decreased during the CEE phase compared with the placebo; (2) mean homocysteine levels during the placebo phase were 9.29 mmol/L (it was within the normal reported range by Stabler *et al*^[91] in 2004; (3) the oestrogen treatment reduced the mean concentration of homocysteine to 8.08 mmol/L; (4) the extent of genomic DNA methylation in peripheral mononuclear cells was noticeably increased after the oestrogen treatment as opposed to the placebo; (5) there was no significant difference in the promoter DNA methylation of the *ERa*, *ERb* and *p16* genes between the oestrogen and placebo; and (6) there were no significant differences in serum folic acid, vitamin B12 and plasma vitamin B6 levels between the two treatment arms. These findings indicate that oestrogen administration could increase the methylation of the genomic DNA. Together with the well-documented data proving that a decreased level of genomic DNA methylation is a common feature of tumorigenesis, that it appears early prior to the DNA mutation that takes place later in the evolution of neoplasm^[45] this means that oestrogen administration has a prophylactic function against the development of cancers by enhancing genomic DNA methylation.

Table 1 Effect of heating and boiling water on trihalomethane content

Compound	Level (µg/L)				
	Original tap water	80 °C 1 min	100 °C 0 min	Boiling 1 min	Boiling 5 min
Chloroform	45.6	23.2	12.3	9.4	4.1
Bromodichloromethane	44.6	24.1	13.5	10.8	4.6
Chlorodibromomethane	42.3	24.1	14.4	12.3	5.5
Bromoform	35.9	21.3	13.9	13.5	6.8

Available from: URL: <http://monographs.iarc.fr/ENG/Monographs/vol52/mono52-6.pdf>.

DISCUSSION AND CONCLUSION

Chlorine is commonly used as a chemical disinfectant in water supplies, in the prevention of algal, bacterial and general slime growths in treatment plants and pipe works, in the control of tastes and odours, and in the removal of iron, manganese and colouring additives^[92].

Trihalomethanes are derivatives of the outcome of the reaction between chlorine/chloride, with contaminants in water supplies, such as organic compounds, bromide and iron.

The associated health threats including colorectal cancers are dependent on the frequency of exposure to and the levels of trihalomethanes in the used water for drinking. These threats could be reduced by restricting the use and contamination by trihalomethanes of public drinking water^[83,93], or by boiling the water^[94] (Table 1), or by adjusting the concentrations of calcium, magnesium and iron^[83-87].

Genomic DNA hypo-methylation could be used as a reliable biomarker for identifying susceptible cases and oestrogen replacement therapy could be used for reversing detected hypo-methylation and consequently reducing the risk of the carcinogenesis^[86,89,90].

However, in cancers of the colon and rectum, like other ER-linked cancers, the ablation of the sex hormones would be necessary, once the disease occurs, for delaying the progress of the disease. It is well-documented that once the disease manifests the role of oestrogen would be altered in that it will enhance global DNA hypo-methylation^[95] and thereby restricting of its availability would be beneficial.

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Retrospective Study

Clinicopathological features of patients with middle third gastric carcinoma

Jin Hong Kim, Jae Kyoong Joo, Seong Yeob Ryu, Ho Gun Kim, Jae Hyuk Lee, Dong Yi Kim

Jin Hong Kim, Jae Kyoong Joo, Seong Yeob Ryu, Ho Gun Kim, Dong Yi Kim, Division of Gastroenterologic Surgery, Department of Surgery, Chonnam National University Medical School, Gwangju 501-757, South Korea

Jae Hyuk Lee, Department of Pathology, Chonnam National University Medical School, Gwangju 501-757, South Korea

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Correspondence to: Dong Yi Kim, MD, Professor, Division of Gastroenterologic Surgery, Department of Surgery, Chonnam National University Medical School, Hakdong, Donggu, Gwangju 501-757, South Korea. dockim@jnu.ac.kr
Telephone: +82-62-2206450
Fax: +82-62-2271635

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Abstract

AIM: To compared the prognosis of middle third gastric carcinoma (MGC) patients with those of patients with proximal/distal gastric carcinoma (PGC/DGC).

METHODS: Of 3299 patients diagnosed with gastric carcinoma who underwent surgery at our hospital over a 15-year period, 919 (27.9%) were diagnosed with MGC. For each patient, the following information was obtained from hospital records: Age, sex, tumor size, depth of invasion, histologic type, nodal involvement, extent of lymph node dissection, hepatic metastasis, peritoneal dissemination, stage at initial diagnosis, operative type, curability, and survival rate.

RESULTS: T1 category tumors were more common in patients with MGC than in patients with PGC ($P < 0.001$). Tumor stage (stage I), N category (N0), and T category (T1) significantly influenced the 5-year survival rates for patients with curatively resected tumors. A multivariate analysis showed that age, tumor size, serosal invasion, lymph node metastasis, and curability were significant predictors of survival in patients with MGC. The survival rate for MGC patients was similar to that for PGC/DGC patients (52.8% vs 44.4%/51.4%, $P = 0.1138$). The 5-year survival rate for MGC patients with curative resection was higher than that for MGC patients with non-curative resection (62.9% vs 8.7%, $P < 0.001$).

CONCLUSION: These results indicate that tumor

location did not affect the prognosis. Curative resection is important for improving the prognosis of patients with MGC.

Key words: Middle third gastric carcinoma; Prognosis; Curative resection

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Core tip: The clinicopathological features of the patients with middle third gastric carcinoma (MGC) were reviewed retrospectively. Tumor location did not affect the prognosis. When the MGC group was divided into patients with or without curative resection, the survival rates were higher for patients with curative resection. Therefore, curative resection is important for improving the prognosis of patients with MGC.

Kim JH, Joo JK, Ryu SY, Kim HG, Lee JH, Kim DY. Clinicopathological features of patients with middle third gastric carcinoma. *World J Gastrointest Oncol* 2016; 8(4): 410-415 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v8/i4/410.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v8.i4.410>

INTRODUCTION

Although the incidence of gastric carcinoma is declining, it remains one of the leading causes of death from malignant tumors worldwide and advanced gastric carcinoma patients still have unfavorable prognoses^[1]. Generally, the prognosis of patients with middle third gastric carcinoma (MGC) is better than that of patients with proximal or distal third gastric carcinoma (PGC/DGC)^[2]; however, few studies have described the follow-up of patients with MGC. Therefore, it is important to analyze the prognostic factors in patients with MGC. We compared the clinicopathological features and outcomes of MGC with those of more proximally or distally located gastric carcinomas.

MATERIALS AND METHODS

Patients

Between 1987 and 2004, 3299 patients with gastric carcinoma were admitted to the Division of Gastroenterologic Surgery, Department of Surgery, Chonnam National University Medical School, Gwangju, South Korea. Of these, 919 (27.9%) had MGC. The clinicopathological features of the patients with MGC were reviewed retrospectively. Patients with carcinomas involving the entire stomach were excluded. Following the Japanese classification of gastric carcinoma outlined by the Japanese Research Society for Gastric Cancer^[3], the location of each tumor was described as the proximal, middle, or distal third of the stomach. The following information about each patient was obtained from hospital records: Age, sex, tumor size, depth of invasion,

histologic type, nodal involvement, extent of lymph node dissection, hepatic metastasis, peritoneal dissemination, stage at initial diagnosis, operative type, curability, and survival rate.

Statistical analysis

The survival rates of the patients were calculated using the Kaplan-Meier method, and the relative prognostic importance of the parameters was investigated using the Cox proportional hazards model. The χ^2 test was used to evaluate the statistical significance of differences, and *P* values less than 0.05 were considered significant.

RESULTS

Of the 3299 patients diagnosed with gastric carcinoma who underwent surgery at our hospital over a 17-year period, 919 (27.9%) were diagnosed with MGC. Table 1 describes the clinicopathological features of these 919 patients and the 2312 patients with PGC/DGC. There was a significant difference in the mean age of the patients with MGC (55.8 years) compared to the patients with DGC (57.6 years) (*P* < 0.001). Of the 919 patients with MGC, 602 (65.5%) were male and 317 (34.5%) were female. There were more males than females in each group, but there was no significant difference in the sex ratio of each group. Carcinomas in the middle third of the stomach were smaller than were carcinomas in the proximal third of the stomach (4.2 cm vs 4.7 cm), and the difference in mean tumor size was significant (*P* < 0.001).

Using the pTNM system, 296 patients with MGC were classified as pT1, 112 as pT2, 410 as pT3, and 101 as pT4. T1 tumors were more common in patients with MGC than in patients with PGC (32.2% vs 13.1%, *P* < 0.001). Using the grade of anaplasia, 324 (35.3%) of the MGC tumors were differentiated and 595 (64.7%) were undifferentiated adenocarcinomas. Of the patients with MGC, 561 (61.1%) had no lymph node metastases (pN0) and 358 (38.9%) had lymph node metastases. Lymph node metastasis was less common in patients with MGC than in patients with PGC (*P* < 0.05).

Hepatic metastases from MGC were found in 20 patients (2.2%), and peritoneal dissemination was present in 81 patients (8.8%). No significant differences were found in the frequency of hepatic metastasis or peritoneal dissemination among the groups. Of the patients with MGC, 396 (43.1%) were classified as either stage III or IV at the initial diagnosis. In MGC patients, 55.6% of the tumors extended to the serosa or adjacent organs (pT3 and pT4), while 72.5% of the PGCs extended beyond the serosa.

Compared with its use in MGC/DGC, total gastrectomy was performed significantly more frequently for the treatment of PGC (85.6% of cases, *P* < 0.001). The curative resection rate for patients with MGC was 83.1%, similar to that for patients with PGC/DGC (81.4%/82.9%,

Table 1 Clinicopathologic findings of middle, proximal and distal third gastric carcinoma patients

Variables	MGC (<i>n</i> = 919) (%)	PGC (<i>n</i> = 312) (%)	DGC (<i>n</i> = 2000) (%)	<i>P</i> value
Age (mean, yr)	55.8 ± 11.7	55.8 ± 12.5	57.6 ± 10.7	< 0.001
Gender				0.277
Male	602 (65.5)	219 (70.2)	1327 (66.4)	
Female	317 (34.5)	93 (29.8)	673 (33.6)	
Tumor size (mean, cm)	4.2 ± 2.8	4.7 ± 2.6	3.7 ± 2.3	< 0.001
Depth of invasion				< 0.001
T1	296 (32.2)	41 (13.1)	648 (32.4)	
T2	112 (12.2)	45 (14.4)	307 (15.4)	
T3	410 (44.6)	177 (56.8)	844 (42.2)	
T4	101 (11.0)	49 (15.7)	201 (10.0)	
Histologic type				< 0.001
Differentiated	324 (35.3)	108 (34.6)	941 (47.1)	
Undifferentiated	595 (64.7)	204 (65.4)	1059 (52.9)	
Lymph node dissection				0.018
< D2	215 (23.4)	51 (16.3)	475 (23.7)	
≥ D2	704 (76.6)	261 (83.7)	1525 (76.3)	
Lymph node metastasis				0.045
N (-)	561 (61.1)	125 (40.1)	1217 (60.9)	
N (+)	358 (38.9)	187 (59.9)	783 (39.1)	
Operative type				< 0.001
Total gastrectomy	297 (32.3)	267 (85.6)	75 (3.8)	
Others	622 (67.7)	45 (14.4)	1925 (96.2)	
Hepatic metastasis				0.068
H (-)	899 (97.8)	300 (96.2)	1914 (95.7)	
H (+)	20 (2.2)	12 (3.8)	86 (4.3)	
Peritoneal dissemination				0.556
P (-)	838 (91.2)	282 (90.4)	1829 (91.4)	
P (+)	81 (8.8)	30 (9.6)	171 (8.6)	
Stage				< 0.001
I	356 (38.7)	71 (22.7)	840 (42.0)	
II	167 (18.2)	66 (21.2)	322 (16.1)	
III	223 (24.3)	109 (34.9)	432 (21.6)	
IV	173 (18.8)	66 (21.2)	406 (20.3)	
Curability				0.796
Curative	764 (83.1)	254 (81.4)	1658 (82.9)	
Non-curative	155 (16.9)	58 (18.6)	342 (17.1)	

MGC: Middle third gastric carcinoma; PGC: Proximal third gastric carcinoma; DGC: Distal third gastric carcinoma.

$P > 0.05$).

The clinicopathological variables tested in our univariate analysis are shown in Table 2. Factors influencing the 5-year survival rate were patient age, sex, tumor size, depth of invasion, histologic type, presence of hepatic metastasis, lymph node invasion, extent of lymph node dissection, and stage at initial diagnosis. When corrected for depth of invasion, tumor stage, and lymph node invasion in the two groups, tumor stage (stage I), N category (N0), and T category (T1) significantly influenced the 5-year survival rates for patients with curatively resected tumors (Table 3). A multivariate analysis showed that age, tumor size, serosal invasion, lymph node metastasis, and operative curability were significant predictors of survival for patients with MGC (Table 4). Figure 1 shows the patient survival rate according to tumor location. The 5-year survival rate for patients with MGC (52.8%) was higher than that for patients with PGC/DGC (44.4%/51.4%), but not significantly ($P > 0.05$). When the MGC group was divided into patients with or without curative

resection, the respective 5-year survival rates were 62.9% and 8.7% ($P < 0.001$) (Figure 2). There were no significant differences in the survival rates among MGC, PGC, and DGC when the patients were divided into early and advanced gastric carcinoma (Figures 3 and 4).

DISCUSSION

The prognosis of gastric carcinoma varies with tumor location^[4-6]. Although MGCs are reported to have relatively better outcomes than carcinomas in other parts of the stomach^[2], there is limited information on the prognostic factors for MGC. Therefore, we compared the clinicopathological features and prognosis of MGC patients with those of patients with PGC/DGC.

Investigators have discussed various prognostic factors for MGC. Serosal invasion, lymph node metastasis, and lymphatic involvement were found to have significant correlations with prognosis in univariate analyses, and serosal invasion and lymphatic involvement were independent prognostic factors in a multivariate analysis^[7].

Table 2 Univariate analysis of prognostic factors in middle third gastric carcinoma patients

Variables	No. of patients	5-yr survival rate	P value
Age			0.0057
< 65	688	56.0	
≥ 65	231	40.0	
Gender			0.0161
Male	602	48.9	
Female	317	59.9	
Tumor size (cm)			< 0.001
< 5	616	68.5	
≥ 5	303	28.0	
Depth of invasion			< 0.001
T1	296	88.3	
T2	112	75.9	
T3	410	37.8	
T4	101	15.4	
Histologic type			0.0294
Differentiated	324	62.8	
Undifferentiated	595	48.2	
Hepatic metastasis			< 0.001
(-)	899	53.7	
(+)	20	10.8	
Operative type			0.4327
Total	297	52.4	
Others	622	63.6	
Lymph node invasion			< 0.001
N (-)	561	77.5	
N (+)	358	32.7	
Lymph node dissection			< 0.001
< D2	215	18.3	
≥ D2	704	60.0	
Stage			< 0.001
I	356	87.5	
II	167	62.5	
III	223	35.2	
IV	173	14.7	

Table 3 Influence of T category, and N category on the 5-year survival rate of patients with middle third gastric carcinoma surgically treated with curative intent

Variables	PGC (n = 254) (%)	MGC (n = 764) (%)	P value
Depth of invasion			
T1	77.6	88.8	0.0477
T2	76.0	76.1	0.7534
T3	44.6	45.1	0.9900
T4	17.3	16.8	0.1698
Lymph node metastasis			
N0	72.3	79.9	0.0270
N1	42.7	49.6	0.6285
N2	33.4	31.0	0.6933
Stage			
I	77.2	87.9	0.0420
II	68.4	63.2	0.5566
III	33.7	36.3	0.5823
IV	17.6	21.8	0.3635

MGC: Middle third gastric carcinoma; PGC: Proximal third gastric carcinoma.

Other authors have reported similar findings^[8,9]. An anterior location was clearly an independent prognostic factor for patients with MGC based on a multivariate

Table 4 Multivariate analysis of survival for middle third gastric carcinoma patients

Variables	Risk ratio	95%CI	P value
Age (< 65 vs ≥ 65)	1.78	1.24-2.55	0.002
Tumor size (mm) (< 50 vs ≥ 50)	1.51	1.03-2.21	0.036
Serosal invasion (negative vs positive)	2.46	1.45-4.15	0.001
Lymph node metastasis (negative vs positive)	2.48	1.59-3.87	0.000
Curability (curative vs non-curative)	3.46	2.29-5.23	< 0.001

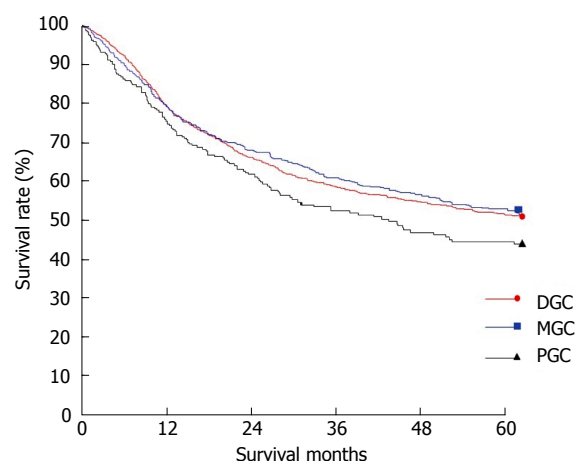


Figure 1 Survival curves for middle third gastric carcinoma, proximal third gastric carcinoma and distal third gastric carcinoma. 5-year survival rate: MGC = 52.8%, PGC = 44.4%, DGC = 51.4%; $P = 0.1138$. MGC: Middle third gastric carcinoma; PGC: Proximal third gastric carcinoma; DGC: Distal third gastric carcinoma.

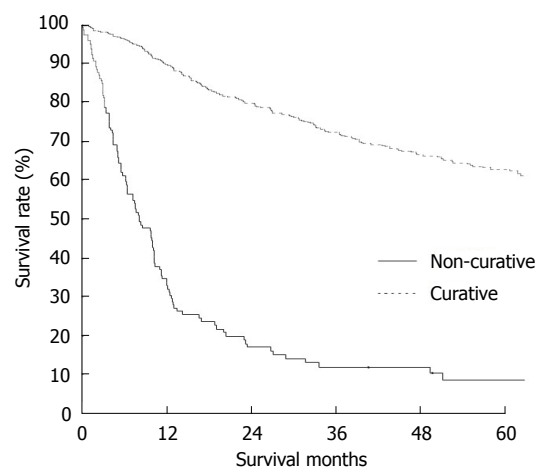


Figure 2 Survival curves for middle third gastric carcinoma according to curability. Five-year survival rate: curative = 62.9%, non-curative = 8.7%; $P < 0.001$.

analysis. It has been postulated that tumors in the anterior wall metastasize more easily to the peritoneum compared with tumors elsewhere because there are no organs on the abdominal side of the anterior wall^[5]. This explanation seems reasonable, although others do not agree^[7]. In this study, we found that age, tumor size, serosal invasion,

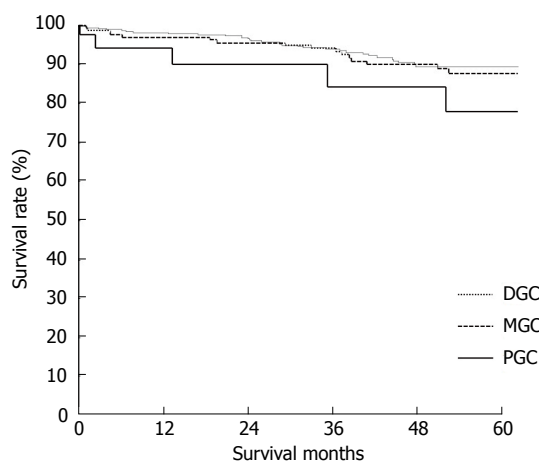


Figure 3 Survival curves of early middle third gastric carcinoma, proximal third gastric carcinoma and distal third gastric carcinoma. MGC = 87.8%, PGC = 77.9%, DGC = 89.5%; $P = 0.0936$. MGC: Middle third gastric carcinoma; PGC: Proximal third gastric carcinoma; DGC: Distal third gastric carcinoma.

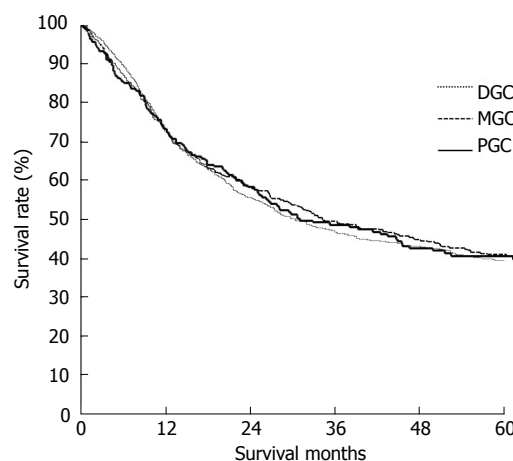


Figure 4 Survival curves of advanced middle third gastric carcinoma, proximal third gastric carcinoma and distal third gastric carcinoma. MGC = 40.8%, PGC = 40.6%, DGC = 39.1%; $P = 0.7586$. MGC: Middle third gastric carcinoma; PGC: Proximal third gastric carcinoma; DGC: Distal third gastric carcinoma.

lymph node metastasis, and curability were independent predictors of survival in patients with MGC in a multivariate analysis.

The operation type for patients with MGC is controversial. One prospective randomized trial conducted in Italy stated that distal gastrectomy was sufficient for treating tumors located in the middle third of the stomach if a cancer-free microscopic margin could be achieved^[10]. However, that study included a relatively small number of MGCs. Therefore, many surgeons still recommend total gastrectomy for MGC because they are concerned about the possibility of local recurrence due to the short proximal resection margin and less extensive lymph node dissection in distal gastrectomy^[11,12]. In a separate report, distal gastrectomy was performed in only 39.3% of patients with a middle third advanced gastric carcinoma for the same reasons, although the authors stated that the type of gastric resection and length of the proximal resection margin did not affect the long-term prognosis. They also reported that distal gastrectomy was sufficient to achieve a tumor-free resection margin in many cases^[13]. Other authors have reported that if curative surgery can be performed, the long-term prognosis of patients with MGC is not affected by the extent of gastric resection, and a distal gastrectomy is feasible^[14-16]. When determining the type of operation for MGC, we also stress tumor-free resection. The statistical analysis in this study showed that operation type was not a prognostic factor.

Generally, the prognosis of patients with MGC is better than that of patients with PGC/DGC^[2]; the present study showed that tumor location did not affect the prognosis. We thought that the possible reason was due to similar curative resection rates. A significant difference in survival between patients with early and advanced gastric carcinomas has been reported^[7]. We also found a significant difference in survival rates between patients with early (87.8%) and advanced (40.8%) gastric carcinomas. The survival rate for patients with MGC was 52.8%, and the cumulative survival rate for patients with

MGC was slightly better than that for patients with PGC/DGC. When the MGC group was divided into patients with or without curative resection, the respective 5-year survival rates were 62.9% and 8.7%. Furthermore, we evaluated the relationship between the survival of patients with gastric carcinoma after curative resection and the depth of invasion. There was no significant difference in cumulative survival between the groups when the depth of invasion was that of T2-T4 tumors.

In patients with MGC, as the tumors progress the lymph nodes around the splenic artery and hilum are also frequently involved^[5]. Several studies have reported that the incidence of lymph node metastasis is 9.7%-20% along the splenic artery and 9.2%-17% at the splenic hilum in advanced PGC and MGC^[17-19]. In our department, splenectomy is not routine for patients with advanced MGC. However, we perform a splenectomy when the tumor invades the spleen directly or when metastasis to the splenic hilar lymph nodes or lymph nodes around the splenic artery is suspected.

Regarding adjuvant chemotherapy, we administered postoperative chemotherapy to select patients according to the pathologic findings instead of tumor location. Since the chemotherapeutic regimen varied during the study period, we did not analyze the effect of postoperative adjuvant chemotherapy.

In conclusion, our results show that tumor location did not affect the prognosis of MGC. When the MGC group was divided into patients with or without curative resection, the survival rates were higher for patients with curative resection. Therefore, curative resection is important for improving the prognosis of patients with MGC.

COMMENTS

Background

The prognosis of patients with middle third gastric carcinoma (MGC) is better

than that of patients with proximal or distal third gastric carcinoma; however, few studies have described the follow-up of patients with MGC.

Research frontiers

The prognosis of gastric carcinoma varies with tumor location. Although MGCs are reported to have relatively better outcomes than carcinomas in other parts of the stomach, there is limited information on the prognostic factors for MGC.

Innovations and breakthroughs

The authors did not find any difference in survival rates according to the tumor location. When the MGC group was divided into patients with or without curative resection, the survival rates were higher for patients with curative resection.

Applications

The study shows the importance of curative resection in patients with MGC.

Terminology

The stomach is anatomically divided into three portions: The upper (U), middle (M), and lower (L) parts. If more than one portion is involved, all involved portions should be described in order of degree of involvement, the first indicating the portion in which the bulk of the tumor is situated.

Peer-review

These authors provided an overall review of the middle third gastric cancer. These authors described several clinico pathological parameters of MGC compared to PGC/DGC. In this article, authors also demonstrated the significant difference between curative resection is one of the prognostic factors for MGC. It is interesting and acceptable for publication.

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TOPIC HIGHLIGHT

- 416 MicroRNA in rectal cancer
Azizian A, Gruber J, Ghadimi BM, Gaedcke J

REVIEW

- 427 Role of Raman spectroscopy and surface enhanced Raman spectroscopy in colorectal cancer
Jenkins CA, Lewis PD, Dunstan PR, Harris DA
- 439 Current adjuvant treatment modalities for gastric cancer: From history to the future
Kilic L, Ordu C, Yildiz I, Sen F, Keskin S, Ciftci R, Pilanci KN

MINIREVIEWS

- 450 Multitarget stool DNA for colorectal cancer screening: A review and commentary on the United States Preventive Services Draft Guidelines
Berger BM, Levin B, Hilsden RJ
- 459 Urinary metabolites as noninvasive biomarkers of gastrointestinal diseases: A clinical review
Sarosiek I, Schicho R, Blandon P, Bashashati M
- 466 Non-surgical factors influencing lymph node yield in colon cancer
Wood P, Peirce C, Mulsow J

ORIGINAL ARTICLE

Retrospective Study

- 474 Intensity modulated radiation therapy with simultaneous integrated boost based dose escalation on neoadjuvant chemoradiation therapy for locally advanced distal esophageal adenocarcinoma
Zeng M, Aguila FN, Patel T, Knapp M, Zhu XQ, Chen XL, Price PD

Contents

World Journal of Gastrointestinal Oncology
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ABOUT COVER

Editorial Board Member of *World Journal of Gastrointestinal Oncology*, Shu-Yu Zhang, PhD, Associate Professor, School of Radiation Medicine and Protection, Medical College of Soochow University, Suzhou 215123, Jiangsu Province, China

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WJGO covers topics concerning carcinogenesis, tumorigenesis, metastasis, diagnosis, prevention, prognosis, clinical manifestations, nutritional support, molecular mechanisms, and therapy of benign and malignant tumors of the digestive tract. The current columns of *WJGO* include editorial, frontier, diagnostic advances, therapeutics advances, field of vision, mini-reviews, review, topic highlight, medical ethics, original articles, case report, clinical case conference (Clinicopathological conference), and autobiography. Priority publication will be given to articles concerning diagnosis and treatment of gastrointestinal oncology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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World Journal of Gastrointestinal Oncology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-85381891
Fax: +86-10-85381893
E-mail: editorialoffice@wjnet.com
Help Desk: <http://www.wjnet.com/esps/helpdesk.aspx>
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PUBLISHER

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2016 Colorectal Cancer: Global view

MicroRNA in rectal cancer

Azadeh Azizian, Jens Gruber, B Michael Ghadimi, Jochen Gaedcke

Azadeh Azizian, B Michael Ghadimi, Jochen Gaedcke, Department of General, Visceral and Pediatric Surgery, University Medical Center Göttingen, 37075 Göttingen, Germany

Jens Gruber, Junior Research Group Medical RNA Biology, German Primate Center, 37077 Göttingen, Germany

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Correspondence to: Jochen Gaedcke, MD, Department of General, Visceral and Pediatric Surgery, University Medical Center Göttingen, Robert-Koch-Straße 40, 37075 Göttingen, Germany. jochen.gaedcke@med.uni-goettingen.de
Telephone: +49-551-3920933
Fax: +49-551-3912550

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rectal cancer, a neoadjuvant chemoradiotherapy (CRT) is recommended before any surgery. However, response to CRT ranges from complete response (responders) to complete resistance (non-responders). To date we are not able to separate in advance the first group from the second, due to the absence of a valid biomarker. Therefore all patients receive the same therapy regardless of whether they reap benefits. On the other hand almost all patients receive a surgical resection after the CRT, although a watch-and-wait procedure or an endoscopic resection might be sufficient for those who responded well to the CRT. Being highly conserved regulators of gene expression, microRNAs (miRNAs) seem to be promising candidates for biomarkers. Many studies have been analyzing the miRNAs expressed in rectal cancer tissue to determine a specific miRNA profile for the ailment. Unfortunately, there is only a small overlap of identified miRNAs between different studies, posing the question as to whether different methods or differences in tissue storage may contribute to that fact or if the results simply are not reproducible, due to unknown factors with undetected influences on miRNA expression. Other studies sought to find miRNAs which correlate to clinical parameters (tumor grade, nodal stage, metastasis, survival) and therapy response. Although several miRNAs seem to have an impact on the response to CRT or might predict nodal stage, there is still only little overlap between different studies. We here aimed to summarize the current literature on rectal cancer and miRNA expression with respect to the different relevant clinical parameters.

Key words: Polymorphism; MicroRNA; Rectal cancer; Response; Chemoradiotherapy; Expression

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Abstract

In rectal cancer, one of the most common cancers worldwide, the proper staging of the disease determines the subsequent therapy. For those with locally advanced

Core tip: In rectal cancer, a proper staging of the disease determines the subsequent therapy. Also, prediction of prognosis or therapy response could serve to individualize therapy. MicroRNAs (miRNAs) are highly conserved

regulators of gene expression, and seem to be promising candidates for biomarkers. Several miRNAs are part of a specific expression profile in rectal cancer tissue, while others have been correlated to clinical parameters and therapy response. However the comparison of different studies shows only little overlap and even partly oppositional results. Differences between analytical methods and tissue storage types can contribute to that. Further functional analyses are needed to fully understand the impact of miRNAs in rectal cancer.

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INTRODUCTION

Colon and rectal cancer

Taken together, colon and rectal cancer is the third most common cancer worldwide, accounting for 1.36 million newly diagnosed colorectal cancers in 2012^[1] with rectal cancer accounting for 30%. The main purposes to differentiate between colon and rectal cancer are anatomical^[2] and molecular differences^[3]. Several studies have also shown that disease-correlated genetic and lifestyle factors differ between colon and rectal cancer^[3-8]. Differences in survival, fewer inherited syndromes, and younger age at diagnosis in rectal cancer patients further strengthen the rationality of separating the two diseases^[8].

Specifically due to the anatomical differences in comparison to colon cancer, local recurrence is a considerable concern in the treatment of rectal cancer. This led to the introduction of radiation in the treatment of rectal cancer patients and represents fundamental therapeutic differences to colon cancer. While treatment of upper rectal cancer provides primary surgical resection and can therefore be compared to colon cancer, the standard treatment of locally advanced cancer in the lower and middle rectum includes preoperative chemoradiotherapy (CRT), followed by total mesorectal excision (TME)^[9]. The introduction of preoperative chemoradiotherapy requires additional challenges in diagnostics and therapy planning which differ from colon cancer, requiring a precise pretherapeutic staging.

The rectal cancer staging can be made according to the TNM staging system from the World Health Organization: The Union for International Cancer Control (UICC). Depending on tumor status, nodal status, and metastases, rectal cancer is subdivided in UICC I - IV. While tumor status is determined by magnetic resonance imaging and trans-rectal endoscopic ultrasound, metastasis status is assessed by computed tomography of thorax and abdomen and ultrasound of the latter. Defining nodal status remains the most challenging and is evaluated today using all aforementioned imaging

techniques. Correct staging of patients with rectal cancer is actually required at two time points: First, before starting any treatment, and second, after neoadjuvant chemoradiotherapy, because response to CRT is heterogeneous; it ranges from resistance to complete pathological response. Response to CRT, measured as tumor regression grade (TRG), correlates significantly with disease-free- and overall-survival. The first staging is crucial for deciding if a preoperative CRT is needed, hence only locally advanced stages receive CRT. The second staging acquires more and more importance with regard to the possibility of organ-preserving strategies, which have been recently suggested as an alternative to TME for patients that responded very well to CRT. Basis for this upcoming approach can be found in the side effects of rectal cancer surgery. However, if lymph node metastases are undetected these have to be considered as origin of local relapse. In this respect molecular markers may play an increasing role as potential predictive marker.

miRNAs

MicroRNAs (miRNAs) are short non-coding RNAs, 20-22 nucleotides in length discovered in 2001^[10,11]. They are highly conserved between vertebrates, invertebrates and plants^[12]. Through base-pairing with their target mRNA, miRNAs induce post-transcriptional gene silencing by mRNA degradation or translational blocking^[13,14]. Consequently, they present master regulators of gene expression and therefore influence many physiological and patho-physiological processes^[15].

Some conservatively estimated 60% of all human mRNAs are regulated by miRNAs, which represent virtually all cellular and molecular functions. Thus, it is not surprising that miRNAs are involved in diverse processes including embryonic development, cell differentiation, cellular proliferation, metabolism, adaptation to environmental stress, and apoptosis^[13]. Thus, miRNAs play important roles in many human diseases, and even in the human aging process^[16]. By now the impact of specific miRNAs is reported not only for almost every cancer type but also for other diseases like diabetes, cardiovascular diseases, neurological diseases and even psychological diseases like schizophrenic disorder. Therefore miRNAs are of great interest as possible biomarkers in various diseases due to their abundance and cell-type specificity.

Many human miRNA loci are located within intronic (miRtrons) regions^[17,18]. While it is a general belief that intronic miRNAs are released from excised introns after the splicing, an interesting study of Kim *et al.*^[19] indicates that intronic miRNAs can be processed from unspliced intronic regions, ensuring both miRNA biogenesis and protein synthesis from a single primary transcript, supporting the assumption that the intronic miRNAs and their hosting genes are co-regulated^[20]. miRNAs are transcribed by RNA Polymerase II (pol II). The primary transcripts (pri-miRNA) are 5'-capped and polyadenylated. They have at least one stem-loop structure that encodes an individual miRNA sequence within the stem. Drosha, a nuclear RNase III type enzyme, and DGCR8, a double-

stranded RNA-binding protein, work as a complex known as microprocessor, which cleaves the primary structure of the pri-miRNA in a process called "cropping"^[10]. The products of this reaction are the pre-miRNAs, which are exported to the cytoplasm by exportin-5^[21,22]. In the cytoplasm the pre-miRNAs are further processed by the cytoplasmic RNase III called Dicer. Only one strand of the produced duplex of RNA is incorporated into the effector complex RNA-induced silencing complex (RISC), acting as the guide strand, while the passenger strand is rapidly degraded. However, either arm of the pre-miRNA can be selected to become the guide strand. The strand-selection differentially or coherently processes mature miRNAs, giving rise of gene regulatory RNAs with distinct target-spectra. This process of flexible arm selection has been reported for many small RNAs, including canonical and intronic miRNAs^[23,24]. Eventually, RISC migrates to P-bodies to scan and bind to the 3' untranslated region of the target mRNA.

The miRNA-mRNA binding is specific due to the sequence complementarity of the "seed" region of the miRNA. The canonical seed is a tract of 7-8 nucleotides usually located at the 5' end of the miRNA molecule, which is fully base pairing one or multiple sites within the sequence of a target mRNA^[25] capable of follow structures of miRNA-5'-seeds or alternative seed architectures.

Hence, the core of the target-region with high complementarity is short, which results in multitude of transcripts with possible binding sites for a given miRNA. Therefore a single miRNA has the potential to regulate hundreds of different mRNA targets^[26], while on the other hand a single mRNA is regulated by diverse miRNAs simultaneously.

Genome-wide miRNA-expression-profiling studies have demonstrated a specific profile of upregulated and downregulated miRNAs in almost all cancer types^[27,28]. In particular, due to their lack of complex post-transcriptional modifications in contrast to mRNAs and other RNA classes (rRNA, tRNA), the potential of miRNAs as biomarkers for cancer diagnosis, prognosis, and response to treatment is expected high. Not only miRNAs can be found in serum or plasma of patients and healthy individuals, but also in other body fluids such as tears, breast milk, bronchial lavage, colostrum and seminal, amniotic cerebro-spinal, pleural and peritoneal fluids^[29]. The diagnostic potential of miRNAs relies in part on their stability to storage handling: miRNAs remain stable even in conditions most RNAs would normally degrade (extreme pH-levels, boiling, etc.)^[30].

Cell-free and circulating miRNAs can be vesicle associated (exosomes and microvesicles), or stable AGO-miRNA complexes and became well accepted biomarkers for non-invasive biomarkers for numerous cancer types^[31-36].

LITERATURE SEARCH

A systematic literature search was conducted using PubMed for "rectal cancer", "miRNA" and "miRNA". A total of 27 studies containing miRNA research from

rectal cancer tissue, normal mucosa tissue, and body fluids were included for review. Five studies were involved in differential expression of miRNAs in rectal cancer, six studies explored specific miRNAs which showed a correlation to clinical parameters, and nine studies analyzed miRNAs concerning alteration during chemoradiotherapy and response prediction. Five studies conducted further *in vitro* analyses for rectal cancer specific miRNAs. Three studies were found to be dealing with polymorphism in miRNAs in rectal cancer patients.

DIFFERENTIAL EXPRESSION OF MIRNA IN RECTAL CANCER

It is widely accepted that tumors share specific oncogenic pathways. *Vice versa* the tissue of origin has also an impact on the molecular features of each tumor. These are of great interest as they may explain cellular processes such as carcinogenesis, progression, or therapy resistance. Accordingly, rectal cancer specimens and normal mucosa tissue were analyzed. In a first analysis Slattery *et al.*^[8] compared colorectal cancer tissue [formalin-fixed paraffin embedded (FFPE)] to normal tissue samples and also compared normal rectal tissue to normal colon tissue using microarray analysis. All samples were further subdivided according to their CpG island methylator phenotype status or the mutational status of *KRAS* or p53, revealing 129, 143, and 136 unique miRNAs respectively. The availability of miRNA expression data of normal colon and rectal tissue samples enabled a comprehensive comparison, which identified 73 differentially expressed genes (based on a two-fold fold change) and thus highlighted also important molecular differences between colon and rectal cancer. A comparable study by Li *et al.*^[37] involving miRCURY Array LNA miRNA chips and technical validation by RT-PCR analyzed expression profiles from six rectal cancer tissues and paired adjacent non-tumor tissue, which identified 67 upregulated and 39 downregulated miRNAs associated with rectal cancer. The number of rectal cancer tissues used ($n = 6$) is extremely low and, by using an array platform with several hundreds of miRNAs, it is required to correct for multiple testing. This did not occur; therefore the findings are potentially inapplicable.

In a larger study, our own group^[38] used LNA-enhanced miRCURY microarrays to map the expression of 2090 miRNAs. Tumor biopsies and matched mucosa samples of 57 patients with locally advanced rectal cancer were profiled. Forty-nine miRNAs differed with high significance between normal and rectal cancer tissue, 20 of these 49 miRNAs were upregulated while 29 were downregulated in rectal cancer vs mucosa. Upon employing a combination of fold-change and *P*-value for selection, the expression of 10 miRNAs was validated using 48 samples (24 matched tumor-mucosa samples) by semi-qRT-PCR; in 8 of the 10 miRNA expression levels correlated very well with miRCURY data as they showed the same alteration in both methods and both sets of tissue.

Studies by Wang *et al.*^[39] could confirm that the expression level of two miRNAs (miR-34a, miR-200c) that were previously found to be differentially regulated in various types of cancer, also were significantly upregulated in rectal cancer by analyzing 72 rectal cancer samples *via* qPCR.

Comparison of different studies to identify overlapping miRNA expression differences, *e.g.*, between rectal cancer and normal tissue is subject of certain restriction: Starting from tissue retrieval (*e.g.*, taking the biopsy during rectoscopy vs tissue excision from the resected surgical specimen that obviously already has a certain ischemia time) over tissue storage (*e.g.*, liquid nitrogen, RNA later, formalin fixation) and tissue work up to the final application of the various techniques that are available for miRNA measurement (*e.g.*, miRNA arrays from different companies, qPCR, sequencing). In this specific application, the reference tissue is of importance. Biases arise depending on whether paired normal mucosa or mucosa from different patients were used as a reference. On the other hand, miRNAs that finally overlap between different studies attract attention as they may be represent basic differences between the compared tissues. In this respect we aimed to identify the overlap between published data sets that were previously introduced, which currently involve only two relevant datasets comparing rectal cancer and normal tissue^[37,38]. Of these, 11 miRNAs were overlapping. Seven miRNAs were significantly upregulated (miRNAs 17, -18a, -21, -31, -135b, -223 and -492) while four were significantly downregulated (miRNAs-29c, -145, 147b and -375). In both studies also the expression of let-7f, miR-148 and -190 were significantly altered in rectal cancer, however they showed an oppositional regulation of these miRNAs comparing with the first two studies questioning their relevance for assessing differential expression. For miR-145 even a third study performed by Wang *et al.*^[40] confirmed a significant in rectal cancer. Figure 1 shows an overview about the differential expression of miRNAs found according to the mentioned studies.

A closer look to the differentially expressed miRNAs reveals a broad range of different function. As a member of the miR-17/92 cluster miR-17 and 18a are both known to be involved in a large number of processes including normal development, tumorigenesis, immune-, cardiovascular-, and neurodegenerative diseases as well as aging^[41]. Renal fibrosis^[42], myelodysplastic syndromes^[43], inflammatory processes^[44], and especially cancer are only a few processes that are regulated by miR-21^[2,45,46]. For miR-31 a decent number of cancer related studies have been published^[47] indicating a more aggressive disease of colorectal cancer^[48] if highly expressed. However, a relation to metastatic disease^[47,49] and an inverse meaning of increased expression status has been shown in other cancer entities such as breast cancer^[48]. The presence of higher expression in different cancer types was reported for miR-135b as well. Nonetheless, it was predominantly analyzed in colorectal cancer and its overexpression by APC loss, PTEN/PI3K

pathway deregulation, and SRC overexpression was demonstrated to promote tumor transformation and progression^[50]. An oncogenic functionally relevant expression has also been found for miR-223 showing a wide range of different tumor entities^[51,52]. In contrast to previous miRNAs, data on the function of miR-492 and its oncogenic relevance are rare. Downregulation of miR-29c - a member of the miR-29 family - is known in several cancer types and its role as a tumor suppressor has been established^[53]. Furthermore, its relevance as antifibrotic miRNA is under debate^[54]. Initial functional relevance of miR-375 was found as a pancreatic islet-specific miRNA. Recently, miR-375 has been found significantly downregulated in multiple types of cancer, targeting several important oncogenes like AEG-1, YAP1, IGF1R and PDK1^[55]. miR-145 is presumed to be a tumor suppressor with apoptosis inhibitor 5, ERK5, K-RAS, and insulin receptor substrate 1 as predicted targets, which are cell cycle and survival regulators^[56]. Data on miR-147 is rare; one study postulates that miR-147 is induced upon Toll-like receptor stimulation and regulates murine macrophage inflammatory responses^[57]. Taken together, the identified miRNAs from both studies revealed functionally characterized regulators that have, in the vast majority, no organ specificity.

CORRELATION OF MIRNA EXPRESSION TO CLINICAL PARAMETERS

Currently, the most reliable tumor marker to assess clinical outcome is the staging system by TNM classification. As this classification is now more than 100 years old, molecular features for different tumor entities are increasing in number markers for a more precise prognosis are expected. In this respect the aforementioned study of Gaedcke *et al.*^[38] identified miR-135b. Its expression correlated significantly with disease-free and cancer-specific survival in an independent cohort of 116 patients. miR-135b was also found by other groups to be of importance. Xu *et al.*^[58] used frozen tissues, performed qPCR analysis, and found miR-135b to have the highest fold-change (17.7-fold) among the upregulated miRNAs in Duke stage IV cases (that are known to be of poor prognosis). They also identified miR-145 to be highly downregulated with a negative fold change between 18 and 23 in stages II, III and IV CRC respectively. Furthermore, they identified significantly decreased expression miR-374a for the identification of patients without metastasis, its effectiveness was confirmed with a sensitivity of 93.33% but a low specificity of only 66.67%. miR-4634 was related to lymph node metastasis in stage III with a sensitivity of 75% and specificity of 83.33%. In this analysis, however, the limitation of a mixed study population of colon and rectal cancer must be acknowledged.

Slattery *et al.*^[59] analyzed data from 1141 CRC cases *via* microarray to identify the impact of 121 miRNAs on disease stage and survival. Five miRNAs were associated with advanced disease stage: hsa-miR-145-5p and hsa-



Figure 1 Differential expression of microRNAs in rectal cancer. The differentially expressed microRNAs (miRNAs) in rectal cancer compared to normal rectal tissue are listed, sorted by studies, respectively. The correlating circles show the number of differentially expressed miRNAs in the mentioned studies and point out the number of miRNAs overlapping between those studies.

miR-31-5p were increased and hsa-miR-200b-3p, hsa-miR-215 and hsa-miR-451a were decreased in advanced stages of CRC. In rectal cancer, 13 miRNAs were significantly associated with mortality after a diagnosis with rectal cancer (Table 1). In addition, they showed that miR-21 expression had an inverse association with mortality in rectal cancer (but not colon cancer patients). However, Nielsen *et al.*^[60] used *in situ*-hybridization and real-time qPCR on FFPE tissue, and identified miR-21 to predict a short disease-free survival in colon cancer, but not in rectal cancer. Interestingly, the *in-situ*-hybridization showed that the miR-21 expression was detected predominantly in the stromal compartment of the tumors. Yang *et al.*^[61] showed in an microarray analysis of samples from 40 patients a significant overexpression of miR-21, miR-155, miR-29a and miR-92a in rectal cancer samples and found only miR-155 had the capacity to discriminate nodal positive from negative cases as well as Duke A/B stages from Duke C/D stages.

Stratmann *et al.*^[62] did not investigate miRNAs directly but the expression level of Dicer - one of the key enzymes in the miRNA generating process - and revealed

that the Dicer expression in rectal cancer is higher than in normal mucosa (and higher than in colon cancer), while Dicer expression in liver metastases was decreased in comparison to either the primary tumor or mucosa. Furthermore, patients with a high expression of Dicer mRNA in the normal mucosa had a worse prognosis (poor survival) than those with a lower expression level.

ALTERATION DUE TO THERAPY AND PREDICTING THERAPY RESPONSE

While imaging techniques (computer tomography, magnetic resonance imaging and ultrasound) manage to diagnose tumor stage, nodal stage or distant metastasis initially in an appropriate manner, their ability to identify the response after chemoradiotherapy is poor, particularly the differentiation between vital tumor cells and scar tissue is challenging for imaging techniques. Response to neoadjuvant chemoradiotherapy measured as TRG is therefore usually determined by pathologists after investigating the operative specimen. An adequate

Table 1 Association of microRNA expression and clinical parameters

microRNA	Clinical parameter	Change of expression	Ref.
miR-17-5p miR-20a/20b-5p miR-21-3p/5p miR-25-3p miR-29a/29c-3p miR-31-5p miR-135b	Association with rectal cancer survival	No further explanation	Slattery <i>et al</i> ^[59]
miR-135b	Association with advanced tumor stage Correlation with disease-free and cancer-specific survival	Increased expression level in advanced tumor stage Patients with a high expression level of miR-135b had a better disease-free and cancer-specific survival	Slattery <i>et al</i> ^[59] Gaedcke <i>et al</i> ^[38]
miR-135b	Correlation with Duke stage IV	Upregulated with the highest fold-change (17.7-fold) among 9 upregulated miRNAs	Xu <i>et al</i> ^[58]
miR-141-3p miR-145	Association with rectal cancer survival Correlation with Duke stage II, III, IV	No further explanation Downregulated with a -18.15, -18.9, -23.8-fold change in stage II, III and IV CRC respectively	Slattery <i>et al</i> ^[59] Xu <i>et al</i> ^[58]
miR-145-5p miR-155	Correlation with advanced tumor stage Correlation with nodal stage and Duke stage	Increased expression level in advanced tumor stage Discrimination of nodal positive from negative cases as well as Duke A/B stages from Duke C/D stages	Slattery <i>et al</i> ^[59] Yang <i>et al</i> ^[61]
miR-200b-3p miR-215 miR-335-5p	Association with advanced tumor stage and survival Association with advanced tumor stage and survival Association between any miRNA expression and survival	Decreased expression level in advanced tumor stage Decreased expression level in advanced tumor stage The expression of miR-335-5p is associated with a better survival	Slattery <i>et al</i> ^[59] Slattery <i>et al</i> ^[59] Slattery <i>et al</i> ^[59]
miR-374a	Correlation with metastasis stage	Decreased expression of miR-374a in tumor of patients without metastasis	Xu <i>et al</i> ^[58]
miR-425-5p miR-451a	Association with rectal cancer survival Association with advanced tumor stage	No further explanation Decreased expression level in advanced tumor stage	Slattery <i>et al</i> ^[59] Slattery <i>et al</i> ^[59]

CRC: Colorectal carcinoma.

evaluation of response before surgery could spare patients with complete response the surgical resection of the rectum (with all the associated disadvantages), but until today there is no validated biomarker for that. Moreover, since patients respond differently to CRT, a biomarker to predict response to neoadjuvant chemoradiotherapy in rectal cancer patients even before CRT could spare the non-responders the CRT. Understandably, there is a great interest to use miRNAs as possible biomarkers to predict therapy response. Some studies analyzed descriptively the changes in miRNA expression after chemoradiotherapy while others were able to identify miRNAs in tumor tissue, which seem to predict the response to therapy.

Svoboda *et al*^[63] performed microarray analysis on tumor biopsies of 31 patients with locally advanced rectal cancer before and 2 wk after chemoradiotherapy with capecitabine (a 5-FU prodrug). They found a significant increase of miR-125b and miR-137 expression levels after 2 wk of chemoradiotherapy. Moreover, they also demonstrated that high levels of miR-125b and miR-137 are associated with a worse response to chemotherapy. However, the sample size is quite short (31 patients), and there is an intertumoral variability described, which should not be neglected. Interestingly, the same group investigated in 2012 in a similar setting 20 patients with locally advanced rectal cancer, whose tumors were classified as most sensible ($n = 10$) or most resistant ($n = 10$). They used TaqMan Low Density Arrays analysis to quantify 667 human miRNAs in the tumor tissue samples (preoperative biopsies of untreated primary tumors) and found 8 miRNAs to be significantly differently expressed between the responders and non-responders: miR-215,

miR-190b and miR-29b-2 were overexpressed in non-responders while let-7e, miR-196b, miR-450a, miR-450b-5p and miR-99a were down regulated in non-responders^[64]; the previously identified miRNAs miR-125b and miR-137 were not mentioned.

Drebbler *et al*^[65] did real-time-PCR analysis to identify the expression of miR-21, miR-143 and miR-145 in macrodissected FFPE tumor tissue of 40 patients before and after chemoradiotherapy. They described a significant upregulation of miR-143 and miR-145 in post-therapeutic tumor tissue compared to pre-therapeutic tumor tissue. In addition, they showed a significant correlation between a low miR-145 expression in the post-therapeutic tumor tissue and a worse response to CRT. However, this result does not address the problem to predict therapy response in advance: The low expression of miR-145 was measured in the post-therapeutic tumor tissue. To predict tumor response miRNA profiles in the pre-therapeutic tissue are needed.

More adequate to this purpose, Della Vittoria Scarpati *et al*^[66] analyzed miRNA expression by microarray and confirmed by qRT-PCR in primary tumor biopsies of patients with locally advanced rectal cancer who underwent neoadjuvant CRT followed by surgery ($n = 38$). Eleven miRNAs were significantly upregulated in patients with a complete response (miR-1183, miR-483-5p, miR-622, miR-125a-3p, miR-1224-5p, miR-188-5p, miR-1471, miR-671-5p, miR-1909, miR-630, miR-765) and two were downregulated (miR-1274b, miR-720). However, the small cohort of patients' needs additional validation in an independent cohort^[66]. Though, none of the mentioned 13 miRNAs was found when Kheirleisid *et al*^[67] performed a similar study by using microarray

analysis of 12 FFPE pre-therapeutic tissue samples of rectal cancer to answer to same question by identifying differentially expressed miRNAs. The promising miRNAs in this study were miR-16, miR-590-5p and miR-153 to predict complete vs incomplete response and miR-519c-3p and miR-516 to discriminate between good vs poor response. Unfortunately, they do not clarify how these miRNAs are altered between the responders and non-responders (downregulated or upregulated).

A possible reason for the different identified miRNAs may be the difference between the tissues used: Della Vittoria Scarpati *et al.*^[66] used fresh biopsies frozen in liquid nitrogen while Kheirleiseid *et al.*^[67] used FFPE. However, if we act on the assumption that the type of preservation (FFPE, Kryo, *etc.*) differs the miRNA expression, the next question posed would be: What is the preservation effect on miRNA expression and which miRNA expression profile derives from the different tumor characteristics? On the other hand, Hotchi *et al.*^[68] used also fresh frozen biopsies from 43 rectal cancer patients before starting CRT and did both microarray analysis and RT-PCR of miRNAs concerning response prediction. They found out that miR-223 was higher expressed in tissue from patients with a good response to CRT and declared miR-223 (which is not mentioned by any other study investigating miRNAs in rectal cancer patients for therapy response prediction) as a promising biomarker for the prediction of response to CRT^[68]. Other studies found other different miRNAs: Lopes-Ramos found miR-21-5p to be over expressed in tumor biopsies of rectal cancer patients with complete response using fresh biopsies frozen in liquid nitrogen^[69], Bhangu *et al.*^[70] found miR-200c as a possible biomarker to predict CRT response as it shows a significantly reduced expression in non-responders using FFPE material. Figure 2 shows the important miRNAs concerning response to CRT in rectal cancer patients.

In a recent study of our own group, we were able to show with qPCR-analysis a significant decrease of miR-18b and miR-20a during CRT in plasma of patients with a negative nodal stage after CRT (ypN0) compared to those with a positive nodal stage (ypN+). This data presents miR-18b and miR-20a as possible candidates for biomarkers predicting nodal stage after CRT^[71]. However, this data requires validation in a larger cohort.

IN VITRO ANALYSES FOR RECTAL CANCER SPECIFIC MIRNAS

Beside the *in vivo* analyses, functional data of specific miRNA that obviously play a role in rectal cancer have been analyzed. One of these is miR-21 that has already been described above. Using tumor biopsies Chang *et al.*^[72] showed an inverse relationship between miR-21 and programmed cell death protein 4 (PDCD4), a known tumor suppressor^[72]. They hypothesized the post-transcriptional modulation of PDCD4 *via* mRNA degradation. These findings were based on data from Asangani *et al.*^[73],

who transfected Colo206f cells with miR-21 and found a significant suppression of PDCD4 proteins *in vitro*.

For miR-182 Amodeo *et al.*^[74] investigated the effect on thrombospondin-1 (TSP-1), a protein inversely correlated with tumor vascularity and metastasis. In CRC, TSP-1 is shown to be downregulated. After transfection with anti-miR-182, expression level of TSP-1 increased. Hence, the authors concluded that anti-miR-182 could be used to restore TSP-1 expression in CRC to inhibit the angiogenic and invasive events in CRC.

For another rectal cancer associated miRNA, namely miR-455, rapidly accelerated fibrosarcoma (RAF1) seems to be a target gene: In 20 mucosa and 20 CRC biopsies miR-455, miR-484 and miR-101 seem to be down-regulated. An overexpression of miR-455 in SW480 cells showed inhibition of proliferation and invasion. Western Blot analyses showed a downregulation of RAF1 in cells with an overexpression of miR-455, although, on mRNA-level, there was no effect shown^[75]. Also the relevance of miRNAs concerning the sensitivity towards CRT could be assessed *in vitro*: Using 12 colorectal cancer cell lines, the miRNA expression profile indicating sensitivity towards an *in vitro* treatment of 5-FU and radiation was established by our own group^[76]. These data were validated by the transfection of let7g, miR-132, miR-224 and miR-320a that led to the expected shift of therapy resistance towards sensitivity. For let-7g the higher expression as a good prognostic marker was validated in patient samples.

POLYMORPHISMS IN MIRNAS

Since miRNAs represent one of the important mechanisms of gene expression control, the relevance of polymorphisms concerning miRNAs has been explored in few studies. Naccarati *et al.*^[77] showed in a case-control study that two single nucleotide polymorphisms within the 3'untranslated regions of target DNA repair genes (nucleotide excision repair genes), hence the miRNA-binding sites, were significantly associated with rectal cancer: rs7356 in RPA2 (predicted binding miRNA: hsa-miR-3149 and hsa-miR-1183) and rs4596 in GTF2H1 (predicted binding miRNA: hsa-miR-518a-5p, hsa-miR-527 and hsa-miR-1205). This study points out that not only the expression levels of miRNAs are relevant, but also their ability to interact with their target gene.

Jang *et al.*^[78] tried to identify polymorphisms in miRNA genes which have a prognostic value in rectal cancer patients and found 196a2C > T (allele of hsa-miR-196a2) polymorphism to be a significant risk factor for the overall survival of rectal cancer patients. The mentioned allele has been reported by other studies to be involved in increased risk of various cancer types^[79-81]. Recently, Mao *et al.*^[82] found miR-146a being decreased in rectal cancer tissue compared to adjacent normal mucosa and they also showed an association between the genetic variant in miR-146a, rs2910164 polymorphism and the risk of CRC.

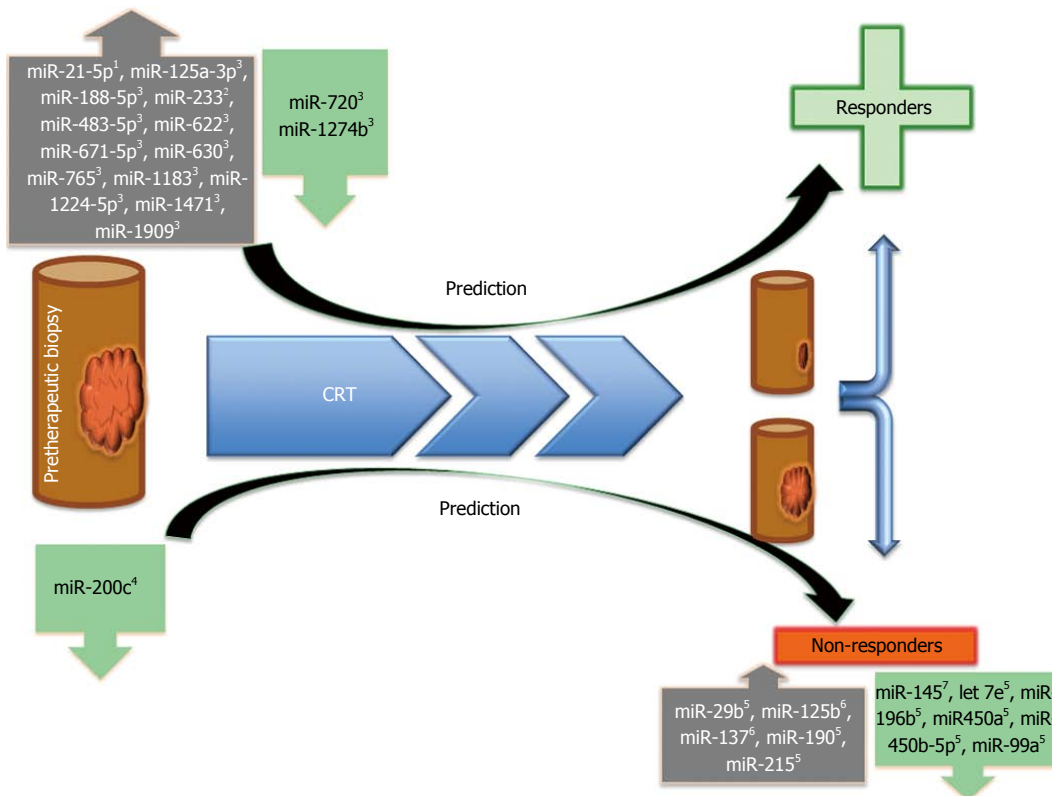


Figure 2 Differential expression of miRNAs dependent on response to preoperative chemoradiotherapy. miRNAs in up arrow callouts are significantly higher expressed; those in down arrow callouts are significantly lower expressed. On the left site there are miRNAs, isolated from pretherapeutic biopsies, which are supposed to predict response or non-response, respectively. The miRNAs in the bottom localized on the right side, are found to be significantly higher or lower expressed in post-therapeutic tumor biopsies of non-responders after chemoradiotherapy compared to pretherapeutic biopsies. ¹Lopes-Ramos *et al.*^[69], 2014; ²Hotchi *et al.*^[68], 2013; ³Della Vittoria Scarpati *et al.*^[66], 2012; ⁴Bhangu *et al.*^[70], 2014; ⁵Svoboda *et al.*^[64], 2012; ⁶Svoboda *et al.*^[63], 2008; ⁷Drebber *et al.*^[65], 2011.

CONCLUSION

miRNAs are widely accepted to play a crucial role in physiological and pathological processes. Interestingly, in contrast to the relevance of rectal cancer and its frequency, especially compared to colon cancer, the number of available studies is rather small. The amount of studies as well as the small number of patients per study may be one of the reasons why only few overlapping miRNAs have been identified. Importantly, a small number of miRNAs were identified with relevance in rectal cancer. Many of these are rather known from cancer specific mechanisms than display rectal cancer specificity. Accordingly, the relevance of miRNAs as a predictive or prognostic biomarker in rectal cancer is questionable. Furthermore, the relevance of functional miRNAs does not appear to be as obvious as in previous studies that are typically cell-line based. However, before ignoring the relevance of miRNAs it should be taken into account that human cancer tissue is functionally a rather complex cell system. The analyses are impeded by the heterogeneity of tumor biopsies that in general include different amounts of non-tumor cells such as stroma or the surrounding tissue. Different analyzing techniques applied to identify miRNA (PCR, microarray, etc.) or the varying fixation media (FFPE, fresh frozen biopsies, etc.) further complicate the comparability of the data. Furthermore, subtle expression differences of a

given miRNA that potentially change complex regulatory mechanism simply may not be identified. This may due to the techniques applied or has simply not been part of the analyses that, in general, focus on expression fold changes.

Specifically for miRNAs, there may be alternative reasons for varying results, such as the highly variability of miRNA expression due to external influences such as nutrition. Humphreys for example, showed that the expression of oncogenic miRNAs can be altered by dietary manipulation: A high red meat intake leads to elevated miR-17-92 (cluster) and miR-21 in rectal mucosa tissue of healthy volunteers. While organ specificity is well known for miRNA, Li *et al.*^[37] identified miRNA expression differences (e.g., miR-182) in CRC between African and Caucasian Americans. Possibly, there are further influences like medications used by the patients, gender differences, or age associated variations that are much higher than currently expected.

Overall, there is a large number of possible reasons as to why a clear identification of miRNAs still failed. However, compared to alternative molecular markers in rectal cancer such as proteins, mRNA or DNA, miRNA are not inferior as there are currently no well established markers. Acknowledging some of the previously listed points, miRNA analyses in rectal cancer aiming to identify regulatory mechanisms or to establish marker for

prediction or prognosis should be endorsed. Furthermore, these efforts should be expanded to blood samples as it has been done in many other cancer types.

FUTURE PERSPECTIVES

Validity of cell free and cellular miRNAs as a prognostic or diagnostic tool remains, at least in parts, elusive. The incomplete understanding of biological processes yielding circulating RNAs and their physiological relevance needs to be addressed in more detail, *e.g.*, by application of less bias-sensitive technologies and combinations of, *e.g.*, high-throughput sequencing, qPCR and microarray techniques^[83]. Functional characterization of altered miRNAs in CRC and surrounding healthy tissue with respect to more recent findings of modifications that impact miRNA processing and target-gene regulation will improve quality and interpretability of the datasets originating from quantitative analysis^[84]. Investigations on differential or coherent expression of miRNAs in affected tissues, changes of strand-selection during tumor progression, and treatment as well as in-deep analyses of the physiological relevance of secreted miRNAs and other non-protein coding RNAs can clarify roles of these and feasibilities to choose particular candidates as markers for prognosis and diagnostics or candidates for therapies^[23,36].

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Role of Raman spectroscopy and surface enhanced Raman spectroscopy in colorectal cancer

Cerys A Jenkins, Paul D Lewis, Peter R Dunstan, Dean A Harris

Cerys A Jenkins, Paul D Lewis, Dean A Harris, Swansea University Medical School, Swansea University, Swansea SA2 8PP, United Kingdom

Peter R Dunstan, Department of Physics, College of Science, Center for Nanohealth, Swansea University, Swansea SA2 8PP, United Kingdom

Dean A Harris, Department of Colorectal Surgery, Singleton Hospital, Swansea SA2 8QA, United Kingdom

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Correspondence to: Dean A Harris, MD, FRCS, MB, ChB, Department of Colorectal Surgery, Singleton Hospital, Sketty Lane, Swansea SA2 8QA, United Kingdom. dean.a.harris@wales.nhs.uk
Telephone: +44-1792-285459

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cancer in the United Kingdom and is the second largest cause of cancer related death in the United Kingdom after lung cancer. Currently in the United Kingdom there is not a diagnostic test that has sufficient differentiation between patients with cancer and those without cancer so the current referral system relies on symptomatic presentation in a primary care setting. Raman spectroscopy and surface enhanced Raman spectroscopy (SERS) are forms of vibrational spectroscopy that offer a non-destructive method to gain molecular information about biological samples. The techniques offer a wide range of applications from *in vivo* or *in vitro* diagnostics using endoscopic probes, to the use of micro-spectrometers for analysis of biofluids. The techniques have the potential to detect molecular changes prior to any morphological changes occurring in the tissue and therefore could offer many possibilities to aid the detection of CRC. The purpose of this review is to look at the current state of diagnostic technology in the United Kingdom. The development of Raman spectroscopy and SERS in clinical applications relation for CRC will then be discussed. Finally, future areas of research of Raman/SERS as a clinical tool for the diagnosis of CRC are also discussed.

Key words: Detection; Colorectal cancer; Spectroscopy; Raman; Surface enhanced Raman spectroscopy

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Core tip: This review focuses of the current role of Raman spectroscopy and surface enhanced Raman spectroscopy (SERS) in clinical applications of colorectal cancer. This includes a review of the current research into *in vivo* endoscopic Raman probes, non-destructive analysis of biofluids and the use of SERS in order to detect low concentration analytes that previously could not be detected with Raman spectroscopy. Both the advantages and disadvantages of the technology are discussed along with possible avenues of future research.

Abstract

Colorectal cancer (CRC) is the fourth most common

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spectroscopy and surface enhanced Raman spectroscopy in colorectal cancer. *World J Gastrointest Oncol* 2016; 8(5): 427-438 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v8/i5/427.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v8.i5.427>

INTRODUCTION

Colorectal cancer (CRC) is the fourth most common cancer in the United Kingdom and is the second largest cause of cancer related death in the United Kingdom after lung cancer^[1]. Currently in the United Kingdom there is not a diagnostic test that has sufficient differentiation between patients with cancer and those without cancer so the current referral system relies on symptomatic presentation in a primary care setting^[2,3]. CRC results from the progressive accumulation of genetic and epigenetic alterations that disrupt normal cellular mechanisms^[4]. The 5-year survival rate for CRC detected in early stages are > 90%, however the 5-year survival rate for later-stage cancers is < 10%^[1,5]. This highlights the need for a simple, reliable diagnostic test that can detect early signs of the disease. The majority of CRCs present symptomatically in a primary care setting so it is important that general practitioners can identify patients who are at highest risk^[2]. The risk associated to patients is based on the referral guideline, in the United Kingdom is this based on a combination of symptoms and age of the patient^[6]. The relationship between initial symptoms and mortality as a diagnostic indicator have previously been discussed in depth^[2,3,7,8]. Unfortunately initial symptoms associated with CRC can also be symptoms of benign diseases such as irritable bowel syndrome^[6], and there is currently no diagnostic test available in primary care that has sufficient differentiation to base referral on^[3]. Furthermore the detection of CRC using symptoms has been shown to be ineffective for decreasing mortality rates in comparison to the European average^[9].

Raman spectroscopy and surface enhanced Raman spectroscopy (SERS) could hold many advantages for use as a diagnostic tool for CRC. These techniques have previously been used to discriminate between cancerous and non-cancerous tissue, biofluid samples, as well as in the development of *in vivo* Raman systems for use in endoscopy. The techniques are non-destructive to samples so can provide molecular information about a sample without the need for staining or in some cases without the need for resection. Raman and SERS have the potential to detect molecular changes in cancerous cells/tissues and biofluids that precede morphological changes such as the development of precursor lesions. The techniques could offer the potential for an early detection tool that detects molecular changes before the stage at which a traditional histopathology would be able to detect. This review paper will discuss the previous applications of Raman spectroscopy and SERS to detect CRC.

CURRENT DIAGNOSTIC PATHWAYS

In United Kingdom, CRC survival outcomes are lower than the European average, this is be attributed to a higher proportion of cancer being diagnosed at late-stage^[9]. In order to combat the NHS rolled out a national screening program in 2006. There are currently two types of screening method available to screen for CRC and late-stage adenoma namely flexible sigmoidoscopy (FS) and measurement of markers in faecal samples^[10]. FS is a procedure involving the insertion of an endoscope into the rectum of a patient in order to examine the distal colon and rectum; the procedure must be carried out by a trained doctor. Atkin *et al.*^[11] (2010) conducted a United Kingdom study into the effectiveness of a single FS procedure as a screening tool. A procedure for patients aged 55-64 years the study saw a 33% reduction in CRC cases and a 43% mortality reduction, however this is only applicable with respect to the distal colon^[11]. However, the cost of a FS is high in comparison to tests that look for markers in faecal samples because it involves trained staff^[12]. Moreover, it can be argued that the results of tests reliant on the opinion of a practitioner can be subjective depending on the experience of the practitioner.

In England and Wales the guaiac faecal occult blood test (gFOBT) is commonly used as a screening tool. The test is simple and cheap compared to other methods^[12]. Problems that occur with gFOBT regarding dietary requirements of patients before taking the test are solved using immunochemical faecal occult blood testing (iFOBT)^[13]. There are different types of iFOBT, all of which detect human specific haemoglobin in faecal samples. Investigations and meta-analysis studies have shown iFOBT to have improved sensitivity in the detection of CRCs and late-stage adenomas in both high-risk, average-risk and populations with no overt rectal bleeding compared to gFOBT without compromising on specificity^[14-17]. However, despite the improved sensitivity of iFOBT tests patient uptake of the screening programme in general is poor. In October 2008 of the 2.1 million people that had been invited to partake in the screening programs in England uptake was just 55%-60%^[18]. Other diagnostic methods available to clinicians in the United Kingdom include double barium enema, computed tomography colonoscopy, MRI imaging and colonoscopy. Outside of the United Kingdom there are other diagnostic tests are also in use such as the tumour M2-pyruvate kinase (tumour M2-PK) test. This is a faecal test that investigations have shown this to also be more sensitive than FOBT^[19]. However, the cost effectiveness and the sensitivity and specificity of the M2-PK especially compared to the iFOBT still needs to be established in the United Kingdom as published results are in disagreement as to whether it is more effective than iFOBT^[20]. There is also a real time polymerase chain reaction based blood test that is available outside of the United Kingdom that detects methylated Septin 9 (mSept9). The blood test

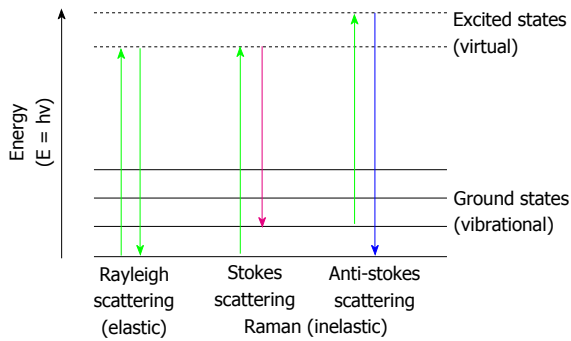


Figure 1 Energy shifts involved in light scattering interactions. Adapted from Lin *et al.*^[23].

has been shown to have sensitivity and specificity ranging from 50%-90% and 88%-91% respectively^[21]. A blood test is a potentially more attractive option for patients compared to faecal and colonoscopy tests so studies are underway to determine if the higher cost of mSept9 would be recovered by higher screening uptake^[22].

Raman spectroscopy could offer a highly sensitive and less invasive alternative/complimentary technique to aid CRC detection. However, Raman spectroscopy has not yet found its way into a routine clinical setting. This review will examine the current research status of the application of Raman spectroscopy for detecting CRC to critique the prospective translation of the technique to a clinical setting.

Methods for review

A systematic literature search (PubMed, MEDLINE, Web of Science) was conducted using the following terms: "Diagnosis", "Raman spectroscopy", "Surface enhanced Raman spectroscopy", "SERS", "CRC", "Tissue", "Biofluids", "Immunoassay", "CEA". Following these searches 30 studies were selected for inclusion based on the criteria: (1) That they are original studies based on the clinical applications of Raman spectroscopy, SERS or both for CRC detection in human tissue, biofluids or cell lines; and (2) in the case where there are large amounts of similar studies that they were the first to report such data. Only studies published up to January 2015 were included in this review.

RAMAN SPECTROSCOPY AND SERS FOR THE DETECTION OF CRC

Introduction

Raman spectroscopy is a type of vibrational spectroscopy that allows the user to gain molecular information about a sample through the scattering of incident light. In general, when light is passed through or onto a sample a small proportion of the photons are scattered (approximately 10^{-5}). The majority of this is Rayleigh or elastic scattering; where the energy of the incoming photon is equal to the energy of the scattered photon (Figure 1)^[23]. Around 1 in 10^7 of the incident photons are in-elastically scattered resulting in the incident photon and the scattered photon

having a difference in energy.

The inelastic scattering is a relatively weak effect which was first observed in 1928 by Sir CV Raman and is known as Raman scattering^[24]. When scattered light is measured with a spectrometer a series of lines are observed, the shift in the energy [measured by wave-number (cm^{-1})] from the Rayleigh line (equal to incident energy) is known as the Raman shift. The shift recorded corresponds to specific vibrational or rotational modes of the sample molecule. The intensity of the "Raman shift" for a particular molecule is directly proportional to the concentration of that molecule within a sample so the resulting spectrum of a sample will be a superposition of Raman response of all the Raman active molecules from within a sample, e.g., proteins, nucleic acids, etc. It is interpreted as the molecular "fingerprint" of the sample, an example of a spectrum can be seen in Figure 2. It is due to the "fingerprint" that Raman is seen to be desirable for application to cancer detection because it offers the possibility of detecting minute differences in analyte concentrations and is a non-destructive technique.

Vibrational techniques

It should be noted that Raman is not the only type of vibrational spectroscopy that has been reviewed for clinical applications^[25,26]. One of the other main areas of vibrational spectroscopy being applied to clinical applications is fourier transform infrared spectroscopy (FTIR) and a type of enhancement infrared attenuated total reflection. FTIR relies on either the absorbance or the transmission of light through a sample, then similar to Raman the difference in the emitted and absorbed or transmitted light is measured to gain molecular information about a sample. This technique has also been used for diagnosis of CRC independently and also coupled with immunohistochemical staining^[27,28]. Compared with other vibrational spectroscopy techniques Raman holds many desirable properties for the application to a screening method. One of the biggest advantages of using Raman spectroscopy is that samples can be in aqueous solutions due to water having a small Raman cross-section at near-infrared wavelengths, water has a high absorbance in FTIR and therefore can interfere with a spectrum. Table 1 shows a comparison table constructed from literature to give an overview of the strengths and weaknesses of FTIR, Raman and traditional hematoxylin and eosin staining (H and E) when used for clinical applications^[25,29,30]. It suggests that for the application to biological samples such as tissues and biofluids Raman could be the most favorable option. Like FTIR it is non destructive and can be performed in real time compared to H and E staining. However, Raman spectroscopy has the advantage over FTIR of being able to scan over a larger wavenumber range than FTIR and with better spatial resolution than both FTIR and H and E. Furthermore, Raman is a technique that relies on scattering so measurements can be taken with single ended endoscopic probes. This is advantageous for *in vivo* applications because it makes it possible to study

Table 1 A comparison of Raman spectroscopy, fourier transform infrared spectroscopy and hematoxylin and eosin staining strengths and weaknesses

	Raman spectroscopy	FTIR	Hematoxylin and eosin staining
Method of detection	Inelastic scattering of monochromatic (laser) light	Absorbance (polychromatic light source)	Combination of basic and acidic dyes
Real time	Yes	Yes	No
Wavenumber range (cm ⁻¹)	50-4000	400-4000	N/A
Spatial resolution	1 µm	5 µm	Cellular
Enhancement techniques	SERS, TERS, CARS, SORS, SRS	ATR	"Special" staining
Effect of water	Minimal	Large absorbance in NIR region	No
Destructive to sample	No	No	Yes

SERS: Surface enhanced Raman spectroscopy; TERS: Tip enhanced Raman spectroscopy; CARS: Coherent anti-Stokes Raman spectroscopy; SORS: Spatially offset Raman spectroscopy; SRS: Stimulated Raman spectroscopy; ATR: Attenuated total reflection; FTIR: Fourier transform infrared spectroscopy; NIR: Near-infrared; N/A: Not available.

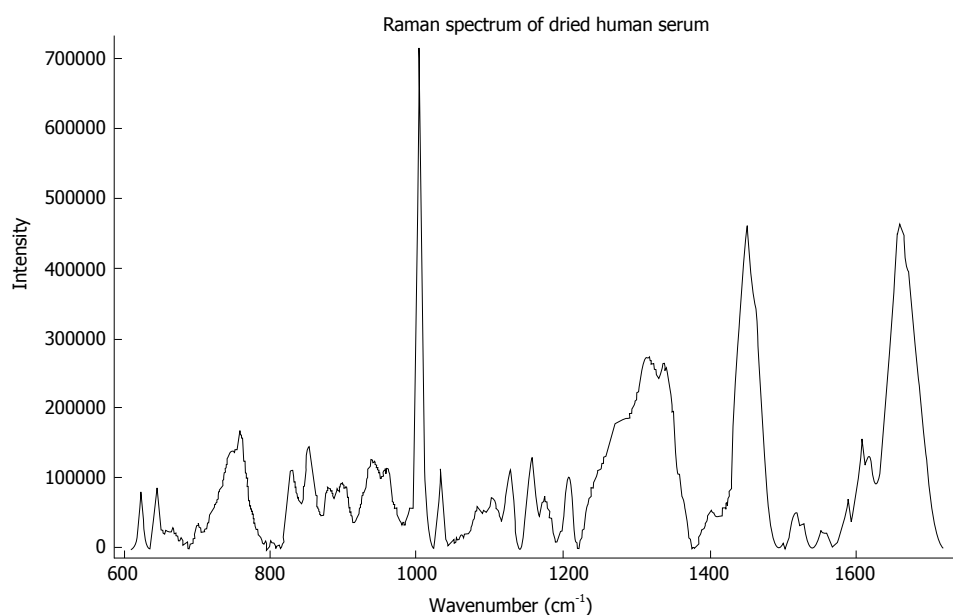


Figure 2 Example Raman spectrum of dried human serum. Spectrum of dried droplet of human serum taken with 785 nm laser excitation, 10 s acquisition time with an InVia Raman spectrometer (Renishaw, United Kingdom).

samples that are optically too thick for transmission techniques^[31]. This, along with the need for a less invasive diagnostic tool that can analyse liquid biofluids leads to the remainder of this review focusing on the application of Raman and SERS technology.

Data manipulation

Many clinical applications of Raman and SERS use chemometric data analysis techniques in order to aid data manipulation and to pick out the small differences that could indicate disease. Details of principle component regression (PCR) and partial least squares can be found in Kramer (1998)^[32]. Principle component analysis (PCA) applications can be found in Shinzawa *et al.*^[33] (2009). This review will only state the technique that has been used in each study.

CLINICAL APPLICATIONS OF RAMAN SPECTROSCOPY IN CRC

Histopathological analysis of tissue biopsies is still con-

sidered the gold standard for the diagnosis of malignant tissues that have been surgically resected. Typically, thin sections of tissue are "fixed" usually using formalin and then mounted onto glass slides and stained using various methods in order to determine TNM stage, tumour type, histologic grade and the level of vascular invasion. However, histopathology is a slow process that requires a trained pathologist, it is also inherently subjective^[34]. Raman spectroscopy offers the possibility of determining the presence of malignancy by detecting differences in Raman spectral features between normal and malignant tissue. Previously, Raman spectroscopy has been applied to *in vivo* probes that have the ability to discriminate multiple tissue types^[35,36], biofluid analysis^[37] and also analysis of cancerous cell lines for both discrimination and characterization^[38,39]. The motivation behind using Raman used *in vivo* is to aid rapid diagnosis and help to identify possible areas of tissue for biopsy that might otherwise be missed. A summary of the literature and different applications of Raman towards clinical applications for CRC can be found in Table 2.

Table 2 A summary of the different clinical applications of Raman spectroscopy to colorectal cancer

Method	Sampling type	Sample number	Ref.	Year	Spectral region (cm ⁻¹)	Laser excitation (nm)	Data analysis
Probe	<i>In vivo</i> (tissue)	20	Shim <i>et al</i> ^[42]	2000	450-1800	785	PCA, PLS, ANN
Probe	<i>In vivo</i> and <i>ex vivo</i> (tissue)	9	Molckovsky <i>et al</i> ^[44]	2003	900-1800	785	PCA, LDA, LOOCV
Micro-spectrometer	<i>In vitro</i> (primary tissue)	10	Chen <i>et al</i> ^[38]	2006	500-1900	782.5	PCA
Probe	<i>Ex vivo</i> (tissue)	59	Widjaja <i>et al</i> ^[45]	2008	800-1800	785	PCA, SVM, LOOCV
Micro-spectrometer	<i>Ex vivo</i> (tissue)	54	Beljebbar <i>et al</i> ^[47]	2009	600-1800	785	SVM, PCA
Micro-spectrometer	<i>In vitro</i> (serum)	120	Li <i>et al</i> ^[37]	2012	800-1800	785	PCR, PLSR, LDA
Micro-spectrometer	<i>In vitro</i> (cell lines)	N/A	Ranc <i>et al</i> ^[39]	2013	400-1800	532	PCA
Probe	<i>In vitro</i> (tissue)	177	Wood <i>et al</i> ^[43]	2014	800-1800	830	PCA, LDA, LOOCV
Micro-spectrometer	<i>In vivo</i> (tissue)	50	Bergholt <i>et al</i> ^[78]	2014	800-1800 and 2900-3600	785	PLS, LDA

PCA: Principle component analysis; LDA: Linear differential analysis; PLS/PLSR: Principle least squares regression analysis; LOOCV: Leave one out cross validation; ANN: Artificial neural network; SVM: Support vector machine; PCR: Principle component regression.

Table 2 shows that most clinical applications of Raman have been in the near-infrared (NIR) region using 785 nm laser excitation analysing tissue samples. This is likely due to reduced fluorescence of biological; samples in the NIR region, furthermore 785 nm laser is less powerful than visible region lasers so is less likely to cause damage to biological samples. The work is generally done in the "fingerprint region" of the Raman spectrum, *i.e.*, 400-1800 cm⁻¹ due to molecular bonds present in biological samples being Raman active in this region. All of the studies use chemometric data analysis so it seems for NR this is essential to differentiate the small differences in the Raman spectra when using biological samples. It is also clear that work in the field has previously been dominated work towards *in vivo* Raman probes for use during endoscopy but more recent work has used both Raman probes and microspectrometers.

Tissue analysis

Reviews dedicated to Raman spectroscopy for the use of *in vivo* probes for clinical use for many types of tissue including tissues of the gastrointestinal (GI) tract are already available^[25,40,41]. Briefly, the *in vivo* probes for use during endoscopy for use with GI tissue was first introduced by Shim *et al*^[42] (2000). In general, the *in vivo* probes use a laser excitation source in the NIR wavelength range (light is non-mutagenic in this region) coupled to an optical fibre probe. The probe can then act as both a source of light and a detector relaying a signal back to a charged coupled device detector and computer analysis. The development of the probes is not a simple process, some materials that the probes are manufactured from have a large Raman cross sections leading to design challenges regarding signal to noise ratio (gaining unwanted noise from the material). Other issues can be caused by tissue fluorescence signal being larger than the Raman signal making data acquisition difficult and spectral acquisition times to be impractical for clinical applications (more than 10 min). Nevertheless, some research groups have been successful in designing probes for specific use with gastrointestinal tissue for

use in routine endoscopy that have short acquisition times^[35,43].

Shim *et al*^[42] (2000) successfully applied an *in vivo* probe to gain Raman spectra of colonic and oesophageal tissues from 20 patients. The pressure spectral acquisitions were made with 5 s exposure time and repeated at normal and malignant sites within both areas of interest. In the colonic tissue subtle differences between spectral area 1100-1800 cm⁻¹ were identified however no significant prominent changes were evident. PCA and LDA analysis was then introduced in order to try and distinguish accuracy for application to GI diagnostics however no specific results relating to diagnostics were published.

Molckovsky *et al*^[44] (2003) were the first to assess the diagnostic potential of near infrared Raman spectroscopy on colonic tissue by using adenomatous polyps as a model for dysplasia. The group used a custom-made fiber-optic Raman probe and used PCA-LDA analysis and LOOCV to analyse a total of 33 polyps from 8 patients. After an initial *ex vivo* study of polypectomy specimens that involved a total of 54 spectra the analysis algorithm identified a sensitivity of 91% and a specificity of 95%. An *in vivo* study was then conducted with a total of 19 spectra from 9 polyps, after spectral analysis the algorithm identified adenomas with a sensitivity of 100% and specificity of 89%. A similar study involving a higher number of specimens for a similar use on *ex vivo* tissues was conducted by Widjaja *et al*^[45] (2008), the group were able to differentiate cancerous tissue with 100% sensitivity and 98.1%-99.7% specificity using a diagnostic algorithm using PCA and LDA.

Raman spectroscopy has also been investigated as a complimentary technique to histopathology. The first application of Raman spectroscopy for characterisation of colonic tissue (among others) to discriminate between cancerous vs normal was by Feld *et al*^[46] (1995). The group looked at the difference spectra between normal and cancerous tissue, this results showed potential that spectral differences in the tissue could be due to higher nucleic acid levels in the cancerous samples^[46].

Beljebbar *et al*^[47] (2009) used a Raman micro

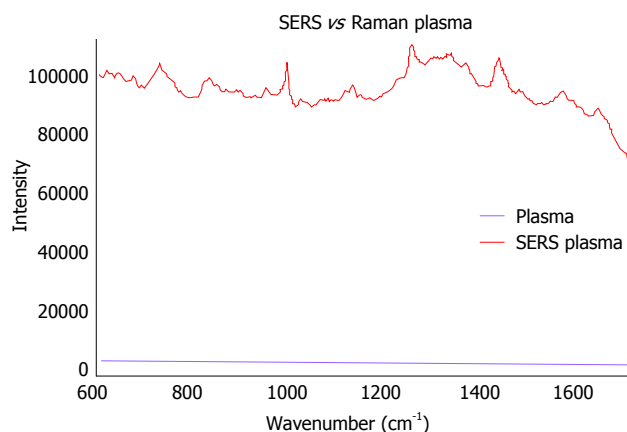


Figure 3 An example spectrum of a surface enhanced Raman spectroscopy response vs Raman response of human blood plasma. Raman spectrum and SERS spectrum of dried human plasma droplet at 10% laser power with 785 nm laser excitation, 10 s acquisition time with an InVia Raman spectrometer (Renishaw, United Kingdom). SERS response gained by 1:1 mixing with 40 nm raw gold nanoparticles (Nanocs, United States). Purple: SERS response of plasma; Blue: Raman response of plasma. SERS: Surface enhanced Raman spectroscopy.

spectrometer on 27 normal and 27 cancerous *ex vivo* frozen tissue samples. Unsupervised hierarchical cluster analysis to differentiate between normal and adenocarcinomatous human colonic tissues was discussed. The technique was based on the spatial distribution of molecular changes in colon constituents such as proteins, lipids and nucleic acids. The spectroscopic data was then used to create pseudo-colour Raman images of tissues for comparison with histopathological slides. The data was then used to create databases for the purpose of comparing unknown specimens. Six extra frozen unknown samples specimens were fed into the database and were correctly identified as either cancerous or normal^[47]. This study showed the potential for Raman spectroscopy to aid histopathology by adding structural molecular information to the visible information gained from H and E staining. This study used frozen tissue samples but there are different fixation methods available. There have been studies on the effect that different fixation methods of both cell lines and tissue samples taken from resection has on the Raman spectral signature but these will not be discussed further in this review^[48-50].

It should be noted that Raman spectroscopy used as an adjunct to histopathology has not just been applied to CRC. Some groups have investigated the use of stimulated Raman spectroscopy (SRS) to create spectral histopathological images for breast, brain and skin tissue among others^[51-54]. For example, Satoh *et al.*^[54] (2014) used SRS and PCA multivariate analysis to produce Raman map images of damaged liver tissue in mice. The images could then be compared to different staining methods used in histopathology.

DETECTION OF CRC IN BLOOD SAMPLES

The advantage of Raman spectroscopy over other vibra-

tional techniques is its ability to analyse samples in aqueous solution with minimal background. This is advantageous for the study of samples such as urine and blood as they can be analysed without changing their composition by drying.

Berger *et al.*^[55] (1999) introduced the idea that Raman had potential for the analysis of biofluids, in particular human blood. Premasiri *et al.*^[56] (2012) found that the NR spectra of whole human blood are dominated by the normal modes of either oxygenated or deoxygenated haemoglobin porphyrin macrocycle and haemoglobin. This leads to the assumption that if NR is to be used for the analysis of blood components such as malignancy specific proteins for CRC other than those associated with red blood cells (RBC) an enhancement mechanism has to be used or blood samples must have RBC removed in order to overcome this issue (Figure 3).

For example, Harris *et al.*^[57] (2009) discussed the potential of peripheral blood samples analysed by NR to provide cancer screening in head and neck cancer. Using Raman spectroscopy and LDA analysis alone the study found this technique to have approximately 65% specificity and sensitivity for the discrimination of cancer in peripheral blood samples. The use of non-enhanced Raman spectroscopy for the discrimination of blood serum between normal and CRC was first reported by Li *et al.*^[37] (2012). Li *et al.*^[37] presents a study using clinical samples from 44 colon, 46 rectum and 30 healthy controls. Raman peak parameters and fluorescence background were used along with multivariate analysis techniques such as PCR and PLSR for the dimension deduction of spectral data. Then, LDA on PC's is used to see diagnostic performance. Three distinct, Raman peaks were found to have significance at 1029 cm⁻¹, 1538 cm⁻¹ and 1170 cm⁻¹. The 1538 cm⁻¹ peak was assigned to beta-carotene and 1170 cm⁻¹ to tryptophan and phenylalanine. The average spectra from the three sample groups were then compared and the latter two peaks were shown to decrease compared to the control group. This is explained as a decrease in anti-cancer related molecules.

This study showed that it is possible to discriminate between serum samples of patients with and without CRC. The result of the PCR-LDA analysis was promising as it identified normal samples with 87.5% accuracy and 96.7% specificity and colon cancer samples with a sensitivity of 84.8%.

SERS detection methods

Raman spectra are subject to interference from samples that exhibit fluorescence; a fluorescence signal is far greater than the Raman response so it can "hide" any Raman signal. Fluorescence is fairly common when using wavelengths less than 785 nm that are commonly used in biological studies. Also, when Raman is used on complex biological samples Raman peaks can be additive, making differentiation between biological markers difficult to resolve, this results in a reliance on data manipulation in order to detect small spectral differences. SERS offers a resolution to some of these

issues as it reduces the effects of inherent fluorescence while increasing the intensity of the Raman response of the sample. Premasiri *et al.*^[58] (2001) demonstrated the need for an enhancement mechanism in order to detect some low concentration analytes in urine analysis by Raman spectroscopy. It was found that urea had a sufficiently high concentration to be analysed by NR in liquid form however, lower-level nitrogen compounds needed enhanced Raman spectroscopy in order to detect these compounds^[58]. Therefore SERS was used as an enhancement mechanism in order to be able to detect the compounds that were in concentrations too small to detect with Raman.

The basic principle of SERS is to amplify the Raman response of a given analyte. The SERS effect was first discovered in 1974, and understood to be an enhancement of Raman scattering in 1977^[59,60]. This is generally achieved by having an analyte attached or close to the surface of a nanoscale metal substrate causing an enhancement factor of up to $10^{14,15,61,62}$. The exact mechanism of SERS enhancement is still an area of active research, however it is generally accepted that two mechanisms contribute to the enhancement^[63]. One is based on electromagnetic field enhancement due to excitation of electromagnetic resonances in the SERS active nanoscale metal substrate. The other is known as "chemical enhancement" which is a result of the metal electrons causing a charge transfer between the metal substrates and the adsorbates. The result of the combined enhancement is an extremely powerful technique that combines ultra sensitive detection limits with the molecular structure information from Raman spectroscopy giving the possibility of single molecule detection^[64].

Clinical applications of SERS for CRC

The main use of SERS in clinical applications for CRC has been as a detection method. Lin *et al.*^[65] (2011) were the first to report SERS serum analysis for the detection of CRC. In their study colloidal gold nanoparticles were simply mixed with serum samples from 38 patients and 45 control samples and dropped onto an aluminium substrate. This technique is known as label-free SERS. It generally relies on blood constituents being adsorbed onto the surface of metallic nanoparticles causing an enhanced Raman response. In the Lin *et al.*^[65] study a Raman micro-spectrometer (Renishaw, Great Britain) fitted with a 785 nm diode laser was used to gain spectra in the $300\text{--}1800\text{ cm}^{-1}$ range. Spectra from the two groups were normalised to the integrated area under the curve in the $350\text{--}1750\text{ cm}^{-1}$ wavenumber range. The mean spectrum for the normal serum and the cancer serum were then compared to isolate wavenumbers that showed the most variation between the two groups. Then an empirical diagnostic algorithm based on peak intensities at 725 cm^{-1} and 638 cm^{-1} was used to classify the normal and the cancer samples. These were chosen based on previous studies by Han *et al.*^[66] (2008) that showed the ratio to be important

disease marker. This technique was compared PCA-LDA multivariate approach. The PCA analysis then used whole spectra to discern the spectral components that had the largest variation. After comparison the group found PCA-LDA to be more effective at detecting CRC. With specificities for detecting cancer to be 68.4% and 97.4% for the empirical approach and the multivariate approach respectively.

This study showed that through simply mixing gold nanoparticles with serum that it is possible to discriminate between normal and cancer samples using SERS along with both empirical and multivariate analysis techniques. The potential for using whole spectra coupled with PCA-LDA to be used as a screening technique for CRC was then discussed using the PCA-LDA methods in a second publication from the same group^[65]. The two publications also included tentative peak assignments and major vibrational bands that have been previously observed in serum samples. The peak assignments are vital to being able to accurately describe what is happening at the molecular level when a patient has a disease. However, due to the additive nature and complex compounds found in serum samples it can sometimes be difficult to be sure of peak assignments. Furthermore, the methods described above such as looking at the 725 cm^{-1} band can be a marker for disease but it is not specific to CRC. In order for the technology to become useful as a diagnostic there is a need to have a Raman/SERS marker that is specific to CRC.

The need for specific detection has motivated the development of "labelled" SERS probes. These probes have previously been used for detecting disease specific proteins in both tissue and serum samples^[67-71]. They have also been used for the detection of circulating tumour cells^[72]. However there is little reported on the specific application of targeted SERS probes for use in detecting CRC^[73]. In general targeted SERS techniques rely on either aggregation of antibody-functionalised nanoparticles after exposure to a protein or they are used to form of sandwich immunoassay similar to that of an ELISA setup but using SERS active probes rather than fluorescent-tagged antibodies (Figure 4).

Both techniques offer advantages and disadvantages; techniques relying on the aggregation of nanoparticles use fewer antibodies and in general have very simple protocols that can be done on cheap substrates. However, when dealing with the aggregation of nanoparticles it can be difficult to "find" the correct spot on a sample where the SERS intensity is greatest. Furthermore, studies relying on aggregation can be susceptible to large variation as controlling the aggregation can be difficult. SERS based immunoassay holds the advantage over aggregation because if the disease specific protein is in a sandwich style assay then the area to probe is easier to locate and the Raman signal is less likely to be variable, as one would expect more even coverage of the protein over the assay area. One advantage of both of these techniques over current fluorescence methods is that Raman bands are much

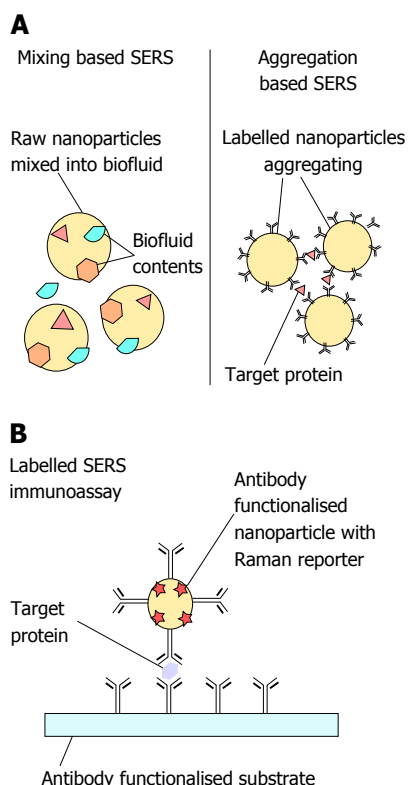


Figure 4 Different methods of producing a surface enhanced Raman spectroscopy response with biological samples. A: Mixing based methods with both non-labeled mixing and labeled or "aggregation" based mixing; B: Labeled surface enhanced Raman spectroscopy based immunoassay. SERS: Surface enhanced Raman spectroscopy.

narrower than those in fluorescence which means there is the potential to use more probes in a multiplex assay than is currently possible with fluorescent probes. Chen *et al.*^[74] (2013) developed a SERS based immunoassay for the detection of carcinoembryonic antigen (CEA) in serum of patients with CRCs. CEA antibody functionalised glass slides were used in conjunction with SERS active probes that were also functionalised with CEA^[74]. A range of concentrations from 5×10^{-3} – 5×10^5 ng/mL CEA were prepared and the SERS response monitored. The SERS intensity was linearly correlated with the concentration of CEA in the characteristic peak or the Raman reporter molecule at 1077 cm^{-1} and hence a calibration curve was established. CEA concentrations in serum samples from 26 patients with CRC were then analysed with both SERS immunoassay and electrochemical luminescence. The results were then compared and it was found that the two techniques had similar agreement. Using the calibration curve for the patient samples a detection limit of 5 pg/mL was achieved. This is the only study specifically using CEA for the detection of CRC, however there is other work in the literature using CEA conjugated antibodies but towards use for other diseases^[69,70].

Ito *et al.*^[75] (2014) developed a SERS based assay that used silver nanoscale hexagonal columns (NHC) on phosphor bronze chips. The chips were negatively ionized and 36 clinical serum samples from patients with benign diseases, gastric and CRCs were dropped

onto the chips. Using a 632.8 nm laser excitation, measurements were taken and two peaks appeared to be prominent in the SERS spectra at 1350 cm^{-1} and 1570 cm^{-1} . Polynomial fitting was then used to determine SERS peak height for each of the samples. In order to validate that the SERS peak height and the concentration of the serum samples were correlated the measurements were repeated at different dilutions of the same serum sample. It was found that 10-fold dilutions were the saturating dilution for the NHC chips so 10 fold dilutions were used throughout the rest of the experiment. Finally the SERS peak height for both of the prominent peaks was compared between the three groups of samples and it was found that the SERS peak heights in the benign samples was indeed significantly lower than in the cancerous samples. This was calculated using the Pearson product-moment correlation coefficient and the non-parametric Spearman's rank correlation coefficient.

Another slightly different approach to using SERS for clinical use for CRC is using SERS as a characterisation/validation tool when developing other nanoscale devices such as the work done by da Paz *et al.*^[76] (2012). In this study SERS was used as a characterisation tool in the development of maghemite nanoparticles as a theragnostic device for CRC^[76]. The SERS active nanoparticles used in this study were functionalised with Anti-CEA antigen in the hope that they can be used to detect primary and metastatic CRC, it was hoped by the authors that these nanoparticles could then be developed for a variety of applications including magnetic resonance imaging (MRI) enhancement and targeted drug delivery.

Limitations of Raman and SERS in clinical applications

Raman and SERS based tools have shown potential that they will have a place as either an alternative or an adjunct to current diagnostic methods. The development of SERS biomarker detection could also lead to its use in personalized medicine. However, there are still some limitations of Raman and SERS techniques that will need to be overcome before they are routinely used in a clinical setting. These include: (1) Many Raman studies involve costly equipment and expensive substrates, there will need to be investigations into cost reduction for large scale applications; (2) Raman and SERS studies that are carried out in a laboratory will require sample handling and storage, the effect of handling samples and storage techniques on the performance of Raman based tools will need to be quantified; (3) Thermal damage thresholds of *in vivo* tissue and *ex vivo* tissue samples from the colon and rectum will need to be established; (4) Many studies for clinical use different analytical techniques, and still require the skill of the user to determine the results of these techniques. User-friendly software for diagnostic analysis of the spectra will need to be developed and tested for multi-user reliability; (5) Inter equipment variability studies will need to be carried out, Raman equipment can often be susceptible to variability from external factors such as room temp, laser stability, etc.; and (6) SERS based techniques have been subject

to reproducibility issues, CRC is a heterogeneous disease so if immunoassay style tools are to be used then large scale studies with clinical samples will need to be carried out.

CONCLUSION

In the field of cancer detection Raman spectroscopy and SERS has gone through a period of rapid progress in the last decade. The use of Raman in clinical applications for CRC has previously been dominated by the discrimination of cancerous vs non-cancerous tissue with only a few studies on the use of Raman with biofluids for CRC detection. There are currently successful *in vivo* Raman tools for real-time use during endoscopy. These tools can be used to gain molecular information through Raman imaging and traditional spectroscopy. Therefore, they aid current endoscopic techniques by giving extra molecular information that could potentially be missed using traditional methods. However, Raman tools are still in general expensive to produce and require specialist knowledge in order to operate the machinery. Furthermore, thermal thresholds for the damage of GI tissue need to be properly established before these tools can be ready for use in a routine clinical setting. In future, national multi-site trials that include large patient numbers are needed to study the thermal threshold of tissues. Future research into the large-scale manufacture (and miniaturisation) of Raman tools needs to be carried out to investigate variability between sites and investigate the cost effectiveness of Raman tools compared to current technology.

In order to detect low concentration analytes SERS has started to become an alternative method to Raman. SERS offers enhanced signals and reduced fluorescence compared to Raman. Current research uses different techniques to gain a SERS response from samples. One of the limitations of SERS based techniques has been that the variations in the plasmon resonance of nanostructures that cause a SERS response are subject to large variability. Therefore, in mixing style SERS methods research into reducing the variability in SERS response even across a single sample will need to be investigated. Another method of gaining a SERS response is through a SERS based immunoassay; this has been successfully used to detect the current accepted biomarker for CRC CEA. The immunoassay design is based on reducing variability by controlling separation of the SERS substrates. SERS immunoassay has the potential to have multiplex detection of analytes in both tissue and biofluids. This could be one of the biggest areas of development if CRC research follows that of diseases such as nasopharyngeal cancer^[77]. Furthermore, if SERS is successfully used to detect different concentrations of biomarkers then this opens up the possibility into research towards personalised medicine and detecting changes in the levels of biomarkers using less invasive methods than are currently available in the United Kingdom (*i.e.*, through blood based testing). There are currently other CRC

detection tests in development that are more advanced than SERS such as mSept9 blood based testing. However, SERS based detection aims to have detection limits below the current available technology; therefore it offers the possibility of research into new biomarkers for CRC based on Raman or SERS spectral signals. Furthermore SERS and Raman based techniques still have the ability to be developed into techniques that are used in conjunction with other developing detection methods.

Both Raman and SERS techniques will also need further research into producing a universal method of background subtraction and analysis of data. Currently many research groups use different methods of data analysis that can be complex and still require clinicians to interpret results using spectral knowledge. In order for Raman and SERS based detection to be implemented into a clinical setting simple, user-friendly programs will need to be produced that remove the need for interpretation of spectra by a user. If the spectral analysis is automated then Raman and SERS techniques have the potential to become "observer-independent" tools.

Raman and SERS techniques are currently still in development with the aim to be in regular use in a clinical setting. If the technological limitations are overcome then the techniques have the potential to produce more specific, affordable detection and screening for CRC that can be routinely used in a clinical setting as an alternative or an adjunct to current methods.

The final limitation to Raman and SERS based techniques will be that of persuading clinicians that the new technology can replace existing techniques, it is possible that national based trials showing the robustness of Raman and SERS techniques will go some way to achieving this.

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Current adjuvant treatment modalities for gastric cancer: From history to the future

Leyla Kilic, Cetin Ordu, Ibrahim Yildiz, Fatma Sen, Serkan Keskin, Rumeysa Ciftci, Kezban Nur Pilanci

Leyla Kilic, Department of Medical Oncology, Acibadem University Hospital, Istanbul 34394, Turkey

Cetin Ordu, Department of Medical Oncology, Istanbul Bilim University, Istanbul 34394, Turkey

Ibrahim Yildiz, Fatma Sen, Serkan Keskin, Rumeysa Ciftci, Department of Medical Oncology, Institute of Oncology, Istanbul University, Istanbul 34394, Turkey

Kezban Nur Pilanci, Department of Medical Oncology, Haseki Training and Research Hospital, Istanbul 34394, Turkey

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Correspondence to: Cetin Ordu, MD, Department of Medical Oncology, Istanbul Bilim University, Büyükdere Cad. No.120, Şişli, Istanbul 34394, Turkey. cetinordu@hotmail.com
Telephone: +90-532-2276179
Fax: +90-212-2889812

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Abstract

The discrepancy between the surgical technique and the type of adjuvant chemotherapy used in clinical trials and patient outcomes in terms of overall survival rates has led to the generation of different adjuvant treatment protocols in distinct parts of the world. The adjuvant treatment recommendation is generally chemoradiotherapy in the United States, perioperative chemotherapy in the United Kingdom and parts of Europe, and chemotherapy in Asia. These options mainly rely on the United States Intergroup-0116, United Kingdom British Medical Research Council Adjuvant Gastric Infusional Chemotherapy, and the Asian Adjuvant Chemotherapy Trial of S-1 for Gastric Cancer and Capecitabine and Oxaliplatin Adjuvant Study in Stomach Cancer trials. However, the benefits were evident for only certain patients, which were not very homogeneous regarding the type of surgery, chemotherapy regimens, and stage of disease. Whether the dissimilarities in survival are attributable to surgical technique or intrinsic biological differences is a subject of debate. Regardless of the extent of surgery, multimodal therapy may offer modest survival advantage at least for diseases with lymph node involvement. Moreover, in the era of individualized treatment for most of the other cancer types, identification of special subgroups comprising those who will derive more or no benefit from adjuvant therapy merits further investigation. The aim of this review is to reveal the historical evolution and future reflections of adjuvant treatment modalities for resected gastric cancer patients.

Key words: Adjuvant chemoradiotherapy; Biomarker; Gastric cancer; Lymph nodes

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Core tip: Despite extensive surgery, gastric cancer will likely recur for most patients. Fortunately, additional treatment modalities either in the perioperative or post-operative setting provide varying degrees of survival

advantage. Although there is considerable data regarding adjuvant chemotherapy and chemoradiotherapy since the Intergroup-0116 study, there is still no established uniform treatment protocol depending on the type of surgery, histological subgroup, or extent of disease. The present review is aimed at identifying the advances in treatment strategies and discussing the pros and cons of each strategy.

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INTRODUCTION

Gastric cancer is the fifth most common cancer in the world, with 952000 new cases being diagnosed in 2012^[1]. The cornerstone for treatment of gastric cancer is surgical resection with lymph node dissection (LND). An extended lymph node resection, which is commonly defined as a D2 resection for gastric cancer, includes the removal of the whole stomach, the greater and lesser omentum, and the N1 and N2 (groups 1-11) lymph nodes. For tumours located in the proximal stomach, resection of the spleen and the tail of the pancreas may be necessary for removing groups 10 and 11 lymph nodes. While a D2 lymph node dissection is considered a standard surgical procedure for resectable gastric cancer in Japan and Korea, the necessity of a D2 dissection still remains a subject of controversy in Western countries. The overall 5-year survival rates in the United States is around 10% to 40%; however, in Japan and South Korea, it is reported to be 50% or higher^[2-4]. It is unclear whether removing additional lymph nodes during the operation contributes to a difference in survival. Additional information on lymph nodes may provide more accurate staging, which is currently only available for patients that undergo a D2 dissection. Other factors such as early diagnosis, case selection, surgical skill, and post-operative care may also contribute to this observed difference in survival.

Despite difficulties with surgical techniques, data from the National Cancer Data Base in the United States points at a 10-year survival rate of 65% for patients with resected stage IA disease and 3%-42% for those with more advanced disease^[5]. Thus, the high rate of both locoregional and distant relapse, even after complete resection, makes adjuvant treatment mandatory for patients with stomach cancer.

The timing and sequence of adjuvant/neoadjuvant strategies and combination therapies have been questioned in numerous phase II and phase III trials. The landmark Intergroup (SWOG 9008/INT-0116) trial demonstrated a survival benefit for resected stage IB-IV, M0 gastric

cancer patients following adjuvant chemoradiotherapy^[6]. However, the extent of surgery and the chemotherapy (CT) regimen, which was associated with high rates of toxicity, was seriously critiqued in this study. Although an extensive (D2) LND was recommended, only 10% of the patients had undergone a D2 dissection. The relative inadequacy of locoregional control with limited LND was supposed to be compensated with adjuvant radiotherapy.

Although there is large amount of data from randomized, controlled trials (RCT) and recommendations from several guidelines, recent analysis from the National Cancer Data Base have revealed that real-life practices somewhat diverge from the evidence-based results^[7]. Trends in American cancer centres have shown that out of stage III patients who received surgery at community hospitals, less than 50% also received adjuvant chemoradiotherapy in 2009. However, the large number of patients involved in the RCTs constitute a heterogeneous population where the benefit from adjuvant therapy may be difficult to interpret for some specific subgroups of patients. Moreover, there is still no phase III data supporting the tailoring of treatment according to stage of disease after surgery, unlike colon and breast cancers, since each stage may benefit from adjuvant therapy to a varying degree. This review will focus on the evidences of adjuvant treatment strategies dependent on the recent RCTs and future directions for optimal approach.

ADJUVANT CHEMOTHERAPY REGIMENS

Surgical resection is the only hope for curative treatment in early stages of gastric cancer. However, only 40% of the patients with gastric cancer will remain disease free after complete resection of their tumour. Therefore, adjuvant and neoadjuvant treatment modalities are crucial for establishing better prognosis for gastric cancer patients. Extensive studies were conducted to determine the efficacy of adjuvant chemotherapy for gastric cancer. Some previous phase III randomized trials did not demonstrate absolute benefit for adjuvant chemotherapy. However, these studies usually did not enrol large datasets and generally included early stage patients^[8-10]. The Adjuvant Chemotherapy Trial of S-1 for Gastric Cancer (ACTS-GC) study was a pioneer phase III study showing a significant survival benefit following adjuvant chemotherapy after D2 LND for gastric cancer patients. The trial documented that one year adjuvant chemotherapy with an oral fluoropyrimidine, S-1 provided a clear survival benefit for stage II and III gastric cancer patients after D2 LND^[3]. Since the publication of the results of the ACTS-GC trial, chemoradiation was excluded from adjuvant treatment modalities for D2 resected gastric cancer in Japan, but this was not the case in Western countries. However, the results of the ACTS-GC study conflicted with a similar large scaled phase III Japanese trial that utilized mitomycin C, fluorouracil (FU), and oral UFT (a combination of tegafur, a prodrug of 5-FU and uracil treatment) as adjuvant chemotherapy^[11]. The investigators considered that this

result was due to high proportion of pT1 gastric cancer patients included in the trial for which surgery alone may yield a good prognosis, and there seemed to be no requirement for adjuvant therapy. Consequently, further trials usually did not include early stage patients (*i.e.*, \leq stage Ib).

Apart from the Asian studies, most of the trials performed with adjuvant chemotherapy have not demonstrated a significant survival benefit^[12-14]. However, the results of the meta-analysis by the Global Advanced/Adjuvant Stomach Tumour Research International Collaboration (GASTRIC) group with an extended follow-up time have revealed a modest but statistically significant survival advantage with adjuvant chemotherapy after curative resection of gastric cancer^[15]. There was an absolute improvement in overall survival (OS) of 6% after 5 years that was maintained at 10 years. The treatment benefit was sustained in the majority of the investigated groups of FU-based regimens, with reductions in the risk of death between 20% and 40%. This meta-analysis pointed out that adjuvant FU-based chemotherapy is associated with improved OS, and combination chemotherapy could be recommended for patients who have not received treatment in the perioperative setting.

S-1, an orally active FU analogue, is a combination of tegafur (a prodrug of 5-FU), gimeracil (an inhibitor of dihydropyrimidine dehydrogenase), and oteracil (inhibitor of the phosphorylation of FU in the gastrointestinal tract). Pharmacokinetics studies have shown that the absorption of FU derived from S-1 is not affected by gastrectomy^[16]. The response rates with S-1 alone were higher than 40% in two phase II trials among patients with advanced gastric cancer^[17,18]. Similarly, S1 demonstrated a clear survival benefit in the ACTS-GC study. After 3 years of median follow-up, the OS in the S-1 group was 33% higher than the surgery-only group^[3]. Grade 3 or grade 4 adverse events occurred in less than 5% of patients in the S-1 group. The OS rate was 80.5% in the S-1 group and 70.1% in the surgery-only group at 3 years. Thus, S-1 was approved as an effective option for adjuvant chemotherapy for patients with resected gastric cancer.

Recently Zhang *et al.*^[19] have published the analysis of 31 RCTs, which included 7120 gastric cancer patients. There was no significant difference in terms of overall mortality among the four chemotherapy regimens including FU + mitomycin (MMC) + adriamycin, FU + MMC (FM), Tegafur and MMC. The evidence for the FM regimen and MMC regimen was not strong enough. According to this meta-analysis, Tegafur was recommended as the first-line adjuvant chemotherapy regimen for patients after complete resection. However, RCTs published after 2000 have consisted of primarily combinations of cisplatin and FU. Collectively, S1 or 5-FU-cisplatin combination regimens in neo-adjuvant, adjuvant, and perioperative settings have yielded a favourable impact on survival^[20-32]. Moreover, chemotherapy seems to provide prolongation of survival for patients with mostly node-positive and T3-T4 disease (Table 1).

The combination of adjuvant with neo-adjuvant chemo-

therapy has proven its value in two randomized trials. As the pioneer study of perioperative chemotherapy for gastric cancer, the British Medical Research Council Adjuvant Gastric Infusional Chemotherapy (MAGIC) trial demonstrated a significant downstaging of the primary tumour and a 10% higher resectability rate with a survival benefit of 13% at 5 years^[31]. The primary goals of the ECF perioperative CT were to increase the likelihood of R0 resection while downstaging the tumour, predicting tumour sensitivity to chemotherapy, improving obstructive symptoms, and eliminating micrometastases. One of the major limitations of the MAGIC trial was that only 42% of patients in the chemotherapy group were able to receive all protocol treatment; 34% of patients who completed preoperative chemotherapy and surgery could not be administered postoperative chemotherapy, possibly due to postoperative complications, early disease progression, or the patients' will. Nevertheless, patients in the perioperative chemotherapy section had a survival advantage when compared with those who underwent surgery alone (5 years OS rate for CT group vs surgery-alone; 36% vs 23%, respectively).

New questions have arisen regarding the optimal adjuvant therapy following the increased acceptance of D2 gastrectomy. The primary goal of design for Capecitabine and Oxaliplatin Adjuvant Study in Stomach Cancer (CLASSIC) study was to answer these questions. The investigators aimed to evaluate the effect of adjuvant capecitabine plus oxaliplatin after D2 gastrectomy^[25]. The CLASSIC study reported a 44% improvement in disease-free survival (DFS) for patients randomly assigned to postoperative capecitabine and oxaliplatin (XELOX) when compared with observation. Subgroup analysis confirmed the beneficial effect of adjuvant capecitabine and oxaliplatin for all disease stages (II, III A or III B), and the extent of nodal involvement correlated with substantially more benefit ($N2 > N1 > N0$) from adjuvant CT. Three-year DFS was defined as the primary endpoint because the majority of the recurrences occur within 3 years of surgery according to the preliminary data from the GASTRIC group. Although not formally validated as a surrogate measure yet, 3-year DFS is strongly correlated with 5-year OS, which is the reference point for judging effectiveness of adjuvant therapy in gastric cancer^[15]. Additionally, after a median follow-up of 62.4 mo, the updated results supported the interim analysis findings; the estimated 5-year DFS was 68% in the adjuvant capecitabine and oxaliplatin group vs 53% in the observation alone group^[33]. The OS data from this study are not yet known; however, the data suggest an improvement in OS with capecitabine and oxaliplatin compared with surgery alone (78% vs 69%).

Whether the results of CLASSIC trial could be adapted to geographical regions where management practices differ is unclear. The CLASSIC trial had considerably better survival outcomes when compared with Western counterparts; the 3-year OS rate in the surgery-only group was 78% in the CLASSIC trial, while it was only 30%-40% in the United States Intergroup-0116 and

Table 1 Randomized trials of chemotherapy for resected gastric cancer, published after 2000s

Ref.	Regimen	LN (+) % (chemo)	T3-T4 % (chemo)	No. of patients	D2 % (chemo)	% 5-yr survival	<i>P</i> value
Neoadjuvant							
Schuhmacher <i>et al</i> ^[20]	5-FU, LV, cisplatin	94.4	100	72	95.7	72.7	0.2
	Surgery alone			72		69.9	
Adjuvant							
Bajetta <i>et al</i> ^[21]	EAP, 5-FU + LV	91	51	137	73	52	0.87
	Surgery alone			137		48	
Chipponi <i>et al</i> ^[22]	5-FU, cisplatin	80	75	101		39.0	NS
	Surgery alone			104		38.7	
Bouché <i>et al</i> ^[23]	5-FU, cisplatin	80.3	77.9	127	55.9	46.6	0.22
	Surgery alone			133		41.9	
Sasako <i>et al</i> ^[24]	S-1	90.4	45.1	529	100	71.7	0.493
	Surgery alone			530		61.1	
Bang <i>et al</i> ^[25]	Capecitabine, oxaliplatin	91	45	520	100	83	0.493
	Surgery alone			515		78	
Kang <i>et al</i> ^[26]	MMC + 5'FDR (MF') <i>vs</i> MF' + CDDP	90	43	424	100	66.5	0.33
				431		65	
Di Costanzo <i>et al</i> ^[27]	PELF	83.8	49.2	130	55	47.6	0.41
	Surgery alone			128		48.7	
De Vita <i>et al</i> ^[28]	ELFE	72	80	112	79	48	0.610
	Surgery alone			113		43.5	
Nitti <i>et al</i> ^[29]	FAMTX or FEMTX	82	61	194	88	43	0.86
	Surgery alone			203			
Neri <i>et al</i> ^[30]	EPI, LV, 5-FU	100	84	69		30 m's (median)	< 0.01
	Surgery alone			68		18	
Perioperative							
Cunningham <i>et al</i> ^[31]	EPI, 5-FU, cisplatin	71	56	250	28	36.3	0.009
	Surgery alone			253		23.0	
Ychou <i>et al</i> ^[32]	5-FU, cisplatin	67	58	113	100	38	0.02
	Surgery alone			111		24	

5-FU: 5-fluorouracil; EAP: Etoposide, adriamycin, cisplatin; LV: Leucovorin; MMC: Mitomycin C; 5' FDR: Doksifluridine; EPI: Epirubicin; FAMTX: Fluorouracil, adriamycin; methotrexate; FEMTX: Fluorouracil, epirubicin; methotrexate; PELF: Cisplatin, epirubicin, leucovorin, 5- fluorouracil; ELFE: Epirubicin, leucovorin, 5-fluorouracil, etoposide; CDDP: Cisplatin; LN: Lymph node.

United Kingdom MAGIC populations. Although patients included in the CLASSIC trial had fewer T3 and T4 lesions (44% in CLASSIC vs 68% in Intergroup-0116 vs 64% in MAGIC), node-positive disease was more frequent (90% vs 85% vs 72%). The differences in survival rates are supposed to be not only due to prognostic differences but also due to intrinsic biological disparities and consistent use of D2 surgery. Since D2 gastrectomy is also a standard of care in Western countries currently, the findings of this study could be remarkable and generalized to the other regions where D2 surgery is performed by experienced surgeons.

ADJUVANT CHEMORADIOOTHERAPY

After surgery with curative intent, local or regional recurrence in the gastric or tumour bed, the anastomosis, or regional lymph nodes occurs in 40% to 65% of patients^[34]. According to the preoperative analysis of the University of Minnesota, locoregional failure was the only evidence of relapse in 29% of patients and as any component of relapse in 88% of patients. Locoregional recurrences occurred in three major locations: (1) Gastric bed (organs and structures in proximity to the primary tumour); (2) regional nodes; and (3) gastric remnant, anastomoses, and duodenal stump^[35]. Autopsies report even higher locoregional failure rates reaching up to

80%-93%^[36]. Thus, radiotherapy is supposed to be an essential adjunct of postoperative treatment in gastric cancer patients.

Two randomized trials evaluating the benefit of adjuvant radiotherapy (RT) alone after resection for gastric cancer revealed conflicting results. The first trial by the British Stomach Cancer Group included 436 patients who were randomized to undergo surgery alone or surgery followed by RT or chemotherapy with mitomycin, doxorubicin, and fluorouracil^[37]. However, more than one third of the patients had gross or microscopic residual disease following surgery. At the 5-year follow-up, there was no additional survival benefit for adjuvant RT or chemotherapy compared with surgery alone. However, there was a significant reduction in locoregional recurrence with the addition of RT to surgery. The second trial by Zhang *et al*^[38] randomized 370 patients to preoperative RT or surgery alone. Survival and resection rates were significantly improved with preoperative RT compared with surgery alone (30% vs 20%; 89.5% vs 79%, respectively). However, there was not a significant reduction in distant recurrence rates (24.3% vs 24.7%). Because only cardiac lesions were included in the trial, it is not clear whether these results can be adapted to distal lesions.

The landmark trial regarding combined modality adjuvant treatment for gastric cancer has been the

Intergroup 0116 study^[6]. A total of 556 patients with resected carcinoma of the stomach or gastroesophageal junction (stage IB through IV, M0) disease were randomized to surgery alone or surgery plus postoperative chemoradiotherapy. The median OS in the surgery only group was 27 mo, as compared with 36 mo in the chemoradiotherapy group ($P = 0.005$). An important issue regarding the surgical procedure was the extent of surgery in this trial. Although the recommendation was an extensive D2 LND, only 10% of the patients underwent a D2 dissection, 36% had a D1 dissection, and more than half had a D0 lymphadenectomy (not all of the N1 nodes were resected). This situation raised the question of whether chemoradiation was compensatory for inadequate surgery. Thus, in high-volume centres where D2 LND is routinely performed, omitting adjuvant radiotherapy has been considered due to high morbidity rates and poor tolerance. However, an observational study including patients with D2 LND has demonstrated that chemoradiotherapy as an adjunct to surgery could be tolerable with acceptable toxicity and good tumour control^[39]. The patients had received a postoperative chemoradiotherapy protocol similar to the Intergroup trial or surgery without further adjuvant treatment. The median duration of OS was significantly longer in the chemoradiotherapy (CRT) group than in the comparison group (95.3 mo vs 62.6 mo). However, these data rely on nonrandomized observation studies with suitable controls or unplanned subgroup analysis.

Updated analysis of the Intergroup trial with longer follow-up has supported the persistent benefit of adjuvant CRT^[40]. The OS and recurrence-free survival data demonstrated continued strong benefit from postoperative radiochemotherapy. Hazard ratios were virtually unchanged since the original report. Moreover, two meta-analyses comparing the efficacy of adjuvant CRT vs CT after R0 resection have confirmed the superiority of the combined modality in terms of disease-free survival^[41,42]. However, there was no OS advantage with the addition of radiotherapy in both analyses.

OPTIMAL CHEMOTHERAPY REGIMEN DURING RADIOTHERAPY

One of the most criticized aspects of the Intergroup trial was the toxicity profile of the FU/LV regimen. Therefore, other investigators have sought alternative postoperative chemoradiation regimens. In a pilot study by Lee *et al.*^[43] patients with stage III-IV (M0) gastric cancer who had undergone extensive D2 LND were administered postoperative chemoradiation with fluorouracil and cisplatin before and after capecitabine and concurrent RT. A total dose of 4500 cGy in 25 fractions over five weeks was delivered to the target volume similar to the INT-0116 trial. This study demonstrated a 3-year disease free and OS of 82.7% and 83.4%, respectively, with the use of adjuvant chemoradiotherapy. Leong *et al.*^[44] reported that postoperative chemotherapy with

epirubicin, cisplatin, and 5-FU (ECF) before and after concurrent chemoradiation with infusional fluorouracil was tolerable and efficient. A similar regimen with ECF before and after radiation with infusional fluorouracil has been compared with the INT-0116 regimen in a randomized phase III trial (CALGB 80101)^[45]. Although the ECF regimen had a more favourable toxicity profile compared with bolus fluorouracil and leucovorin, there was no significant improvement in survival.

Alternative regimens for concomitant and adjuvant treatment have also been experienced. The efficacy of paclitaxel - cisplatin and 5-FU regimen against oesophageal and gastroesophageal junction adenocarcinomas has been demonstrated previously^[46]. Results of a phase I trial conducted among patients with locally advanced gastric cancer using weekly cisplatin and RT with paclitaxel as a 96-h continuous infusion were also promising^[47]. Another trial from MD Anderson included patients with gastric cancer who received two cycles of induction chemotherapy with infusional 5-FU, cisplatin on days 1 to 5, and paclitaxel over 24 h on day 1 of each 28-d cycle^[48]. During the 5-wk course of RT, infusional 5-FU and paclitaxel were administered weekly. Complete and partial response rates were 22% and 15%, respectively, which were quite promising. In the light of these findings, a phase II trial (RTOG-0114) was designed to integrate paclitaxel and cisplatin with or without 5-FU concomitantly through radiotherapy. However, the paclitaxel, cisplatin, and 5-FU (PCF) arm were closed due to the high gastrointestinal toxicity rates, which were significantly worse than INT0116 results^[49]. For the paclitaxel and cisplatin (PC) arm, the 2-year DFS was 52% (95%CI: 36%-68%). Although the PC arm was tolerable, the DFS failed to exceed the predefined lower bound of DFS at 2 years. Thus, this regimen could not be recommended as an adjuvant modality for future randomized phase III studies. These trials suggested that intensification of adjuvant chemotherapy or chemoradiation regimens may not be as effective as expected. Table 2 summarizes the major trials evaluating adjuvant CRT.

Whether an intensified CRT regimen prolonged survival after D2 dissection was another subject of debate. The ARTIST trial was designed to answer this question; comparing six cycles of capecitabine and cisplatin (XP) chemotherapy with two cycles of XP followed by concurrent capecitabine and RT followed by two additional cycles of XP after D2 dissection^[4]. However, the study failed to demonstrate a significant DFS benefit with the addition of radiotherapy to XP (3-year DFS rates 78.2 vs 74.2% for CRT and CT arms, respectively). Treatment was completed as planned by 75.4% of patients in the chemotherapy arm and 81.7% in the CRT arm. The updated analysis with longer follow-up did not reveal DFS or OS benefit either^[50]. However, the subgroup of patients with pathologic lymph node metastasis in the CRT arm had superior DFS when compared with those who received CT alone (3-year DFS; 76% vs 72%). Besides,

Table 2 Features of major adjuvant chemoradiotherapy trials for gastric cancer

Ref.	CT Regimen without RT/with RT	n (total)	D2 rates	G3-G4 toxicity (hem/GI)	Completeness of treatment	RT technique
Macdonald <i>et al</i> ^[6]	Bolus 5-FU + LV/bolus 5-FU + LV	556	10%	54%/33%	64%	2D
Lee <i>et al</i> ^[43]	FP/capecitabine	31	100%	50.2%/12.8%	74.20%	2D
Zhu <i>et al</i> ^[57]	Bolus 5-FU + LV/bolus 5-FU + LV	380	100%	5.9%/7.5%	NA	IMRT
Leong <i>et al</i> ^[44]	ECF/inf 5-FU	54	NA	66%/28%	NA	3D
Schwartz <i>et al</i> ^[49]	PC (PCF arm closed)/PC	78	NA	24%/33% (for PC arm)	NA	3D
Lee <i>et al</i> ^[4]	XP/capecitabine	458	100%	48.4%/19%	81.7%	3D

CT: Chemotherapy; RT: Radiotherapy; D2: D2 lymph node dissection; G3-G4: Grade 3-grade 4; hem: Hematologic; GI: Gastrointestinal; NA: Not available; 5-FU: 5-fluorouracil; LV: Leucovorin; inf: Infusional; FP: 5-FU, cisplatin; ECF: Epirubicin, cisplatin, 5-FU; PC: Paclitaxel, cisplatin; PCF: Paclitaxel, cisplatin, 5-FU; XP: Capecitabine, cisplatin; 2D: Two-dimensional; 3D: Three-dimensional; IMRT: Intensity modulated radiotherapy.

intestinal-type gastric cancer derived more benefit from CRT (3-year DFS rates were 83% and 94% in the CT and CRT arms, respectively).

The most commonly encountered nonhaematologic grade 3 to 4 side effects were stomatitis, hand and foot syndrome, diarrhoea, and vomiting, each of which occurred in 1% to 12% of patients in both arms. The rate of grade 3 and 4 neutropenia was 39% in the CT arm and 48% in the CRT arm; however, the rate of febrile neutropenia was quite low in both arms (< 1%), suggesting that postoperative treatment with cisplatin and capecitabine and is tolerable following a D2 LND. In conclusion, capecitabine at a dose of 1650 mg/m² per day with RT was well tolerated.

In the light of the studies mentioned above, combination regimens other than 5-FU/LV are still under investigation for gastric cancer. The NCCN guidelines, however, do not recommend the standard bolus 5FU/LV regimen utilized in the INT0116 trial. Relying on the data from gastric cancer trials, such as the ARTIST trial and colorectal cancer studies, capecitabine or infusional 5-FU is recommended concomitantly with RT by the NCCN due to their better toxicity profile and tolerability^[51].

RADIOTHERAPY TECHNIQUE

Since the publication of the INT0116 results, the radiotherapy technique and planning of the target volume has changed over time. Patients involved in this trial received 45 Gy of radiation in 25 fractions to the surgical bed, regional lymph nodes, and preoperative tumour volume. The regional lymph nodes included perigastric, splenic, hepatoduodenal, pancreatoduodenal, celiac, and local paraaortic lymph nodes based on patterns of failure after a D0/D1 dissection. In the Intergroup-0116 trial, two-dimensional (2D) radiation therapy was utilized and the CRT arm was associated with high rates of toxicity, with nearly three-quarters of patients experiencing grade 3/4 toxicities. Only 64% of patients in the CRT arm completed the planned treatment program, and 17% discontinued treatment due to toxicity. However, treatment-related mortality was low (1% on the chemoradiation arm vs 0% on the surgery alone arm). In addition, overall chemoradiation appeared tolerable.

Fortunately, technology has improved over time to

allow conformal radiation therapy, sparing normal tissues and allowing dose escalation. Three-dimensional (3D) conformal radiotherapy reduces the damage to normal tissues to some extent and is considerably superior to 2D radiation^[52]. Currently, modern 3D RT techniques are applied for the resected gastric cancer patients at most of the oncology centres in the world. 3D planning enables exact description of the target volume and organs at risk by visualization of anatomic changes in the internal organs after surgery^[53].

Whether or not to change the RT target volumes for patients undergoing D2 dissection is another subject of debate. The findings of the study by Chang *et al*^[54] have revealed that the most prevalent sites of regional recurrence after D2 dissection were the lymph nodes around the superior mesenteric vessels, the abdominal aorta from the upper margin of celiac trunk to the lower margin of aortic bifurcation, and the hepatoduodenal lymph nodes, which were primarily in the nodal basin outside the D2 dissection field. Consistent with these findings, the RT target volume in the ARTIST trial did not involve lymph nodes in the perigastric region and splenic hilum^[4]. The investigators have noted higher locoregional relapse rates in the CT arm (13% vs 7%, $P = 0.0033$) which supports the addition of CRT even in the presence of D2 dissection with the modified RT target volume.

Intensity modulated radiotherapy (IMRT) is a more sophisticated radiotherapy technique, with capability of delivering high doses of radiation to a targeted area with high geometrical accuracy. According to the recent studies, IMRT for gastric cancer is dosimetrically superior to conventional therapy, because IMRT is able to decrease the radiation dose to organs at risk, especially the spinal cord and kidney, while providing the intended radiation dose to the target areas^[55,56]. The most recent phase III trial comparing concomitant CT with IMRT and chemotherapy alone investigated the role of IMRT among gastric cancer patients with D2 LND^[57]. The IMRT plus CT arm was tolerable with a significant improvement in DFS (5-year DFS, 45% vs 36%); however, the results of this trial could not point at an OS benefit like the previous comparative studies (5-year OS, 24% vs 27%, $P > 0.05$). According to some investigators, IMRT appears to provide only limited advantages when compared with sophisticated 3D conformal RT planning^[58]. Moreover, the risk of a second cancer induced by radiation is reported

to increase in some patients^[59,60]. Whether 3D conformal RT or IMRT provides better protection of organs at risk remains controversial.

SELECTING PATIENTS FOR ADJUVANT CHEMORADIOOTHERAPY

Stage

Although the patients involved in the INT0116 trial were stage IB-IV (M0), the majority had advanced disease, whereas up to 60% of patients in the ARTIST trial were stage I / II. Furthermore, in the subgroup analysis of the ARTIST trial, improved DFS ($P < 0.05$) was observed in stage III and IV patients in the CRT group. The proportion of stage III/IV (M0) patients enrolled in the study by Zhu *et al.*^[57] was 71%, which demonstrated a DFS benefit for CT with IMRT after D2 dissection. The subset (node-positive) analyses of the ARTIST trial and the DFS advantage for stage III and IV (M0) patients in the Chinese trial supported that the use of adjuvant CRT for the whole stage IB to stage IV (M0) population may be overtreatment. Similarly, adjuvant CT alone may be inadequate for resected stage III-IV patients. Subgroup analysis of 5-year OS in the ACTS-GC trial from Japan showed an insufficient survival benefit of S1 for N3a and N3b stages (HR = 0.77, 95%CI: 0.53-1.13 and HR = 0.92, 95%CI: 0.47-1.79, respectively). The results indicated the necessity of adjuvant RT in these patients who were at high risk for locoregional relapse. Accordingly, in our study, which included D2 dissected pN3(M0) gastric cancer patients, the addition of RT to CT did not provide a statistically significant improvement in DFS or OS, but there was an evident difference between the CT and CRT arms numerically (median DFS 12.5 and 15.2 mo; median OS, 26.8 mo vs 34.2 mo for CT and CRT arms, respectively)^[61]. Another retrospective study from China including stage III gastric cancer patients with D2 dissection showed OS and DFS advantage for stage IIIC patients undergoing adjuvant CRT compared with CT^[62]. These studies indicate that patients with relatively advanced disease stages (III or IV) would benefit the most from adjuvant CRT.

The presence of lymphovascular invasion (LVI) or perineural invasion (PNI) have been demonstrated as significant prognostic factors for both OS and DFS among patients undergoing adjuvant treatment^[63]; however, thus far, there have not been any randomized trial data evaluating the administration of CT or CRT depending on the stage or presence of LVI or PNI, unlike for breast and colon cancers. However, the ARTIST II trial is on track to evaluate the efficacy of all available adjuvant treatment modalities after D2 dissection for node positive patients (clinicaltrials.gov NCT01761461); chemotherapy with S-1 for 1 year vs chemoradiotherapy involving two cycles of SOX followed by S-1/radiotherapy and then four additional cycles of (SOX) vs combination chemoradiotherapy with S-1 and oxaliplatin (SOX) for 6 mo. Patients were stratified according to stage, type of surgery, and the

Lauren classification.

Another phase III study is currently recruiting stage IB gastric cancer patients for evaluating adjuvant capecitabine vs observation (clinicaltrials.gov NCT01917552). The results of these trials are expected to answer the question regarding tailoring treatment to disease stages.

Histology and biomarkers

The main carcinogenic event for the evolution of diffuse type of gastric cancer is loss of expression of E-cadherin, a key cell surface protein for establishing intercellular connections. Biallelic inactivation of the gene encoding E-cadherin, CDH1, can occur through germline or somatic mutation, allelic imbalance events (e.g., loss of heterozygosity), or epigenetic silencing of gene transcription. Diffuse type cancers are highly metastatic and characterized by rapid disease progression and a poorer prognosis than intestinal cancers^[64]. Thus far, there has been no adjuvant therapy trial designed according to histological subtype. However, exploratory subgroup analysis of randomized trials point at varying degrees of benefit according to histologic subtype. The investigators of the INT0116 study reported their observation of a reduced treatment benefit in patients with diffuse histology in their updated analysis^[40]. Similarly, the patients with intestinal type gastric cancer were found to be more prone to benefit from CRT than those with diffuse type in subgroup analyses of the ARTIST trial^[50]. Patients with intestinal type histology showed a significant improvement in DFS in the CRT arm compared with the CT arm (94% vs 83%, $P = 0.01$, respectively). Whether or not this is a random observation of an unplanned subset analysis or reflective of the biologic variations is unknown, but if chemoradiotherapy is less effective in diffuse gastric cancer, future clinical trials may consider different adjuvant strategies based on histological subtype. A phase II/III study is currently recruiting patients with resectable signet-ring cell gastric carcinoma to perioperative treatment similar to MAGIC trial or surgery followed by six cycles of ECF (clinicaltrials.gov NCT01717924). This study may help determining the efficacy of intense CT with cisplatin for diffuse type gastric cancer cases.

In addition to morphologic appearance and clinical behaviour, the two distinct types of gastric adenocarcinoma differ with respect to their pathogenesis and genetic profiles^[65]. For the intestinal subtype, there is meticulous evidence for the role of *Helicobacter pylori* in the initiation of the events that lead from chronic active gastritis to atrophic gastritis, intestinal metaplasia, dysplasia, and finally adenocarcinoma. Many gene changes have been described in various stages of the preneoplastic/neoplastic cascade, but the alterations do not generally follow a sequential arrangement. Some changes are seen in early preneoplastic lesions but are not present in more advanced lesions. Therefore, it is not easy to develop an appropriate target for treatment.

Approximately 50% of intestinal-type gastric cancers have alterations in tumour suppressor genes, including

TP53, *TP73*, *APC*, *TFF*, *DCC*, and *FHIT*^[66]. In addition, epigenetic alterations, such as DNA methylation of gene promoters, can silence the expression of certain genes, including *CDH1* (the E-cadherin gene), in not only diffuse type but also in intestinal-type cancers^[67,68]. Unlike the complex molecular pathway for intestinal type, diffuse carcinomas display a discriminative molecular abnormality: Defective intercellular adhesions through the loss of expression of the cell adhesion protein E-cadherin as mentioned below. Although this knowledge has not resulted in a specific targeted therapy for diffuse gastric cancer yet, the recognition of germline *CDH1* mutations in families helps identify high-risk individuals and encourage them to receive prophylactic gastrectomy.

The struggle to identify specific biomarker for predicting a treatment benefit has not resulted in success in the adjuvant setting so far. In the ARTIST trial, the different status of the *EGFR*, *HER-2*, *MET*, *MLH1*, and *CDH1* genes were considered; however, differences in the expression of these genes between the CRT and CT groups had no effect on DFS^[50]. Inhibition of HER-2 overexpression *via* trastuzumab in metastatic disease has revealed a median of 2.7 in OS benefit in the ToGA trial, and currently this strategy is being evaluated in the adjuvant setting (clinicaltrials.gov NCT01130337, NCT01748773). Previously, the amplification of mesenchymal-epithelial transition (MET) receptor has been linked to poorer clinical outcome in patients with gastric cancer^[69]. There are some conflicting case reports on attempts to target MET in patients with gastric cancer^[69,70]. However, it seems feasible to wait until the results of the trial, which is testing a MET antibody in the metastatic setting, are reported (clinicaltrials.gov NCT01662869).

CONCLUSION

Currently, there is no doubt that adjuvant chemotherapy or chemoradiotherapy after resection of gastric cancer offers survival benefits. The major challenge for the clinicians is how and where to place the additional treatment modality (*i.e.*, CT or CRT; adjuvant or perioperative setting). The selection of the appropriate patient who will provide more or no benefit from therapy further complicates the situation. Obviously, there is lack of data to compare perioperative CT vs adjuvant CRT. However, the evidence for adjuvant CT with XELOX or S1 after D2 dissection is satisfactory. Although the evolution of the RT technique since the Intergroup study promises better tolerability, the addition of CRT after D2 dissection merits further investigation in the light of the findings from the ARTIST trial. Instead of the bolus 5-FU regimen or 5-FU combinations, infusional 5-FU or capecitabine concomitantly with RT may be preferred due to the improved toxicity profile. Nevertheless, the struggle to individualize treatment strategies for a robust combat with the resistant subgroups, such as the diffuse type of gastric cancer, should continue until optimal targets for therapy are defined.

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Multitarget stool DNA for colorectal cancer screening: A review and commentary on the United States Preventive Services Draft Guidelines

Barry M Berger, Bernard Levin, Robert J Hilsden

Barry M Berger, Medical Affairs, Exact Sciences Corporation, Madison, WI 53719, United States

Bernard Levin, Scientific Advisory Board, Exact Sciences, New York, NY 10025, United States

Robert J Hilsden, Departments of Medicine and Community Health Sciences, University of Calgary, Calgary AB T2N 4N1, Canada

Author contributions: Berger BM contributed to the writing and data analysis and coordinated the writing of the paper; Levin B and Hilsden RJ contributed to the writing and data analysis.

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Correspondence to: Barry M Berger, MD, Chief Medical Officer, Medical Affairs, Exact Sciences Corporation, 5801 Research Park Blvd, Madison, WI 53719, United States. bberger@exactsciences.com
Telephone: +1-608-5358553

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Abstract

Multitarget stool DNA (mt-sDNA) testing was approved for average risk colorectal cancer (CRC) screening by the United States Food and Drug Administration and thereafter reimbursed for use by the Medicare program (2014). The United States Preventive Services Task Force (USPSTF) October 2015 draft recommendation for CRC screening included mt-sDNA as an "alternative" screening test that "may be useful in select clinical circumstances", despite its very high sensitivity for early stage CRC. The evidence supporting mt-sDNA for routine screening use is robust. The clinical efficacy of mt-sDNA as measured by sensitivity, specificity, life-years gained (LYG), and CRC deaths averted is similar to or exceeds that of the other more specifically recommended screening options included in the draft document, especially those requiring annual testing adherence. In a population with primarily irregular screening participation, tests with the highest point sensitivity and reasonable specificity are more likely to favorably impact CRC related morbidity and mortality than those depending on annual adherence. This paper reviews the evidence supporting mt-sDNA for routine screening and demonstrates, using USPSTF's modeling data, that mt-sDNA at three-year intervals provides significant clinical net benefits and fewer complications per LYG than annual fecal immunochemical testing, high sensitivity guaiac based fecal occult blood testing and 10-year colonoscopy screening.

Key words: Colorectal cancer screening; Multitarget stool DNA; Stool DNA; The United States Preventive Services Task Force; Cancer Intervention Surveillance Modeling Network; Fecal immunological technique; Modeling; Interval

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Core tip: Multi-target stool DNA (mt-sDNA) testing was approved for average risk colorectal cancer (CRC) screening by the United States Food and Drug Administration (2014). The evidence supporting mt-sDNA for routine screening use is robust. The clinical efficacy of mt-sDNA every three years, measured by life-years gained, and CRC deaths averted, is similar to that of other screening strategies more specifically recommended by the United States Preventive Services Task Force. In an irregularly screened population, however, tests with the highest point sensitivity and reasonable specificity like mt-sDNA are more likely to reduce CRC related morbidity and mortality than less sensitive tests that depend on annual adherence to achieve high programmatic sensitivity.

Berger BM, Levin B, Hilsden RJ. Multitarget stool DNA for colorectal cancer screening: A review and commentary on the United States Preventive Services Draft Guidelines. *World J Gastrointest Oncol* 2016; 8(5): 450-458 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v8/i5/450.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v8.i5.450>

INTRODUCTION

In its October 5, 2015 draft recommendation statement for colorectal cancer (CRC) screening, the United States Preventive Services Task Force (USPSTF) includes multi-target stool DNA (mt-sDNA) as an "alternative" screening test that "may be useful in select clinical circumstances"^[1]. However, the evidence supporting mt-sDNA for routine screening use is robust. The clinical efficacy of mt-sDNA as modeled for life-years gained (LYG), and CRC deaths averted is similar to other options more specifically recommended in the draft statement. This paper reviews the evidence supporting mt-sDNA clinical validity^[2-4], analytical validity^[5-7], and the USPSTF CRC screening modeling^[8-10]. This body of evidence supports the use of mt-sDNA at three-year intervals (mt-sDNA3y) to provide significant clinical net benefits and fewer complications per LYG than annual fecal immunochemical testing (FIT), annual high sensitivity guaiac based fecal occult blood testing (hsFOBT), and screening colonoscopy every 10 years (Colo10y). In comparison to biennial or triennial FIT (FIT2y, FIT3y) or hsFOBT (hsFOBT2y, FSFOBT3y), both of which are adherence intervals more typically achieved in clinical practice^[11,12], only mt-sDNA3y is at or within 98% of the screening test efficiency frontier^[10] as measured by the ratio of LYG to colonoscopies generated. Additionally, mt-sDNA3y generates greater than 90% of the LYG by Colo10y in the simulation model of colorectal cancer (SimCRC)^[10].

CRC is the second leading cause of cancer death in the United States. In 2015, an estimated 133000

people will be diagnosed with the disease and about 50000 will die from it^[13]. When detected early, CRC can be treated with positive outcomes. The Centers for Disease Control and Prevention estimate that 42% of Americans are not currently up to date with colon cancer screening, including millions of Americans who have avoided screening completely, and that if everyone age 50 or older was regularly screened, at least 60% of CRC deaths could be avoided^[14]. These statistics leave much room for improvement in routine CRC screening; effective, broad implementation of a non-invasive high sensitivity, low risk screening strategies like mt-sDNA could immediately help to address this serious public health issue.

THE mt-sDNA TEST

mt-sDNA uses a single random stool sample, collected by patients at home, without requiring any preparation, or change in medications or diet. The test identifies 10 biomarkers known to be associated with CRC and pre-cancerous lesion, including altered human DNA and hemoglobin. The test combines all biomarker results with the normalizing gene beta-actin in an algorithm that generates a composite, single, "Negative" or "Positive" patient result. Several detailed reviews describing the biology that underlies this screening approach and the design features of the test have been published^[5-7]. The test was systematically designed to lower patient burdens with respect to ease of sample collection and specimen handling with a custom-designed collection kit. High CRC, high-grade dysplasia, and large adenoma point sensitivity and the prolonged pre-malignant phase of colorectal carcinogenesis allow for longer screening intervals between screening tests with mt-sDNA, which significantly lowers the burdens of annual testing. The American Cancer Society currently recommends mt-sDNA at three-year intervals^[15]. Burdens on patients, providers and health care systems are further reduced through an included United States 24 h, 7 d-a-week telephonic patient navigation system. This system assists patients throughout the testing process to maximize the number of successful screening events and reports results to directly to ordering medical providers.

The mt-sDNA analytic process and biomarker selection were used to create a test with greater sensitivity for CRC and significant premalignant lesions than fecal hemoglobin as a single marker. All tests based on fecal hemoglobin alone are biologically limited in their ability to detect colorectal neoplasia, especially precancerous lesions and early stage CRC, which may bleed intermittently or not at all. The random sampling and small sample size used in FIT and hsFOBT tests contribute to sampling error and further limits detectability. In contrast, mt-sDNA detects DNA alterations, both mutations and aberrant methylation, in DNA released from cells that are constantly shed from premalignant lesions and early and late stage cancers, enhancing their detectability by mt-sDNA. Stool sampling error with mt-sDNA is significantly diminished through the

use of an aliquot of the entire stool sample homogenate and the laboratory's automated, uniform processing protocol^[2].

mt-sDNA is primarily being used in the United States and has been approved by the United States Food and Drug Administration for average risk CRC screening^[16] and is reimbursed by the United States Centers for Medicare and Medicaid Services once every three years^[17]. It was awarded a unique Clinical Procedural Terminology code of 81528 by the American Medical Association. Cologuard[®] (multi-target sDNA) has been CE marked for use in Europe, though there is limited availability to date through laboratories in England and Dubai. Additional studies to determine local efficacy in a number of Asian countries are under discussion and studies are ongoing in Italy, the United Kingdom and the Netherlands. The test cost is USD649 (USD509 for Medicare), which includes a United States patient navigation/compliance system supporting over 70 languages.

In the United States, mt-sDNA is used in both opportunistic and local invitational settings. The test, requiring no change in diet, medication or any preparation and a specimen collection at home, is appropriate for population-based use. However, the size of the specimen container, allowable 72 h transit time back to the laboratory, and cost may mitigate use in that manner, especially in low resource countries. Countries with limited colonoscopy capacity for evaluating positive tests may be challenged by somewhat lower specificity (90%) of mt-sDNA compared to FIT, measured in patients requiring no biopsies when examined with colonoscopy, in any given year. However, overall, mt-sDNA results in fewer negative colonoscopic follow-up examinations for a positive screening test than result from the compounded programmatic specificity failure of annual FIT or hsFOBT at 95% specificity^[8].

mt-sDNA PERFORMANCE IN SCREENING POPULATIONS

Three studies in screening populations show consistent results. Studies demonstrate significant greater single test application sensitivity of mt-sDNA over FIT for advanced colorectal neoplasia detection, though at lower specificity.

DeeP-C study

Results from this pivotal, prospective 90-site, 10000 patient cross-sectional clinical study were published in the New England Journal of Medicine in April 2014^[2]. The DeeP-C study compared mt-sDNA and FIT, using colonoscopy as the reference standard on all cases. The study demonstrated that mt-sDNA was significantly more sensitive than FIT (Table 1) for detecting CRC, especially early stage CRC and advanced and non-advanced adenomas. Specificity in the target screening age of 50-74 years was 92.3% compared to 97.0% for FIT in patients where no biopsy was required during

colonoscopy. Matching FIT specificity to that of the mt-sDNA test only increases FIT sensitivity 2 percentage points. Thus the lower specificity of mt-sDNA did not account for the increased sensitivity of the mt-sDNA assay over FIT.

Alaska study: This prospective study of 661 Alaska native people compared mt-sDNA to FIT with colonoscopy as the reference, the same design as DeeP-C^[3]. In the overall study population ($n = 661$), which included both higher risk and average risk individuals for routine screening (screen subgroup) mt-sDNA detected 49% of advanced colorectal neoplasms (colorectal cancer plus advanced adenoma) vs 28% for FIT ($P < 0.001$), including the identification of 100% (10/10) colorectal cancers vs 80% (8/10) for FIT. In the screen sub-group ($n = 464$), mt-sDNA detected 50% advanced colorectal neoplasia vs 31% for FIT ($P = 0.01$), including the detection of 100% (4/4) of colorectal cancers vs 75% (3 of 4) for FIT. In subjects with no adenomas detected on colonoscopy, specificity was 93% for mt-sDNA vs 96% for FIT ($P = 0.034$). mt-sDNA may provide an attractive approach to provide high sensitivity screening for populations where routine travel for colonoscopy is challenging or where colonoscopy capacity itself is limited. Similarly, individuals participating only irregularly in screening care may accrue a greater benefit using a higher sensitivity tests when provided with an opportunity for screening.

Netherlands study: mt-sDNA was compared to FIT (OC Sensor) in prospectively collected frozen archived samples ($n = 1047$)^[4] from an invitational screening cohort (COCOS) collected in the Netherlands^[18]. The study compared the performance of mt-sDNA and FIT to colonoscopy on all subjects for the detection of advanced colorectal neoplasia. mt-sDNA detected 49% (50/102) and FIT 25% (26/102) of cases of advanced colorectal neoplasia ($P < 0.001$) at specificities of 89% and 96% respectively. The findings are consistent with those of the DeeP-C and Alaska studies.

PATIENT PREFERENCES AND TEST PERFORMANCE IN DIFFERENT POPULATIONS AFFECT SCREENING PROGRAM EFFICACY

The effectiveness of a test is a function of its performance, its availability, and patient adherence. The USPSTF noted in their draft statement that "clinicians should consider engaging patients in informed decision-making about the screening strategy that would most likely result in completion, with high adherence over time, taking into consideration both the patient's preferences and local availability"^[1]. Data has begun to accrue from studies that the mt-sDNA performance and process may appeal to previously unscreened patients

Table 1 Findings from the DeeP-C cross-sectional study^[5], comparing multitarget stool DNA with fecal immunological test using colonoscopy as the reference standard on all cases (*n* = 9989 subjects)

	Colonoscopy findings <i>n</i> detected	mt-sDNA % detected	FIT % detected
Sensitivity			
Colorectal cancer (stages I-IV)	65	92.3%	73.8%
Early stage colorectal cancer (stage I and II)	50	94.0%	70.0%
AA	757	42.4%	23.8%
High grade dysplasia	39	69.2%	46.2%
Sessile serrated adenoma/polyp ≥ 1.0 cm	99	42.4%	5.1%
Specificity			
Specificity (only CRC and AA excluded)	9167	86.6%	94.9%
Specificity, no adenomas, no biopsy done	4457	89.8%	96.4%
Age-adjusted (50-74 yr) ^[31]	4032	92.3%	97.0%

FIT: Fecal immunological test; AA: Advanced adenoma.

and lead to increased screening rates. A study by Berger *et al.*^[19] surveyed a random sample of almost 3000 average-risk patients (99% participation rate) who had been prescribed mt-sDNA (Cologuard) by their physician and found that 42% of the patients, aged 50-74 years had not been previously screened.

A second study by Cole *et al.*^[20] of 675 average-risk patients, aged 50-75 years who had never been screened for CRC showed that when patients were informed regarding screening alternatives they preferred the noninvasive, mt-sDNA option by more than 50% over colonoscopy or FIT. The study went on to note that educating patients about the noninvasive mt-sDNA option and involving the patient in the shared decision-making process about test choice can increase the likelihood that noncompliant patients will get screened.

A third study by Abola *et al.*^[21] of 423 individuals (617 invited, 69% participation rate) found that 75% considered mt-sDNA more suitable for screening than colonoscopy with no significant difference between Caucasian and African American respondents. The authors concluded that "intervention to increase the uptake of sDNA (mt-sDNA) testing may reduce racial disparities in CRC".

The availability of mt-sDNA allows patients who would be screened but who will not use colonoscopy for personal or cultural reasons or who reside in rural areas where there is less access to colonoscopy, to have a high sensitivity noninvasive test option. This is especially relevant for those who will not adhere to an annual screening regime.

Addressing disparities resulting from test access and patient preference are important. Studies show that multiple screening choices increase the overall level of screening and that patients will gravitate to their preferred option. Inadomi *et al.*^[11] showed that offering colonoscopy alone was less effective (38% screened) than offering a choice of colonoscopy or gFOBT screening (69% screened) for obtaining a successful screening event within 12 mo of a physician's recommendation for screening. A large prospective randomized screening study comparing FIT to colonoscopy, conducted in Spain, by Quintero *et al.*^[22], allowed post-randomization cross-

over. Approximately 23% (1706/7355) of patients randomized to colonoscopy crossed over to FIT, with 1.2% (117/9353) of patients randomized to FIT crossing over to colonoscopy. This demonstrates a preference for non-invasive testing in a significant number of subjects. If this occurred in a clinical situation outside of a clinical trial, 23% of patients would be selecting a screening approach with much less sensitivity and a more burdensome screening requirement. These issues could be significantly mitigated by using high sensitivity mt-sDNA as a routine non-invasive choice. Additional studies have shown that test preferences for non-invasive screening over invasive screening are most attractive to certain populations related to cultural preferences, a population area where high sensitivity non-invasive screening may also improve screening efficacy.

Finally, for patients who do choose non-invasive testing, sensitivity for CRC and its precursors in the proximal colon are key to screening efficacy. African American patients and elderly patients in general have an increased prevalence of proximal colorectal neoplasia^[23-25]. In contrast to FIT which is more sensitive for lesions in the distal colon, mt-sDNA has equivalent sensitivity for CRC in the proximal and distal colon. Importantly, and key for decreasing proximal CRC incidence when using noninvasive screening, mt-sDNA has significant sensitivity for sessile serrated adenoma/polyps, the pre-cursor lesion for approximately 25% of CRC and a common cause of missed or interval cancers arising in the proximal colon. In the DeeP-C study, mt-sDNA identified 42.4% of patients with sessile serrated adenomas ≥ 1 cm in diameter whereas FIT detected only 5.1% ($P < 0.001$) as these lesions are non-hemorrhagic whereas they exfoliate aberrantly methylated DNA in the stool^[2].

MODELING mt-sDNA PERFORMANCE AS AN APPROACH FOR SETTING AN INITIAL INTER-TEST INTERVAL

Establishing an initial inter-test interval using vetted, well designed and calibrated CRC screening models is recommended for new tests^[8]. Modeled intervals can be

tempered over time to accommodate accumulating clinical experience, patient preferences and medical delivery system impacts. Comparative prospective randomized longitudinal studies with mortality endpoints are large, complex, lengthy, and expensive. Currently, only screening with low sensitivity guaiac FOBT and flexible sigmoidoscopy are supported by prospective randomized control trials (RCT's) with mortality endpoints. The use of models allows for virtual prospective studies to be done on large cohorts. These provide comparative performance of multiple tests simultaneously. Such modeling and its limitations are described below.

The predictive power of modeling has limitations related to the degree that the biology of colorectal cancer, clinical practice related factors, test uptake, and performance assumptions accurately reflect the clinical screening "ecosystem". One concern is establishing a comparative baseline by only using 100% uptake and adherence for each screening test. The USPSTF technical report^[8] assumes 100% compliance and adherence for each strategy, despite strong evidence that initial uptake of FIT/FOBT in the United States is low (10.4%)^[26,27] and adherence for FIT and FOBT declines significantly over time^[28]. A recent review of a large cohort of patients continuously insured for ten years showed that of patients who were screened according to guidelines, only 0.3% (268/97518) were current with screening as a result of completing ten consecutive annual FIT/FOBT tests^[29]. Patients who were non-adherent with colonoscopy and non-adherent to annual test use completed an average of 2.6 FIT/FOBT during the 10-year study period. Forty-six percent completed only a single FOBT/FIT test during the 10-year study period. Ninety-nine point six percent (97801/97518) of patients who were current with screening had received a colonoscopy during the 10-year study period. In a three-year follow-up^[28] of the Inadomi study^[11] reported by Liang *et al.*^[28], the annual adherence for patients in the group assigned to FOBT screening fell from 67% the first year to 27% at year two, and to 14% by year three. In the group that chose FOBT over colonoscopy, adherence dropped from 38% in the first year to 19% at year two and to 12% at year three. In highly resourced integrated health system with patient navigation infrastructure, improved programmatic adherence with annual FIT has been shown, though initial uptake remains < 50%^[30]. Despite evidence of poor year-over-year adherence, the USPSTF continues to recommend routine screening with annual FIT, hsFOBT, or annual FIT with flexible sigmoidoscopy every 10 years, based on modeling 100% adherence while acknowledging that "In practice, such high adherence is not observed either for initial or repeat screening^[8]" and considers high sensitivity mt-sDNA an "alternative test" only for use in selected patients.

This USPSTF draft CRC screening recommendation^[1] is informed by the results of three independently-developed microsimulation models of CRC that are funded by the National Cancer Institute's Cancer Intervention and Surveillance Modeling Network (CISNET) - SimCRC,

microsimulation screening analysis (MISCAN) for CRC, and colorectal cancer simulated population model for incidence and natural history. The performance of the various CRC screening tests were evaluated using these models to predict LYG, decreases in CRC incidence, CRC related mortality, number of screening tests required, and complications arising from screening^[8].

For comparative purposes, CISNET assumed 100% perfect adherence to all screening and surveillance procedures and performed no sensitivity analysis around adherence that would more accurately reflect actual test use. Further, their analysis grouped FIT, gFOBT, and mt-sDNA together because they are "exclusively stool-based screening modalities with comparable burden"^[8]. However, mt-sDNA has higher single-event CRC, and advanced adenoma sensitivity, including sensitivity for sessile serrated adenomas. It has significantly lower patient burdens given the need for far fewer test events with the recommended three-year screening schedule, a specifically designed patient collection process to minimize sample handling, and an embedded patient navigation support system. Based on the lower patient burden and the great biological differences in the test approach, mt-sDNA could have been considered in its own category for interval effect analysis, similar to all other non-stool based strategies. At the least, mt-sDNA3y could have been grouped with FIT2y and FIT 3y and hsFOBT2y and hsFOBT3y as a more clinically representative grouping for evaluation. This is an important consideration as, under CISNET modeling rules, only one strategy per "group" could ultimately be "recommended" for routine screening^[8].

This grouping and arbitrary rule led to the finding that "annual mt-sDNA" was less efficient with respect to the number of colonoscopies generated per LYG than annual FIT and hsFOBT and precluded a consideration of the multi-year interval (3 years) for which the test is already recommended by others^[9,15]. According to the draft recommendation statement, the CISNET modeling of mt-sDNA at a one-year interval (mt-sDNA1y) would "potentially yield approximately the same number of life-years gained as the recommended strategies previously listed" but when "compared with other stool-based screening tests and screening with colonoscopy every 10 years, FIT-DNA (mt-sDNA) requires a larger number of lifetime colonoscopies (a proxy for the harms of screening) per LYG"^[1]. When calculating lifetime colonoscopies using the intervals for each screening test as recommended by the American Cancer Society (Table 2)^[15], the data shows that mt-sDNA3y has the fewest lifetime colonoscopies (COL) and an equivalent number of colonoscopies per LYG compared to FIT and hsFOBT at one-year intervals (FIT1y and hsFOBT1y) (Table 3)^[8]. Colonoscopy itself generates approximately twice as many colonoscopies per LYG as any of the non-invasive strategies.

Overall, mt-sDNA3y is associated with less burdens and harms than FIT1y and gFOBT1y. In clinical practice the comparative benefits of mt-sDNA may be even greater given the lack of adherence to annual FIT or

Table 2 American Cancer Society recommended colorectal cancer screening test frequency intervals for average risk individuals

Test	Frequency (yr)
Colonoscopy	10
CT colonography	5
Flexible sigmoidoscopy	5
Multi-target stool DNA test (Cologuard, mt-sDNA)	3
High sensitivity guaiac-based fecal occult blood test	1
Fecal immunochemical test	1

CT: Computed tomography.

hsFOBT screening^[11,28-30]. A comparison reflecting actual clinical practice experience would have included a comparison of mt-sDNA3y with FIT/FOBT at 2y and 3y, which is detailed below.

A sensitivity analysis exploring non-annual adherence demonstrated more clinically relevant benefits and harms for stool-based strategies. The CISNET modeling data on FIT and hsFOBT at two-year intervals^[8] (FIT2y and hsFOBT2y) and mt-sDNA3y (Table 4), show mt-sDNA3y to be the only strategy generating greater than 90% LYG by screening colonoscopy 10y (% of COL 10y LYG) (SimCRC) in any of the models. While the colonoscopies per LYG are similar for hsFOBT and somewhat lower for FIT2y, overall LYG, CRC incidence, and related deaths are notably lower and more lives will be saved with mt-sDNA3y than with either FIT2y or gFOBT2y^[8].

The CISNET modeling data on FIT and gFOBT at three-year intervals^[8] (FIT3y, hsFOBT3y) reflects a second scenario supported by clinical experience^[8-10]. Compared to FIT3y and hsFOBT3y, mt-sDNA benefits are notably better with 19-22 CRC deaths averted, 43%-68% CRC incidence reduction, and 68%-78% mortality reduction across the three models (Table 5)^[8]. At three-year intervals, FIT and hsFOBT generate only 68%-77% of the life years gained by Colo10y vs 84%-91% for mtsDNA3^[8,10].

BALANCING BENEFITS AND HARMS

The specific harms associated with the non-invasive testing process are held to be minimal. Paradoxically, the USPSTF^[1] uses colonoscopies as a proxy for harms for the non-colonoscopy screening tests, but not for colonoscopy based screening itself. If colonoscopy related harm is a greater concern than screening benefit, especially where differences in the balance of harms and benefits is very small among non-invasive tests, mt-sDNA3y appears favorable when compared to Colo10y. mt-sDNA3y generates far fewer colonoscopies per 1000 people screened (1701-1827) across the three CISNET models^[8] than Colo10y (4007-4101) or annual hsFOBT (2230-2287) and similar numbers to annual FIT (1739-1899) (Table 3)^[8].

There are no direct harms or complications from mt-sDNA beyond those associated with a follow-up colonoscopy for a positive mt-sDNA screening test. No additional investigation is indicated for a positive mt-

sDNA test outside a careful structural examination of the colon in a well prepared patient, generally by optical colonoscopy. The aggregate contribution of other cancers of the aerodigestive tract and inflammatory diseases to the mt-sDNA false positive rate is two cases per 10000 screened patients, precluding the need for additional studies in an otherwise asymptomatic patient on the basis of a positive mt-sDNA test alone^[31]. Like all tests, mt-sDNA may be associated with false positive results and false negative results, wherein advanced colorectal neoplasia is not identified on a single screening event. Colonoscopy, however, may be associated, though rarely, with significant adverse events^[31].

The USPSTF technical report calculated complications for all model outputs. These complications are based on the serious adverse event rates summarized in the USPSTF evidence synthesis^[25] and are dependent on patient age and type of lesion removed. Table 3 shows the complications per 1000 patients screened, the LYG, the CRC deaths averted and screening related complications across the three models. mt-sDNA3y has the lowest rate of complications per LYG (0.036-0.044) vs annual FIT (0.038-0.045), annual hsFOBT (0.042-0.047) or colo10y (0.051-0.060). With respect to complications per death averted, mtsDNA3y (0.41-0.50) outperforms colonoscopy (0.58-0.68) and in two of three models, annual FIT (0.43-0.50) and annual hsFOBT (0.48-0.55)^[8].

Finally, the total number of screening tests required itself is an indicator burden. Fewer stool tests and clinical encounters are required for mt-sDNA3y than with FIT1-3y or hsFOBT1-3y (Tables 3-5). mt-sDNA3y provides significantly fewer burdens on patients, physicians, and healthcare systems than other fecal tests. Notably, this factor was not accounted for as a "burden" in the USPSTF analysis^[8].

mt-sDNA CLINICAL UTILITY CAN BE INFERRED FROM PREVIOUS RCT'S OF FOBT

The clinical utility of mt-sDNA3y with respect to reducing both CRC related mortality and CRC incidence can be inferred from previous RCT's of annual and biennial screening with the less sensitive FOBT test. No CRC screening test recommended by the USPSTF has been shown empirically to decrease CRC related mortality^[25]. Only low sensitivity guaiac based FOBT (gFOBT, *e.g.*, Hemoccult II), used annually or biennially, and flexible sigmoidoscopy alone have been shown to decrease CRC mortality in well-designed RCTs, but these are no longer widely used in the United States for screening, nor recommended by the USPSTF^[1,8,25]. However, the USPSTF infers decreases in CRC related mortality and the CRC incidence for both FIT and hsFOBT from the mortality benefit demonstrated for Hemoccult II gFOBT in these RCT's^[8]. These benefits can also be applied to mt-sDNA similarly through the same logical inference, as given mt-sDNA's superior sensitivity over FIT (Table 1) and by

Table 3 Burdens, harms, benefits, and efficiencies for 100% perfect adherence for colorectal cancer screening tests at current recommended intervals, ages 50-75, per 1000 people screened

CISNET model	Test	Burdens and harms			Benefits							
		Stool tests	Total COL	Complications	LYG	CRC DA	CRC incidence reduction	CRC mortality reduction	% of COL 10y LYG	COL per LYG	Complications per LYG	Complications per DA
SimCRC	COL 10y	0	4007	14	275	24	81%	87%	100%	15	0.051	0.58
	FIT1y	15778	1739	10	260	23	67%	81%	95%	7	0.038	0.43
	hsFOBT1y	12914	2230	11	261	23	69%	82%	95%	9	0.042	0.48
	mt-sDNA3y	5990	1701	9	250	22	63%	78%	91%	7	0.036	0.41
MISCAN	COL 10y	0	4101	15	248	22	62%	79%	100%	17	0.060	0.68
	FIT1y	15843	1757	10	231	20	47%	72%	93%	8	0.043	0.50
	hsFOBT1y	12927	2287	11	232	20	49%	73%	94%	10	0.047	0.55
	mt-sDNA3y	5779	1714	9	215	19	43%	68%	87%	8	0.042	0.47
CRC-SPIN	COL 10y	0	4049	15	270	24	88%	90%	100%	15	0.056	0.62
	FIT1y	15444	1899	11	244	22	72%	81%	90%	8	0.045	0.50
	hsFOBT1y	13026	2253	11	247	22	75%	82%	92%	9	0.045	0.50
	mt-sDNA3y	5927	1827	10	226	20	68%	76%	84%	8	0.044	0.50

CISNET: National Cancer Institute's Cancer Intervention and Surveillance Modeling Network; SimCRC: Simulation model of colorectal cancer; MISCAN: Microsimulation screening analysis; CRC-SPIN: Colorectal cancer simulated population model for incidence and natural history; COL: Colonoscopies; LYG: Life-years gained; DA: Deaths averted; COL 10y: Colonoscopy at a 10-year interval.

Table 4 Burdens, harms, benefits, and efficiencies at 2-year adherence rates for fecal immunological technique/fecal occult blood testing compared to recommended intervals for colonoscopy and mt-sDNA, ages 50-75, per 1000 people screened

CISNET model	Test	Burdens and harms			Benefits							
		Stool tests	Total COL	Complications	LYG	CRC DA	CRC incidence reduction	CRC mortality reduction	% of COL 10y LYG	COL per LYG	Complications per LYG	Complications per DA
SimCRC	COL 10y	0	4007	14	275	24	81%	87%	100%	15	0.051	0.58
	FIT2y	9326	1215	7	234	20	53%	72%	85%	5	0.030	0.35
	hsFOBT2y	8388	1597	9	235	21	56%	73%	86%	7	0.038	0.43
	mt-sDNA3y	5990	1701	9	250	22	63%	78%	91%	7	0.036	0.41
MISCAN	COL 10y	0	4101	15	248	22	62%	79%	100%	17	0.060	0.68
	FIT2y	9342	1243	8	200	17	35%	62%	81%	6	0.040	0.47
	hsFOBT2y	8408	1636	9	200	18	37%	63%	81%	8	0.045	0.50
	mt-sDNA3y	5779	1714	9	215	19	43%	68%	87%	8	0.042	0.47
CRC-SPIN	COL 10y	0	4049	15	270	24	88%	90%	100%	15	0.056	0.62
	FIT2y	9241	1346	9	207	18	58%	68%	77%	6	0.043	0.50
	hsFOBT2y	8448	1626	9	212	19	62%	70%	78%	8	0.042	0.47
	mt-sDNA3y	5927	1827	10	226	20	68%	76%	84%	8	0.044	0.50

CISNET: National Cancer Institute's Cancer Intervention and Surveillance Modeling Network; CRC: Colorectal cancer; SimCRC: Simulation model of CRC; MISCAN: Microsimulation screening analysis; CRC-SPIN: Colorectal cancer simulated population model for incidence and natural history; COL: Colonoscopies; LYG: Life-years gained; DA: Deaths averted; COL 10y: Colonoscopy at a 10-year interval; FIT: Fecal immunological test.

inference, superiority to low sensitivity gFOBT^[2]. CISNET modeling supports the proposition that mt-sDNA3y is more efficient^[10] and efficacious than hsFOBT2y and therefore similar clinical utility can be ascribed to mt-sDNA3y as the four RCTs of low sensitivity gFOBT2y provide for the clinical utility of hsFOBT2y^[8]. Using comparable clinical efficiency^[10] to allow clinical utility to be inferred from the RCTs of biennial FOBT obviates concern around small differences in specificity between the tests. In practical terms, even if patient uptake of mtsDNA is the same as that of FIT or hsFOBT, patients are more likely to see a greater net benefit from higher sensitivity mt-sDNA screening than from intermittent FIT/FOBT use, especially given the low rate of serious colonoscopy related complications.

CONCLUSION

CRC is the second leading cause of cancer mortality in

the United States with nearly 50000 deaths per year. mt-sDNA provides a colorectal cancer screening test with high sensitivity for the detection of CRC and the most significant pre-malignant lesions in a non-invasive format. It is approved as safe and effective for routine screening of asymptomatic individuals by the United States FDA, CE marked in Europe, and vetted and approved for coverage by the United States Centers of Medicare and Medicaid Services at three year intervals. mt-sDNA at three-year intervals is included in the American Cancer Society guidelines. Clinical experience demonstrates that patients formerly non-compliant with screening, ages 50-74, comprise a significant proportion (42%) of mt-sDNA users, which is consistent with patient screening preference studies.

Multiple studies support the superior point sensitivity of mt-sDNA over FIT, an important attribute of a screening test with limited harms that appeals to patients hesitant to pursue screening by other methods. The data

Table 5 Burdens, harms, benefits, and efficiencies for fecal immunological technique and fecal occult blood testing at 3-year adherence rates compared to recommended intervals for colonoscopy and mt-sDNA, ages 50-75, per 1000 people screened

CISNET model	Test	Burdens and harms			Benefits							
		Stool tests	Total COL	Complications	LYG	CRC DA	CRC incidence reduction	CRC mortality reduction	% of COL 10y LYG	COL per LYG	Complications per LYG	Complications per DA
SimCRC	COL 10y	0	4007	14	275	24	81%	87%	100%	15	0.051	0.58
	FIT3y	6887	971	6	212	18	45%	65%	77%	5	0.028	0.33
	hsFOBT3y	6456	1286	7	212	18	47%	66%	77%	6	0.033	0.39
	mt-sDNA3y	5990	1701	9	250	22	63%	78%	91%	7	0.036	0.41
MISCAN	COL 10y	0	4101	15	248	22	62%	79%	100%	17	0.060	0.68
	FIT3y	6795	995	7	176	15	28%	55%	71%	6	0.040	0.47
	hsFOBT3y	6302	1296	8	175	15	30%	55%	71%	7	0.046	0.53
	mt-sDNA3y	5779	1714	9	215	19	43%	68%	87%	8	0.042	0.49
CRC-SPIN	COL 10y	0	4049	15	270	24	88%	90%	100%	15	0.056	0.62
	FIT3y	6857	1081	7	178	16	49%	59%	66%	6	0.039	0.44
	hsFOBT3y	6498	1317	8	183	16	53%	61%	68%	7	0.044	0.50
	mt-sDNA3y	5927	1827	10	226	20	68%	76%	84%	8	0.044	0.50

CISNET: National Cancer Institute's Cancer Intervention and Surveillance Modeling Network; COL: Colonoscopies; COL 10y: Colonoscopy at a 10-year interval; LYG: Life-years gained; CRC: Colorectal cancer; MISCAN: Microsimulation screening analysis; CRC-SPIN: Colorectal cancer simulated population model for incidence and natural history; SimCRC: Simulation model of colorectal cancer; DA: Deaths averted; FIT: Fecal immunological test.

provided by the USPSTF technical report^[8] from three separate models supports the efficacy of mt-sDNA3y and demonstrates across 1000 screened individuals, age 50-74, that it yields a median of 226 life-years gained (range 215-250), averts 20 CRC deaths (range 19-22), reduces CRC mortality by 76% (range 68%-78%, and produces the most benefit (LYG) per complication (harm). The three CISNET models demonstrate that in terms of the number of colonoscopies per LYG, mt-sDNA3y (7-8) is equivalent to annual FIT (7-8) and lower than hsFOBT (9-10)^[8].

The USPSTF draft recommendation states "Screening for CRC is a substantially underused preventive health strategy in the United States... Accordingly, the best screening test is the one that gets done" and that maximizing the total proportion of the eligible population that receives screening will "result in the greatest reduction in deaths due to CRC"^[1]. As such, the clinical and modeling evidence and societal need for improved noninvasive screening strategies support a USPSTF recommendation for mt-sDNA for routine screening at three-year intervals, a conclusion consistent with the recommendations of others.

Failure to include a clear recommendation of mt-sDNA3y in the final USPSTF guideline may limit access to mt-sDNA for Americans not covered by Medicare. By only recommending the same tests as were recommended in 2008, the USPSTF draft recommendation limits significant progress in improving United States screening rates^[13,14] by affirming the current approaches only. In order to increase the screening rate, we must offer more efficacious choices. mt-sDNA3y for routine screening provides an opportunity to expand the pool of screened patients and to increase the quality of screening among those choosing non-invasive approaches.

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Urinary metabolites as noninvasive biomarkers of gastrointestinal diseases: A clinical review

Irene Sarosiek, Rudolf Schicho, Pedro Blandon, Mohammad Bashashati

Irene Sarosiek, Mohammad Bashashati, Division of Gastroenterology, Department of Internal Medicine, Texas Tech University Health Sciences Center, El Paso, TX 79905, United States

Rudolf Schicho, Institute of Experimental and Clinical Pharmacology, Medical University of Graz, 8010 Graz, Austria

Pedro Blandon, Division of Nephrology, Department of Internal Medicine, Texas Tech University Health Sciences Center, El Paso, TX 79905, United States

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Correspondence to: Mohammad Bashashati, MD, Research Scientist, Division of Gastroenterology, Department of Internal Medicine, Texas Tech University Health Sciences Center, 4800 Alberta Ave, El Paso, TX 79905, United States. bashashati.md@gmail.com
Telephone: +1-915-2155148
Fax: +1-915-5456210

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Abstract

The diagnosis of gastrointestinal (GI) disorders is usually based on invasive techniques such as endoscopy. A key important factor in GI cancer is early diagnosis which warrants development of non- or less-invasive diagnostic techniques. In addition, monitoring and surveillance are other important parts in the management of GI diseases. Metabolomics studies with nuclear magnetic resonance and mass spectrometry can measure the concentration of more than 3000 chemical compounds in the urine providing possible chemical signature in different diseases and during health. In this review, we discuss the urinary metabolomics signature of different GI diseases including GI cancer and elaborate on how these biomarkers could be used for the classification, early diagnosis and the monitoring of the patients. Moreover, we discuss future directions of this still evolving field of research.

Key words: Metabolomics; Gastrointestinal diseases; Cancer; Inflammatory bowel disease; Metabolome; Urine

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Core tip: Scientists are always searching for new disease biomarkers. An acceptable biomarker could help us in early diagnosis and classification of the diseases as well as the prediction of disease outcome. The diagnosis of gastrointestinal (GI) diseases is usually based on techniques such as upper or lower GI endoscopy, while highly sensitive and specific non-invasive diagnostic or screening tools are usually lacking. In this review, we have discussed the potentials of urinary metabolomics study as a future tool for the screening, diagnosis, classification and surveillance of GI diseases including inflammatory bowel disease and cancer.

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INTRODUCTION

The rapid growth of high-quantity technologies and computational contexts allows the analysis of organic systems in distinctive details. New technologies such as DNA sequencing and mass spectrometry have permitted observing thousands of molecules concurrently instead of a few components that have been analyzed in old-fashioned research^[1].

By considering epigenetic ruling and posttranslational alterations, metabolites serve as direct signatures of biochemical activity in biological systems. Moreover, beyond genes and proteins, they are usually in direct association with disease phenotypes^[2]. Metabolomics or metabolic profiling is based on comprehensive and rapid analysis of thousands of metabolites simultaneously in biological samples including plasma and urine and is a feasible strategy for biomarker discovery^[3].

The routine urine analysis is often used for the diagnosis of diseases in the urinary tract. However, more than 3000 metabolites are detectable in the urine and their levels may be used as the signature of systemic diseases^[4]. These signatures are affected by energy and nutrient intake, body and cellular metabolisms and the environmental factors such as microbiota which have close cross-talk with the gastrointestinal (GI) system. Therefore, any disease in the GI tract may change the metabolic profile of the body that can be reflected in the bodily fluids including blood and urine.

This review provides an insight to the urinary metabolic profile of the GI diseases and its potential application in the clinical diagnosis and predicting their clinical as well as treatment outcome.

TECHNIQUES AND EVALUATION OF URINARY METABOLOMES

Assessment of some GI diseases requires the use of endoscopic methods which are not without risks. Determination of disease biomarkers in easily obtainable biofluids like urine, therefore, would be a valuable adjunct or even an alternative to conventional methods. Many serological markers for inflammatory bowel diseases (IBD) already exist, however, they are less helpful in determining disease subtypes (*i.e.*, Crohn's disease and ulcerative colitis) or forms of indeterminate colitis^[5]. Biomarkers or biomarker profiles that can predict and discriminate these subtypes with high probability are therefore desirable. Various studies pursuing this goal have been performed in the past couple of years and

have increased the list of metabolites found in higher or lower concentrations in body fluids, including urine, during IBD^[6]. These metabolites have been measured in IBD patients by highly sensitive techniques, for instance, by ¹H nuclear magnetic resonance (NMR) spectroscopy^[7-9], ion cyclotron resonance-Fourier transform mass spectrometry^[10] and by ultra-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry (MS) in rodents with experimental colitis^[11]. While the latter techniques are characterized as extremely sensitive, ¹H NMR spectroscopy is maybe less sensitive but known to produce highly reproducible results. A recent study compared different techniques for the detection of urine metabolites in humans and concluded that the NMR technique is the best method for identifying and quantifying urinary compounds^[4].

For the discrimination of IBD subtypes and the determination of severity and progress of GI diseases, many metabolites need to be identified. To organize and correctly interpret the large number of data, statistical methods, like multivariate analysis (*e.g.*, principal component analysis and orthogonal partial least squares projections) are applied. In the case of NMR, a method called "targeted or quantitative metabolic profiling" has been used to detect new possible sets of biomarkers^[12]. Here, the spectra of already characterized metabolites are stored in a database, and spectra measured in a new biofluid sample are compared with those from the database and thus identified and quantified. The determined metabolites not only may have importance as potential biomarkers but they can, at the same time, provide a link to the pathophysiology of the disease. In this respect, knowledge on the role of the determined molecule within the metabolic pathway is important. A urine metabolome database that allows researchers access to the types, structures and concentrations of urinary metabolites in different diseases has been therefore introduced by the Metabolomics Innovation Centre (<http://www.metabolomicscentre.ca/>; a platform hosted by the University of Alberta, Canada)^[4].

Taken together, urinary metabolites can be evaluated in GI diseases by different experimental methods of high or low sensitivity. Irrespective of the method used, detection of a unique metabolic fingerprint either for diagnosis, treatment, or detection of disease mechanisms is the primary goal.

URINARY METABOLOMICS IN IBD

IBD affecting over 1 million individuals in the United States and 2.5 million in Europe is a common chronic gastrointestinal disease with substantial costs for health-care. Moreover, it is estimated that the absolute number of IBD patients in newly industrialized countries may approximate that in the Western world until 2025^[13]. Despite this increase in the burden of IBD, gold standard tests for its diagnosis, monitoring and management are

usually invasive and sometimes inconclusive. Therefore, biomarkers including noninvasive methods such as urinary metabolomics studies might be useful for the management of patients with IBD^[14,15].

Urinary metabolomics has been studied in different mouse models of IBD. Interleukin-10 (*IL-10*) gene-deficient mice which are genetically susceptible to inflammation and colitis have shown different urinary metabolomics compared to non-inflamed animals. For instance, Murdoch *et al.*^[16] showed that several urinary metabolites such as trimethylamine (TMA) and fucose are changed dramatically in the *IL-10* gene-deficient mice after 8 wk of age which is the timeline for development of severe histological injury and colitis. These alterations in the metabolomics are majorly mediated by commensal microflora which play a key role in the disease process.

In another study on *IL-10* gene deficient mice, Lin *et al.*^[17] showed an association between 15 metabolites including fucose, xanthurenic acid, and 5-aminovaleric acid with intestinal inflammation. Elevated urinary xanthurenic acid in gene deficient mice was linked to increased plasma levels of kynurenine^[17]. In a further study, the same group validated these findings by showing that feeding *IL-10* gene-deficient and wild-type mice with Kiwifruit increases Kiwifruit-derived urinary metabolites more significantly in *IL-10* gene-deficient mice compared to wild-type mice without affecting urinary metabolites levels previously associated with inflammation^[18].

In another study, Otter *et al.*^[19] showed association between the concentrations of xanthurenic acid, α -CEHC glucuronide, and an unidentified metabolite m/z 495(-)/497(+) with inflammation in *IL-10* gene deficient mice.

Overall, studies on *IL-10* gene deficient mice generally agree with changes in urinary xanthurenic acid, a product of tryptophan catabolism through the kynurenine pathway. Bacterial lipopolysaccharides and pro-inflammatory cytokines are the activators of this pathway and its metabolites act as the moderators of T-cell tolerance to intestinal microbiota. As colitis does not usually develop in germ-free *IL-10* gene deficient mice, the role of intestinal microbiota looks considerable in the induction of urinary metabolomics alterations during colitis^[17,19-21].

Although, overall studies indicated that *IL-10* gene deficient mice have different urinary metabolomics profile compared to wild-type mice, Tso *et al.*^[22] showed that these differences are gender and age specific.

Schicho *et al.*^[23] expanded metabolomics study to an acquired model of chemical colitis induced by dextran sodium sulfate (DSS). After studying 69 urinary metabolites, they showed that urinary creatine, carnitine, and methylamines (including TMA and TMAO) were increased whereas antioxidant metabolites were decreased in DSS mice.

Another study on trinitrobenzene sulphonic acid-induced acute colitis in rats indicated that urinary tryptophan metabolites [4-(2-aminophenyl)-2,4-dioxobutanoic acid and 4,6-dihydroxyquinoline], gut microbial metabolites (phenyl-acetyl-glycine and p-cresol glucuronide), and the

bile acid 12 α -hydroxy-3-oxocholadienic acid which are associated with damage of the intestinal barrier function, microbiota homeostasis, immune modulation and the inflammatory response are altered during experimental colitis^[11].

Moreover, in a naïve T cell adoptive transfer experimental model of colitis, Martin *et al.*^[24] showed decrease in Krebs cycle intermediates in urine (succinate, α -ketoglutarate) indicating reduction in the glutaminolytic pathway related to overall loss of energy homeostasis during colitis.

Besides studies on animal models of colitis, studies on IBD patients have confirmed the diagnostic potentials of urinary metabolomics. By studying 206 Caucasian subjects [86 Crohn's disease (CD) patients, 60 ulcerative colitis (UC) patients, and 60 healthy controls], Williams *et al.*^[9] showed that urinary metabolites, which were in correlation with intestinal microbiota, were different in IBD patients compared to controls. In brief, urinary hippurate differed significantly between the three groups with the lowest level in CD patients. Moreover, 4-cresol sulfate levels were lower and formate levels were higher in CD patients compared to UC patients or controls. This study could significantly differentiate CD from UC^[9].

Another study compared the urinary metabolomic signature of patients with active UC, quiescent UC, and controls. In this study no significant difference in the urinary metabolomics profile of these 3 groups was observed^[8]. On the other hand, based on a recent study, a significant partial least squares discriminant analysis model was obtained through measuring urinary metabolomics in patients with active IBD vs a group with IBD in remission. Based on this study, glycine was increased in urine and acetoacetate decreased in urine during active IBD. Moreover, in active IBD, urinary citrate, hippurate, trigonelline, taurine, succinate and 2-hydroxyisobutyrate were decreased compared to the controls. Despite mentioned observations, this study could not clearly differentiate CD and UC patients based on the analysis of urine samples. Interestingly, contrary to the serum samples, up-regulation of acetoacetate and down-regulation of citrate, hippurate, taurine, succinate, glycine, alanine and formate in the urine samples of patients with IBD in remission could distinguish them from healthy controls^[25].

Another study showed that urinary metabolomics including tricarboxylic acid (TCA) cycle intermediates, amino acids, and gut microflora metabolites are different in patients with IBD compared to healthy controls. Comparison of CD and UC patients revealed different metabolomics fingerprints, but removal of patients with the surgical intervention revealed that CD could not be differentiated from UC^[26].

Schicho *et al.*^[23] expanded their findings in DSS mice by studying human subjects with IBD. Their study showed an increase in mannitol, allantoin, xylose, and carnitine in the urine and a decrease in urinary betaine and hippurate during IBD. However, the same as above mentioned studies^[25,26], they could not differentiate CD

and UC based on their metabolomics profile^[7].

Putting together, based on the metabolomics studies in IBD, the absolute urinary metabolomics signature of IBD is not yet clear. However, the body of literature supports the diagnostic role of urinary metabolites in IBD. More specifically, it seems that microbiota derivative metabolites are altered in IBD and are involved in the pathophysiology of this chronic inflammatory condition. Future multicenter studies on larger sample sizes and with considering confounders such as age, gender and medications should clarify whether urinary metabolomics could be used to: (1) Differentiate UC from CD; (2) predict outcome of the treatments; and (3) define the stage and severity of inflammation.

URINARY METABOLOMICS IN GI CANCERS

GI cancers are common and their burden is huge. Based on a global study in 2013, colorectal, stomach and esophageal cancer are ranked third, fifth and ninth for cancer incidence and fourth, second and sixth for cancer deaths, respectively^[27].

Despite available screening method for colorectal cancer which are usually costly and invasive, screening tests for upper GI cancers have not been well developed. Early detection of cancer or pre-cancerous lesions is always desirable. This could benefit from a urine-based cost-effective diagnosis and noninvasive screening assay whereby patients with undiagnosed cancer could be screened.

By analyzing urine samples from esophageal cancer patients and a control healthy group, Hasim *et al.*^[28] showed that mannitol, glutamate, γ -propanine, phenylalanine, acetate, allantoin, pyruvate, tyrosine, β -glucose and guinolate were higher in the urine of patients with esophageal cancer; however, N-acetylcysteine, valine, dihydrothymine, hippurate, methylguanidine, 1-methylnicotin- amide and citric acid were lower. Based on this study, urinary metabolomics could differentiate cancer and control groups. In addition, different pattern of metabolites were positively correlated with the rate of lymph node metastasis and clinical stages. Moreover, unsaturated lipids were a unique marker in differentiating late stages ($> 1b2$) and early stage ($\leq 1b2$) diseases^[28].

Based on another study, urinary metabolomics signatures clearly distinguished both Barrett's esophagus and esophageal cancer from controls. Although some overlaps were detected, the metabolomics profile of esophageal cancer was different than Barrett's esophagus^[29].

Metabolomics studies in gastric cancer are also promising. In a model of gastric adenocarcinoma-bearing mice, the urinary levels of TMAO and hippurate were significantly decreased, although the levels of 3-indoxylsulfate, 2-oxoglutarate, and citrate were significantly increased^[30].

Another animal study of implanted human gastric

cancer detected significant metabolic differences among normal, non-metastatic and metastatic groups. Based on this study, 10 selected metabolites were different between cancer and control groups. Briefly, the level of lactic acid, butanedioic acid, malic acid, citric acid and uric acid were higher in cancer indicating increase in aerobic glycolysis, respiration (mainly TCA cycle) and the impairment of mitochondrial enzymes. Moreover, glycerol and hexadecanoic acid as indicators of adipocyte lipolysis were higher in cancerous animals. Seven metabolites were also different between non-metastasis and metastasis groups. Alanine and glycerol (as substrates for glycolytic pathway) and L-proline were lower in cancerous animals with metastasis possibly due to a higher level of consumption. On the other hand, the level of myoinositol in the urine of metastasis group was higher^[31].

In a recently published article, the urinary metabolomics of gastric cancer patients was compared to healthy individuals. Based on this study, urinary metabolomics related to amino acids and lipid metabolism was significantly different in cancer vs control and could successfully discriminate both groups. Interestingly, the metabolomics signature of cancer showed much higher sensitivity compared to carbohydrate antigen 19-9 and carcinoembryonic antigen. 4-hydroxyphenylacetate, alanine, phenylacetylglutamine, mannitol, glycolate, and arginine levels were significantly correlated with cancer T stage. Together with hypoxanthine level, the above mentioned metabolites were tended toward control after surgical treatment^[32].

In a study by Chen *et al.*^[33], urinary lactic acid, arginine, leucine, isoleucine and valine were significantly higher, while citric acid, histidine, methionine, serine, aspartate, malic acid, and succinate were remarkably lower in the gastric cancer patients vs controls. In addition, the urinary valine and isoleucine levels were lower in advanced stages compared to early-stages of cancer^[33].

Another study also showed that urinary metabolomics could effectively differentiate gastric cancer patients from controls^[34]; however, the metabolites which were distinctive, were different than previously mentioned studies^[32,33], suggesting complexity in interpreting metabolomics results.

A study on urine metabolites of a colorectal cancer group of patients and their age-matched healthy controls as well as a rat model of chemically induced precancerous colorectal lesion revealed good separations between cancer patients or rats with pre-cancerous lesions and their healthy equivalents. Moreover, altered TCA cycle as well as gut microflora metabolisms were detected in cancer patients and the rat disease model. After surgery, the urinary metabolomic profile of cancer patients altered significantly compared to the preoperative stage since gut microflora metabolism and TCA cycle were down-regulated. In addition, 5-hydroxytryptophan significantly decreased after surgery suggesting an improvement of the tryptophan metabolism^[35].

The findings of the above mentioned study in colorectal

cancer were confirmed in a further study which also showed that a panel of urinary metabolite markers composed of citrate, hippurate, p-cresol, 2-aminobutyrate, myristate, putrescine, and kynurenate was able to discriminate colorectal cancer subjects from their healthy counterparts^[36].

Studies on the urinary metabolomics of GI cancers reveal alterations in microbiota, proteins and lipid mediated metabolites which are involved in the initiation and dissemination of cancer as well as the cellular overgrowth and proliferation, although no unique signature has been yet recognized. As a huge amount of variability is attributed to between-individual differences, future studies on larger sample sizes of GI cancer patients are required in order to detect associations with moderate effect sizes^[37].

URINARY METABOLOMICS IN OTHER GI CONDITIONS

Although many of the metabolomics studies have focused on GI conditions such as cancer and IBD, a few studies have assessed the roles of urinary metabolomics in other diseases.

Based on a study which compared the urinary metabolomics of 34 patients with celiac disease and 34 healthy controls, patients with celiac disease had a significantly lower levels of mannitol, glutamate, glutamine and pyrimidines, and higher levels of indoxyl sulfate, choline, glycine, acetoacetate, uracil, meta-hydroxyphenyl propionic acid, and phenylacetylglycine. This metabolomic signature is consistent with the hypothesis of small bowel dysbiosis in these patients^[38]. A further study hypothesized that the metabolomic signature of patients with potential celiac disease, defined as patients with the immunological abnormalities of celiac disease who lack jejunal biopsy findings consistent with their disease, is similar to those with overt celiac disease. Surprisingly, although these patients shared similar metabolomic profile in their serum, no clear joined signature was found in their urine, suggesting that defective small intestinal histology is needed for the development of a urinary metabolomic fingerprint of celiac disease^[39].

Studies on the urinary metabolomics of other GI diseases are limited. An animal study has shown the value of urinary metabolomics in the assessment of NSAIDs induced GI ulcer. Based on this study, a panel of urinary metabolites including 2-oxoglutarate, acetate, taurine and hippurate were significant biomarkers for the gastric damage induced by indomethacin in rats and could successfully predict the degree of GI damage, suggesting that NSAIDs induced gastric damage can be possibly screened in the preclinical stages by using urinary metabolomics^[40].

CONCLUSION

Urinary metabolomics studies show altered signature

in patients with GI disorders compared to healthy controls. The body of literature in this area has majorly focused on IBD and GI cancers. What is shared in all of these disorders is the alteration of urinary metabolites which are in association with GI microbiota and possibly dysbiosis in these chronic conditions. In addition, in cancer patients, the metabolomes which define cell proliferation and differentiation are altered. In IBD, differentiating UC and CD based on urinary metabolomic profile does not look simple at this stage, since confounders such as the clinical severity of the disease and medications may interfere with the metabolism in the body and the metabolomics profile of these patients. The most important use of urinary metabolomics in GI cancer is for early detection of pre-cancerous lesions. Whether the metabolomics signature in patients with pre-cancerous lesions such as Barrett's esophagus and colon polyps can predict the future outcome, *i.e.*, the possible chance of progressing to cancer is still under debate. Predicting the outcome of the diseases in response to medical or surgical therapies is also important in this area. In conclusion, although literature supports the role of urinary metabolomics in the diagnosis of some GI conditions, the fingerprints of these diseases are not unique and usually have overlaps.

LIMITATIONS OF URINARY METABOLOMICS IN GI DISORDERS

In 2009, Scalbert *et al.*^[41] extensively reviewed the limitations of mass-spectrometry-based metabolomics studies. Confounding effects of the diet, large Inter- and intra-individual variations, variations induced by sample collection, handling and storage and inconsistency in data extraction, interpretation and analytical methods were proposed as the major limitations of metabolomics studies. These limitations still affect the metabolomics studies. Moreover, the technology used for the measurement of metabolomics has limitations. For example, NMR is able to measure approximately 8% and gas chromatography MS is able to measure approximately 7% of the human urine metabolomes^[41]. For the urinary metabolomics, effects of the kidney function as well as the metabolic function of the body which may affect secretion and reabsorption of the circulating metabolites may confound the final results^[42].

FUTURE DIRECTION

Both organic and functional GI disorders usually lack well-defined noninvasive biomarkers which can help us with the diagnosis, treatment and the prediction of their outcome. In functional disorders like irritable bowel syndrome, the diagnosis is not usually definite and is based on exclusion. Moreover, the diagnosis of organic GI disorders usually relies on invasive techniques. Although, the urinary metabolomics signature shows alterations in different GI conditions compared to healthy subjects,

no unique signature has been yet defined. IBD, GI cancers and celiac disease have all shown alterations in the urinary metabolomics which are associated with possible GI dysbiosis, but to our knowledge, no study has systematically evaluated the GI microbiota profile concurrently. Studies on the urinary metabolomics profile of GI diseases have not usually considered confounding factors and the ways of analysis which have been used in these studies are not similar and sometimes cause different results in a single disease setting. Future studies should focus on the validation of the methods and should enhance our knowledge of metabolomic profiles which are in association with different metabolic pathways. The same as breath testing for helicobacter pylori and small bowel bacterial overgrowth, future urinary metabolomics studies may focus on metabolomic profiles induced through the consumption of labeled specific agents. Metabolomics of volatile vs non-volatile compounds is also an important area which should be considered. In addition, the effects of urinary diseases on GI system and microbiota as what has been recently observed in patients with chronic kidney diseases^[42] should be taken into account when interpreting urinary metabolomics studies.

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Non-surgical factors influencing lymph node yield in colon cancer

Patrick Wood, Colin Peirce, Jurgen Mulsow

Patrick Wood, Colin Peirce, Jurgen Mulsow, Department of Colorectal Surgery, Mater Misericordiae University Hospital, Dublin 7, Ireland

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Correspondence to: Jurgen Mulsow, Consultant Colorectal Surgeon, Department of Colorectal Surgery, Mater Misericordiae University Hospital, Eccles Street, Dublin 7, Ireland. jmulsow@mater.ie
Telephone: +353-1-8545091
Fax: +353-1-8034023

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Abstract

There are numerous factors which can affect the lymph node (LN) yield in colon cancer specimens. The aim of this paper was to identify both modifiable and non-modifiable factors that have been demonstrated to

affect colonic resection specimen LN yield and to summarise the pertinent literature on these topics. A literature review of PubMed was performed to identify the potential factors which may influence the LN yield in colon cancer resection specimens. The terms used for the search were: LN, lymphadenectomy, LN yield, LN harvest, LN number, colon cancer and colorectal cancer. Both non-modifiable and modifiable factors were identified. The review identified fifteen non-surgical factors: (13 non-modifiable, 2 modifiable) which may influence LN yield. LN yield is frequently reduced in older, obese patients and those with male sex and increased in patients with right sided, large, and poorly differentiated tumours. Patient ethnicity and lower socioeconomic class may negatively influence LN yield. Pre-operative tumour tattooing appears to increase LN yield. There are many factors that potentially influence the LN yield, although the strength of the association between the two varies greatly. Perfecting oncological resection and pathological analysis remain the cornerstones to achieving good quality and quantity LN yields in patients with colon cancer.

Key words: Lymph node; Number; Factors; Yield; Colon cancer

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Core tip: Surgeons, pathologists and patients alike must appreciate that there are many factors which influence lymph node (LN) yield in resected colon cancer specimens. Clinicians must strive for the perfect oncological operation and pathological analysis. However, clinicians should be aware that despite optimal surgery and pathological analysis, other factors may influence the LN yield following colonic resection for cancer.

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INTRODUCTION

The American Joint Committee on Cancer/Union Internationale Contre le Cancer utilises the TNM system to stage colon cancer. The stage of disease is dependent on the depth of penetration into the intestinal wall (T1-T4), the presence of localized lymph node (LN) metastases (N0-N2) and the presence of distant metastases (M0-M1). This system potentially lends itself to “understaging” of disease since accurate staging is closely linked to both adequate and high quality LN evaluation. Indeed, numerous studies have demonstrated an association between the number of LNs examined and patient survival, with the consistent finding that an increased number of evaluated LNs leads to improved survival^[1-9]. Furthermore, the decision to administer adjuvant chemotherapy is highly dependent on the presence or absence of LN metastases: When present, patients are classified as stage III and typically receive chemotherapy while those with stage II disease and no adverse features routinely undergo surveillance only.

The “gold” standard of at least a 12 LN examination following resection for colon cancer was initially proposed in 1990^[10]. The National Institute of Clinical Excellence suggested that when more nodes are examined the tumour is significantly more likely to be classified as node positive. Conversely, when few nodes are examined, there is a substantial risk of understaging^[11]. This standard has now been adopted in multiple guidelines for both colon and rectal cancer resection specimen analysis^[12-14]. More recently, the analysis of ≥ 12 LNs has been adopted as a standard quality indicator for colorectal resection specimens in the United States by the National Quality Forum^[13,15], the National Comprehensive Cancer Network (NCCN), the American Association of Clinical Oncology and the American College of Surgeons^[16-18]. However, the literature is variable on the subject with some groups suggesting that a harvest of 9 LNs is sufficient to stage node negative tumours^[19], others agreeing that harvesting more than 12 LNs is adequate for staging colon cancer^[4,6,20] and others still suggesting that there is no clear cut-off value and that as many LNs as possible should be harvested and analysed^[2,8].

Irrespective of the agreed and accepted LN cut-off, it should be appreciated that multiple factors may be influence nodal yield. Undoubtedly, surgical technique and pathological analysis are the cornerstones for adequate LN examination, however other, principally patient factors may be of relevance and lead to reduced LN yield despite optimal surgery and specimen analysis. This study aimed to review both modifiable and non-modifiable non-surgical factors that have been shown to influence colonic resection specimen LN yield and to summarise the pertinent published literature.

LITERATURE REVIEW

A literature review of PubMed for the period 1991-2015 was performed to identify the potential factors which may influence the LN yield in colon cancer resection specimens. The terms used for the search were: LN, lymphadenectomy, LN yield, LN harvest, LN number, colon cancer and colorectal cancer. Both non-modifiable and modifiable factors were identified (Table 1) and the individual papers reviewed. Further relevant publications were identified by cross reference of the reviewed papers.

NON-MODIFIABLE FACTORS

Ethnicity

There have been a number of studies which have assessed the influence of ethnicity on LN yield. The rationale as to why ethnicity would potentially affect the LN yield remains unclear.

In a large study Cone *et al.*^[21] interrogated the Surveillance Epidemiology and End Results (SEER)-Medicare database in the United States, evaluating all colonic cancer resections between the years 2000 and 2003. Their analysis included nearly 33000 patients, 62.5% of whom had less than 12 LNs in the resected specimen. Multivariate analysis showed that Hispanics were less likely than Caucasian patients to have ≥ 12 LNs resected (OR = 0.61, 95%CI: 0.5-0.74). Hispanic patients were younger (although all patients in the analyses were older than 65 years), lived in more populated areas and had a lower income status than their Caucasian counterparts. The reduced LN yield did not confer a negative outcome with no significant difference in survival between the groups.

Other smaller single institution studies such as that by Valsecchi *et al.*^[22] failed to show an association between LN yield and ethnicity.

Age

Numerous studies have assessed the influence of patient age on LN yield in resected colon cancer, the hypothesis being that younger patients are more likely to have a more aggressive oncological procedure, or conversely that older patients frequently undergo less aggressive lymphadenectomy. A national United Kingdom study from 2006 reported that increasing age was associated with a significant reduction in the number of harvested LNs ($P < 0.001$)^[23]. Moreover, for every 10 years increase in age in their cohort, there was an associated reduction in LN harvest by 0.9 nodes (95%CI: 0.7-1.1). The authors also noted that as the patient age increased there was also a significant increase in variability of LN harvest between the 79 participating centres ($P < 0.001$), which included both peripheral and tertiary referral centres. These findings were felt most likely to be due to a wider lymphadenectomy being performed in younger, medically fitter (and elective) patients as opposed to older patients with co-morbidities. An alternative

Table 1 Factors influencing lymph node yield in colon cancer resection specimens

Non-modifiable	Modifiable
Ethnicity	Tumour tattooing
Age	Neoadjuvant therapy
Gender	
Socioeconomic class	
Tumour location	
Tumour size	
Tumour histological subtype	
ASA grade	
Tumour classification and stage	
Tumour microsatellite instability	
Lymph node positivity/negativity	
Lymphovascular invasion	
Body mass index	

ASA: American Society of Anesthesiologists.

hypothesis is that LNs undergo a process of involution with increasing age^[23].

Stocchi *et al*^[24], in their study from the Cleveland Clinic, Ohio, also reported an association in 901 patients with stage II colon cancer between increasing age and fewer examined LNs ($P < 0.001$)^[24]. Patients younger than 65 years had a mean of 35.1 (range 15-44) nodes examined compared to a mean of 22.2 (range 12-28) nodes in patients older than 65 years.

Another population-based study by Chou *et al*^[15] analysed 127927 patients who underwent resection for stages I - III colon cancer between 1994 and 2005 in the United States. Of note, in 4.6% of patients, no regional LNs were examined and thus, these individual patients were not staged. Once again, age was shown to be a consistently important determinant of LN yield and for every 10-year incremental increase in patient age, there was an associated average reduction of 9% in the number of harvested LNs ($P < 0.01$). It should be noted that over the timeframe of the study, there was not only an increase in the average LN yield, but also a decrease in the mean age at diagnosis for patients with colon cancer - from 70.3 years in 1994 to 68.8 years in 2005. The rationale for this significant association between age and LN yield in this paper was thought to be as a result of a "complex interplay of patient and surgeon factors". The authors explained this by hypothesising that older patients are likely to be considered higher-risk operative candidates and thus a suboptimal surgical dissection (with resultant inadequate lymphadenectomy) may be performed with a view to reducing operative time. Nathan and colleagues also interrogated the SEER database for patients operated with curative intent for stage I - III colon cancer between 1998 and 2005^[25]. In the 27101 patients analysed, increasing patient age was again significantly associated with a decreased LN yield ($P < 0.001$). Finally, Baxter *et al*^[26] also interrogated the SEER database, specifically focusing on patient who had undergone colonic resection for pT3 lesions. They identified 11044 patients and once again were able to show that older patients had fewer LNs examined ($P < 0.001$). Several other studies have mirrored these

findings^[22,27-34], however, 2 smaller studies of 341 and 223 patients respectively, failed to demonstrate an association between colonic LN yield and patient age^[35,36].

Overall, it appears that increased patient age significantly influences LN yield in colon cancer.

Patient gender

A number of studies have shown a significant association between male sex and reduced LN yield^[25,28,36]. The largest of these included over three hundred thousand patients in a United States population based study analysing factors influencing LN yield in patients with gastrointestinal cancer^[28]. The reasons underlying this association are poorly understood. Dubecz *et al*^[28] suggested that men are more likely to be uninsured and thus may be less likely to receive "state-of-the-art" treatment which might include adequate lymphadenectomy as performed in a high-volume colorectal centre.

Socioeconomic class

It has been suggested that patients with lower socioeconomic class may be less likely to be treated in specialised centres and to receive the most up to date management with the result that their LN yield following resection for colon cancer is lower. A population-based analysis of all patients with gastrointestinal (GI) adenocarcinomas treated surgically in the United States between 1998 and 2009 ($n = 326243$) was performed by Dubecz *et al*^[28]. They aimed to evaluate time trends in lymphadenectomy for GI cancer and to identify factors associated with inadequate LN yield. Adequate lymphadenectomy was defined by the NCCN recommendations as a LN yield of > 12 in colon and rectal cancer. Throughout the study period it was found that the LN yield increased over time for all of the sub classifications of GI cancer. The median number of LNs retrieved for colon cancer increased from 9 in 1998 to 16 in 2009. However, only 49% of patients with a GI adenocarcinoma diagnosis underwent adequate lymphadenectomy. The rate of adequate evaluation was higher in colon cancer (77%) than in rectal cancer (42%). Patients living in areas with higher poverty rates were more likely to undergo inadequate lymphadenectomy. The socioeconomic data was based on county of residence which was linked to United States Census data. The first quartile, Q1 (most well off), had an adequate lymphadenectomy in 49% of cases, Q2 in 51% adequate while Q3 and Q4 (least well off) had adequate lymphadenectomy in 47% of cases. Patients of lower socioeconomic class were most likely to have an inadequate lymphadenectomy with the authors postulating that this finding most likely reflected a high proportion of uninsured patients who may be less likely to receive state of the art treatment. In a separate study, Rajput *et al*^[29], also showed that insurance status was associated with LN yield across patients identified from the NCCN and SEER databases. On multivariate analysis, patients with Medicare and Medicaid plans had lower yields than patients covered by commercial plans ($P = 0.007$). The authors' belief was that this finding

was secondary to the age profile of the patients, with those in the Medicare population tending to be older than those with private insurance coupled with a demonstrable decrease in LN yield with increasing age across all patients in the study.

In summary, lower SE status may be associated with reduced LN yield following resection for colon cancer, however there are multiple factors that may underlie this association.

Tumour location

There is consistent evidence that the location within the colon of the primary is strongly associated with the number of LN examined by the pathologist, with the length of the specimen often implicated as the causative factor. Stocchi *et al*^[24] reported that a 12 LN harvest was more likely with right sided as opposed to left sided carcinomas (85% vs 72%, $P < 0.001$). Similarly, in the study from Baxter *et al*^[26], patients with a left sided colon cancer (and rectal cancer) were less likely to have an adequate LN evaluation compared with patients with right sided lesions. In a separate study, Wright *et al*^[37] reported a median number of 12 LNs for right sided cancer and 9 LNs for left sided colonic tumours. Chou *et al*^[15] also reported a similar trend: In a sub-analysis of right and left sided colon cancers, tumours located in the ascending colon and hepatic flexure had, on average, 34% more LNs retrieved than those in the sigmoid and rectosigmoid. However, the authors acknowledged that the SEER data on which their study was based did not record the length of the resected specimen and speculated that the observed differences in LN yield may in fact be due to longer specimen lengths following right sided resection. The association between specimen length and LN yield has been repeatedly demonstrated. Stocchi *et al*^[24] showed that specimens less than 30 cm in length had a median LN harvest of 17 nodes whereas those longer than 30 cm had a median harvest of 24 nodes ($P < 0.001$)^[24]. Shen *et al*^[31] also reported variability in LN yield depending on both tumour site and specimen length. They studied 365 resected colon cancers and demonstrated an increased LN yield of 17.8 for caecal and ascending colon lesions vs 14.3 for sigmoid lesions ($P < 0.01$). Descending colon lesions were associated with the longest specimens at 29.2 cm and there was a clear association between the length of the specimen and LN yield, with an average of 11 LNs in specimens of 10 cm or less in length compared with 18.3 LNs when the specimens were over 30 cm in length. Numerous studies have shown similar patterns of decreased LN yield for left-sided vs right-sided colonic cancer^[22,27,29,33,34,38-40]. Two smaller studies, analysing 137 and 48 colon specimens respectively, failed to show an association between LN yield and primary tumour site^[41,42].

In summary, the published literature supports the hypothesis that tumour location influences LN yield in colon cancer.

Tumour size

It has previously been proposed that larger tumours elicit an intense antigenic response within the surrounding regional LN basin. This "response" may potentially make them more visible to pathologic examination and may thus lead to an increased LN yield^[37]. In a study by Chou *et al*^[15], for every 1 cm increase in tumour size, there was a corresponding average 2% increase in the number of examined LNs in colon cancer specimens. Tumour size was also shown to be a significant predictor of LN yield in univariate analysis in a study by Valsecchi *et al*^[22] ($P < 0.01$). There have been 2 recent studies from the Memorial Sloan Kettering group, both reporting a strong association between tumour size and the nodal yield^[27,43]. In the first study, tumour size of 4 cm or less resulted in a mean nodal harvest of 19.7 as compared to a mean nodal harvest of 23.3 when the tumour measured over 4 cm ($P = 0.02$)^[27]. In the second and more recent study, analysis of 256 colectomy specimens demonstrated a linear relationship between tumour size and LN yield ($P < 0.0001$)^[43]. Søreide *et al*^[39] also showed that LN harvest is related to tumour size. Tumours greater than 5 cm had adequate LN yield in 50% of cases, compared to 24%, when tumour size were less than 5 cm.

Colon cancer histological subtype and tumour differentiation

Tekkis *et al*^[23], in a study including more than 5000 patients, showed that the tumour differentiation was one of eight factors which had a significant influence on the number of LNs examined. Poorly differentiated tumours had significantly increased LN yield when compared to well or moderately differentiated lesions. In the same study, the tumour subtype was not shown to significantly influence nodal yield.

A number of other studies have reported an association between tumour differentiation subtype and LN yield, with the consensus being that the more poorly differentiated the tumour the greater the LN yield compared to well differentiated lesions^[25,27,35,37].

ASA grade

The evidence to support an association between American Society of Anesthesiologists (ASA) grade and LN yield is limited. The rationale behind linking ASA grade and LN yield is similar to that for increasing age. Patients with higher ASA are often older and may undergo emergent surgery, which may lead to less radical dissection in order to complete the operation in a timelier manner. A national United Kingdom study published in 2004^[23] did show that patients with higher ASA grade were less likely to have adequate LN harvesting when compared to patients with lower ASA grades: ASA III vs I ($P < 0.001$) and ASA IV-V vs I ($P = 0.036$).

LN positivity

The available literature shows conflicting findings with respect to the influence of LN positivity on LN yield. Any

association, positive or otherwise, should be interpreted with some caution due to the potential for underlying bias. An association between increased LN yield and nodal positivity, as shown by Tekkis *et al.*^[23] for example, may simply reflect a more comprehensive search for nodes. On the other hand, a finding of multiple involved nodes may lead to a less thorough search for further nodes leading to a lower overall nodal yield. In a study by Nash *et al.*^[27], no correlation was demonstrable between the total number of LNs examined and the number of LNs with metastatic disease ($P = 0.32$). However, there was a trend towards finding one fewer LN in each specimen for every 2 metastatic LNs.

Lymphovascular invasion

Lymphovascular invasion (LVI) is a surrogate marker for tumour aggressiveness and is associated with a poorer outcome. The limited available data shows no association between LVI and LN yield. Gelos *et al.*^[35] performed a retrospective analysis of 341 patients who underwent colorectal cancer resection with curative intent between 2000 and 2005 and investigated the impact of a number of factors including LVI on LN yield. There was a median of 15.17 LNs retrieved per patient, with 82.8% of the 341 patients having a LN harvest greater than 12, however the presence of LVI did not influence tumour LN yield. In another smaller study (48 patients) with a mean LN count of 14.1, no statistically significant relationship existed between the number of LNs and the presence of LVI ($P = 0.64$)^[42].

Microsatellite instability

An association between LN yield and microsatellite instability (MSI) has been put forward by a number of authors. MSI tumours are considered less aggressive than their microsatellite stable (MSS) counterparts and may demonstrate an enhanced host inflammatory reaction^[44-47].

An association between a high rate of MSI and a high total LN count in colorectal cancer has been demonstrated in a number of small studies. Higher LN retrieval may in part explain the improved survival seen in patients with MSI. Søreide *et al.*^[39] studied 121 patients under the age of 75 with the aim of determining whether proximal tumour location and MSI improved LN yield. One thousand two hundred (1200) LNs were retrieved from 121 patients and of these, 96 were positive (0.8%). Median LN harvest was 10 and only 36% of patients had an adequate harvest (*i.e.*, 12 or more LN). MSI was found in 33 out of the 121 patients (27%) and this was associated with a greater median LN yield of 12 vs 9 in the MSS group. Fifty-four percent of patients with MSI had adequate LN harvest vs 29% in the MSS group and 36% in the study as a whole [OR = 2.9 (1.3-6.5), $P = 0.011$]^[39].

Eveno *et al.*^[48] reported a smaller series of 82 patients with stages I and II colon cancer and also showed a significantly increased LN yield in the MSI group (mean 23.6 vs 13.7 LN).

A separate study investigated the association between MSI and LN yield but did not show a significant association^[49]. Of 168 patients with stage III colon cancer the mean total LN yield for MSI and MSS tumours was 15.9 and 16.9 respectively ($P = 0.664$). The authors concluded that increased survival in the MSI group ($P = 0.026$) could not be explained by differences in LN yield.

Body mass index

Studies performed in patients with gastric and rectal cancers have shown an association between obesity and reduced LN yield^[50,51]. Damadi *et al.*^[52] retrospectively reviewed 191 patients who underwent a resection for colon cancer between 1999-2006. They hypothesized that obese patients with a body mass index (BMI) > 30 kg/m² would have a smaller yield of LNs compared to non-obese patients with a BMI < 30 kg/m², however they found no significant difference between the groups (mean LN yield 12.7 in obese vs 12.4 in non-obese, $P > 0.2$).

Linebarger *et al.*^[53] performed a retrospective review of 401 patients, and stratified them into six groups based on BMI: Underweight (< 18.5 kg/m²), normal (18.5-24.9 kg/m²), overweight (25-29.9 kg/m²), stage I obesity (30-34.9 kg/m²), stage II obesity (35-39.9 kg/m²) and stage III obesity (> 40 kg/m²). They found no significant difference in the number of LNs harvested for each of the groups.

Kuo *et al.*^[54] retrospectively analysed 645 patients with stage III colon cancer from Taiwan who underwent colectomy. Patients were again placed into four groups based on their BMI: Obese (BMI > 27 kg/m²), overweight (BMI 24-27 kg/m²), normal (BMI 18.5-24 kg/m²) and underweight (BMI < 18.5 kg/m²). The mean BMI of the patients in the study was 23 kg/m². The authors showed a significantly increased mean LN yield in the underweight patient group (28.1 vs 23 in the normal BMI group, 19.5 in the overweight group and 19.8 in obese patient group respectively). A 2010 study analysed a cohort of 718 NCCN patients with stage I-III colon cancer and found three factors were associated with not meeting the quality standard of a 12 LN evaluation: Left-sided tumours, stage I disease and a BMI > 30 kg/m²^[28].

The impact of BMI on LN yield is overall unclear, with some studies pointing to a reduced in patients with a higher BMI.

MODIFIABLE FACTORS

Tumour tattooing

Endoscopic tattooing is frequently performed in order to facilitate tumour localisation during laparoscopic resection. Tumour tattooing may inadvertently map the sentinel node and associated draining nodes and thus make them more readily identifiable for pathological evaluation. A retrospective case controlled trial conducted between 2005 and 2009 aimed to determine if colonoscopic tumour tattooing could be utilised to increase staging accuracy by increasing the LN yield^[55]. The

authors assessed two groups of patients: The first group contained a series of 95 consecutively tattooed patients and the second group a series of 210 non-tattooed patients. All patients underwent surgery for colorectal cancer within the same time period. There was a higher LN yield in patients with pre-operative tattooing compared to the non-tattooed control group (median LN yield of 15 vs 12 nodes, $P = 0.014$). Multivariate analysis showed that the presence of carbon-containing LNs (with a detection rate of 71%) was an independent predictor for an increased LN yield ($P = 0.002$), although the reason for the lack of a predictive characteristic for the group in which tattooing did not result in carbon containing LNs was not clear.

The potential role of preoperative tattooing was also reported by Nash *et al.*^[27]. Their study was designed to develop a predictive model of LN yield in colon cancer. One hundred and fifty-two specimens from patients who had undergone resection for colon cancer were used, with detailed anatomical and surgical technique documentation on each specimen. A linear regression analysis was performed and this identified both predictors and confounders of the quantity of the LN harvest. Of the 15 variables analysed, it was found that tumour size, tumour location, number of resected pedicles and use of pre-operative tattoo had significant linear/quadratic relationships on the LN yield. When controlling for the 14 other variables, patients who underwent endoscopic tattooing had 3.1 more LNs harvested. This data further suggests that endoscopic tattooing may be used pre-operatively to maximise LN yield and increase the accuracy of disease staging. The authors acknowledged that as they did not record the proportion of LNs which harboured grossly apparent dye at the time of LN identification, they could not make a definitive conclusion as to the mechanism by which preoperative colonic cancer tattooing might increase LN yield Dawson *et al.*^[56] also hypothesised that pre-operative tattooing with India ink might increase the subsequent LN yield from the resected specimens. Their retrospective study included 174 patients who underwent surgery for colon cancer between 2006 and 2009. Sixty-two patients had pre-operative tattooing. The mean number of LNs harvested in the tattooed group was 23 compared to 19 in the non-tattooed group ($P = 0.03$). In the tattooed colon cancer group a 12 LN minimum was achieved in 87.1% patients vs 72.3% in the non-tattooed group. These results were mirrored in a separate analysis, within the same study, of 35 patients with rectal cancer. Once again, the results from this study suggest the routine utilisation of pre-operative colonoscopic tattooing may increase the LN yield in resected colonic malignancy.

Neoadjuvant chemotherapy

The role of neoadjuvant therapy in the setting of colon cancer remains in evolution. Data from studies performed in patients with rectal cancer has shown that neoadjuvant therapy may result in a decreased LN yield, however

this is in the context of both radiotherapy and chemotherapy. The initial data from the United Kingdom based FOxTROT trial reported on 150 patients in 35 centres^[57]. All patients had either T3 (with > 5 mm invasion into the muscularis propria) or T4 colon tumours and were randomised to either preoperative and postoperative chemotherapy or standard postoperative chemotherapy alone (2:1 randomisation). Overall, the authors reported that preoperative chemotherapy was a viable option with acceptable toxicity in this cohort. When the LN data were examined, 85 of 98 patients (87%) and 43 of 50 patients (86%) had 12 or more LNs examined in the combined preoperative and postoperative chemotherapy and postoperative chemotherapy groups respectively. Indeed, 46% and 54% of patients in both groups had greater than 20 LNs examined with median values of 21 and 22 nodes respectively ($P = 0.2$). The apical node was positive in 1 of 98 patients in the combined group (1%) and 10 of 50 patients in the postoperative chemotherapy only group (20%). Thus, in this study neoadjuvant chemotherapy did not result in a lower LN yield however more data is needed before definitive conclusions can be made.

CONCLUSION

There are many factors that can potentially influence the LN yield following resection for colon cancer and the relationship between these factors remains poorly understood. High quality oncological surgery and pathological analysis are the most important factors in ensuring optimal LN yield. However, the current review has highlighted a number of additional modifiable and non-modifiable factors that may also influence the number of LNs harvested. Older age, obesity, and male sex may be associated with reduced LN yield. Similarly, studies have shown an association between ethnicity and lower socioeconomic class and reduced LN harvest. Rather than being true associations, however, it is likely that these findings reflect, at least in part, external modifiable factors such as the surgeon's attitude to older patients undergoing surgery or the quality of care received by patients in lower SE groups. LN yield appears to be increased in patients with right-sided cancer, bulky tumours, or poor tumour differentiation. Again, these associations may reflect other factors known to influence nodal yield such as the length of the resection specimen. Nonetheless, these variables should be taken into consideration when evaluating the completeness of the LN harvest for individual patients.

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Retrospective Study

Intensity modulated radiation therapy with simultaneous integrated boost based dose escalation on neoadjuvant chemoradiation therapy for locally advanced distal esophageal adenocarcinoma

Ming Zeng, Fernando N Aguila, Taral Patel, Mark Knapp, Xue-Qiang Zhu, Xi-Lin Chen, Phillip D Price

Ming Zeng, Fernando N Aguila, Taral Patel, Mark Knapp, Xue-Qiang Zhu, Xi-Lin Chen, Phillip D Price, Department of Radiation Oncology, Mount Carmel Health System, Columbus, OH 43219, United States

Ming Zeng, Xue-Qiang Zhu, Cancer Center, Sichuan Academy of Medical Sciences, Sichuan Provincial Hospital, Chengdu 610072, Sichuan Province, China

Fernando N Aguila, Central Ohio Surgical Associates, Inc., Columbus, OH 43219, United States

Taral Patel, Mark Knapp, Department of Hematology and Oncology, Zangmeister Cancer Center, Mount Carmel Health System, Columbus, OH 43219, United States

Xi-Lin Chen, Department of Oncology, 307 Hospital, Beijing 100071, China

Author contributions: Zeng M, Aguila FN, Patel T and Knapp M involved the study protocol design; Zhu XQ and Chen XL collected and analyzed the data; Zeng M, Patel T and Chen X draft the manuscript.

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Correspondence to: Ming Zeng, MD, PhD, Department of Radiation Oncology, Mount Carmel Health System, 3100 Plaza Properties Blvd, Columbus, OH 43219, United States. miller2002@yahoo.com
Telephone: +1-614-2168721

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Abstract

AIM: To evaluate impact of radiation therapy dose escalation through intensity modulated radiation therapy with simultaneous integrated boost (IMRT-SIB).

METHODS: We retrospectively reviewed the patients who underwent four-dimensional-based IMRT-SIB-based neoadjuvant chemoradiation protocol. During the concurrent chemoradiation therapy, radiation therapy was through IMRT-SIB delivered in 28 consecutive daily fractions with total radiation doses of 56 Gy to tumor and 5040 Gy dose-painted to clinical tumor volume, with a regimen at the discretion of the treating medical oncologist. This was followed by surgical tumor resection. We analyzed pathological completion response (pCR) rates its relationship with overall survival and event-free

survival.

RESULTS: Seventeen patients underwent dose escalation with the IMRT-SIB protocol between 2007 and 2014 and their records were available for analysis. Among the IMRT-SIB-treated patients, the toxicity appeared mild, the most common side effects were grade 1-3 esophagitis (46%) and pneumonitis (11.7%). There were no cardiac events. The R0 resection rate was 94% ($n = 16$), the pCR rate was 47% ($n = 8$), and the postoperative morbidity was zero. There was one mediastinal failure found, one patient had local failure at the anastomosis site, and the majority of failures were distant in the lung or bone. The 3-year disease-free survival and overall survival rates were 41% ($n = 7$) and 53% ($n = 9$), respectively.

CONCLUSION: The dose escalation through IMRT-SIB in the chemoradiation regimen seems responsible for down-staging the distal esophageal with well-tolerated complications.

Key words: Intensity modulated radiation therapy; Esophageal adenocarcinoma; Simultaneous integrated boost; Neoadjuvant chemoradiation; Dose escalation; Resection rate

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Core tip: There are more data supporting neoadjuvant chemoradiation for locally advanced esophageal cancer. The best regimen of neoadjuvant chemoradiation remains to be defined, current available data using three-dimensional vs intensity modulated radiation therapy deliver modest dose to downstage the tumor. In this report, we reviewed our experience using dose escalation technique to Gross Tumor Volume with compromising dose to organ at risk, the high R0 resection rate results suggest the feasibility of using this approach for future prospective study.

Zeng M, Aguila FN, Patel T, Knapp M, Zhu XQ, Chen XL, Price PD. Intensity modulated radiation therapy with simultaneous integrated boost based dose escalation on neoadjuvant chemoradiation therapy for locally advanced distal esophageal adenocarcinoma. *World J Gastrointest Oncol* 2016; 8(5): 474-480 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v8/i5/474.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v8.i5.474>

INTRODUCTION

Distal esophageal cancer is a commonly lethal malignancy, with the annual death rate in the United States about 15590^[1]. Most single-modality treatment regimens provide poor cure rate. For example, surgical treatment alone has a 5-year survival of approximately 15% to 20%^[2,3]. Radiation therapy alone for resectable highly selected squamous cell cancer results in a 5-year survival

rate of approximately 34%^[4]. In recent decades, more data have emerged for locally regional esophageal cancer management. There are reports that suggest triple-modality treatment regimens provide better local control, better control of distant metastases, and better overall survival^[5]. Meta-analysis showed a survival benefit for patients treated with preoperative chemoradiotherapy (CRT) compared with surgery alone^[6]. We have been using triple-modality approach for locally advanced distal esophageal cancer. Although the results from CROSS studies demonstrated the triple-modality approach to be well-tolerated with survival benefit, the absolute benefit for adenocarcinoma remains small and the survival benefit for adenocarcinoma is not statistically significant^[7], with a 17% pathological complete response (pCR). There is clear evidence correlating pCR and better local control with improved survival^[8]. However, there are few reports regarding the role of radiation dose variation in triple-modality therapy. Understanding dose change and its impact on tumor response provides insight into the effectiveness of the combined treatment and may lead to improved survival. Therefore, we analyzed our experience with dose escalation through the intensity modulated radiation therapy with simultaneous integrated boost (IMRT-SIB) technique, with emphasis on the relationship of the site of pCR to the radiation dose escalation delivered to the gross tumor volume.

MATERIALS AND METHODS

Our Institutional Review Board approved this study. The patients included in this study were treated at our institution for locally advanced esophageal cancer between January 2007 and December 2014. The inclusion criteria were pathologically confirmed adenocarcinoma and no past or current history of malignancy or radiation treatment in the chest or abdomen. Written informed consent was obtained from all patients before starting treatment. All patients underwent staging and those with no distant disease and medically operable patients received neoadjuvant CRT for 5-6 wk. The resectable disease included stage cT1N1M0 or cT2-3N0-1M0 (Table 1)^[9].

Pretreatment staging included elaborate history taking, physical examination, routine blood studies, pulmonary function tests, an upper gastrointestinal endoscopy with biopsies, and computed tomography (CT) of chest, abdomen and neck. Endoscopic ultrasound was used routinely for staging of esophageal tumors if technically possible. All patients in this analysis underwent positron emission tomographic (PET) scanning as part of staging to better define gross tumor volume.

All treatment began with concurrent chemoradiation. Chemotherapy consisted of five cycles of concurrent platin-based weekly chemotherapy or 5-fluorouracil (5-FU)-based daily chemotherapy. The platin-based chemotherapy was given at a dose of 40-100 mg/m², starting on days 1, 8, 15, 22 and 29. The 5-FU-based chemotherapy was either continuous infusion CIV 5-FU (225 mg/m²) or oral capecitabine (capecitabine,

Table 1 Characteristics of 17 patients

Characteristic	Value
Mean age (range)	65 yr (45-76)
Men/women (n)	14/3
ECOG PS (n)	
0	14
1	3
2	0
3	0
T stage (n)	
T1	0
T2	2
T3	15
T4	0
N stage (n)	
N0	12
N1	5
Concurrent chemotherapy (n)	
5-FU/cisplatin	11
Carboplatin/Taxel	6
Tumor location: < 35 cm/> 35 cm (n)	14/3
Mean gross tumor volume (cm ³) (range)	43.7 (33.3-62.4)
Mean clinical tumor volume (cm ³) (range)	503.2 (419.3-577.1)

ECOG PS: Eastern Cooperative Oncology Group performance status; 5-FU: 5-fluorouracil.

Genentech, San Francisco, CA) 750 mg/m² twice daily starting on day 1 to day 28.

The radiation therapy could be delivered through four-dimensional (4D) plus IMRT with SIB. All patients who received radiation therapy started with CT-based 4D treatment simulations. The simulation was performed with the patient in the supine position using immobilization with the patient's arms over the head. The 4D simulations were performed if respiratory gating was feasible, otherwise, free-breathing 3D CT acquisition data would be obtained during simulation, the patients then were excluded from the IMRT-SIB protocol. All treatment planning in this series was performed by the same radiation oncologist. During the treatment planning, two target volumes were drawn gross tumor volume and clinical target volume, PET/CT imaging obtained within 1-3 wk prior to simulation data. Patients who did not have PET/CT data were excluded from the IMRT-SIB protocol. Clinical target volume and the clinical internal target volume reflected the microscopic sites of highest risk. The treatment planning target volume (PTV) for clinical target volume is about 5 mm beyond clinical target volume; the clinical target volume was contoured based on the Radiation Therapy Oncology Group consensus study protocol^[10]. The treatment PTV for gross tumor volume had no margins.

There are total 17 patients received SIB technique and included in this study, after resection the postoperative T stages of the analyzed tumors were as follows: ypT0 (n = 8, 47%); ypT1 (n = 4, 23.5%); ypT2 (n = 3, 17.6%); ypT3 (n = 2, 11.7%). Nodal disease was confirmed in three patients (17.6%) by pathological staging and the median number of assessed lymph nodes was 13 (range 3-27) (Table 2). There were five pulmonary complications (29%), one cardiac complication (5.8%), and six surgical

Table 2 Pathological staging post simultaneous integrated boost based neoadjuvant chemoradiation

Pathological staging	Patient n (%)
ypT	
0	8 (47)
1	4 (23)
2	3 (18)
3	2 (12)
4	0
ypN	
0	13 (76.5)
1	4 (23.5)

complications (35%). There were no treatment-related or operative deaths (Table 3).

The time required to finish radiation treatment ranged from 28-35 d. Clinical tumor volume (CTV) represents the conventional dose coverage, 5040 in 28 fractions and PET-positive alone target area will receive SIB to 5600 in 28 fractions, which is labeled as gross tumor volume (Figure 1). The PTV of 180 cGy per fraction provided a proximal and distal margin of 5 cm and a radial margin of 7 mm around the CTV volume except to the heart with approximately 3-5 mm margins. The average beam number was 6.3 (range, 5-9). All organs at risk met their dose constraints. The daily prescription dose of 2 Gy was specified at the International Commission on Radiation Units and Measurement reference point, and at least the 95% isodose had to encompass the entire PTV. The maximum dose to the PTV was not to exceed the prescription dose by 7%. Tissue density inhomogeneity correction was used. The 4D plan using respiratory gating technique applied to all patients.

Patients were followed routinely after finishing neoadjuvant chemoradiation. Surgical resection was performed between 6-8 wk after completion of CRT. The operative technique consisted of a transthoracic approach with a two-field lymph node dissection or a transhiatal approach, depending on tumor localization. A wide local excision of the N1 lymph nodes, including standard excision of the celiac nodes, was carried out in both techniques. Continuity of the digestive tract was restored by gastric tube reconstruction or colonic interposition procedure with cervical anastomosis.

For grading of the therapy response, the degree of histomorphologic regression was classified into four modified categories, as described by Mandard *et al*^[11]. Surgical margins were designated in accordance with the criteria of the AJCC staging manual. All resection margins, including circumferential margins, were evaluated for vital tumor with a cutoff point of 1 mm. Margin status was confirmed by frozen and permanent sections and the close distance to the nearest millimeter between cancer cells, and the margin was measured microscopically and recorded prospectively. The operation was defined as an R0 resection if there was no microscopic tumor found at the margin and as an R1 resection if a margin was positive microscopically.

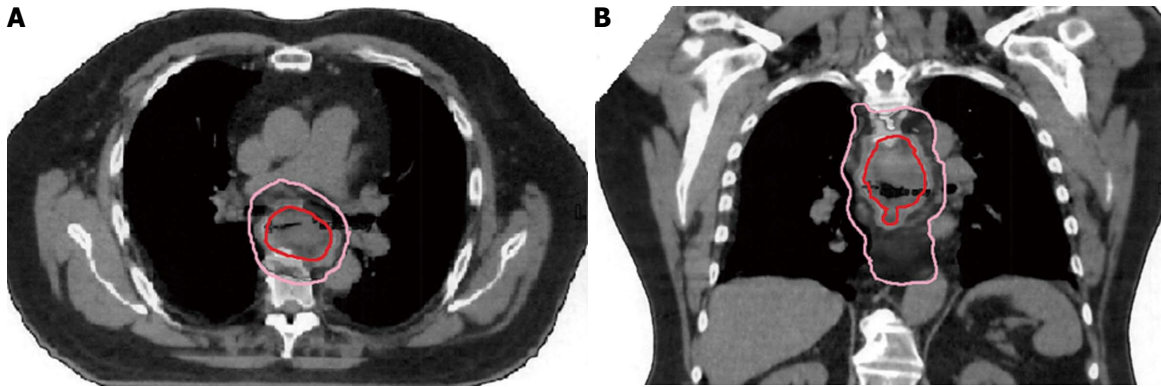


Figure 1 Transverse (A) and coronal (B) images of representative four-dimensional intensity modulated radiation therapy with simultaneous integrated boost. The isodose lines for 5040 cGy (pink) and 5600 cGy (red) were labeled.

Table 3 Postoperative toxicity after neoadjuvant treatment followed by surgery

Pulmonary	5
Pneumonia	3
Pneumonitis	2
Cardiac ¹	1
Surgery related	6
Anastomotic leakage	4
Anastomotic stricture	1
Wound infection	1
Death within admission	0

¹Cardiac toxicities include heart attack, cardiac rhythm changes, pericardia effusion.

Duration of follow-up was defined as the interval between the day of completed surgery, death, or the last follow-up visit or telephone call. The Kaplan-Meier method was used to calculate survival probabilities. Survival analyses were performed using Prism Graph Pad Version 5.00 (GraphPad Software, Inc., LaJolla, CA).

RESULTS

During the study period, 57 patients underwent neoadjuvant chemoradiation therapy followed by esophagostomy. After exclusion of patients with insufficient data, those who did not meet study criteria, and those without adequate follow-up ($n = 22$), the records of 35 patients were reviewed. Of the 35, 19 patients were eligible and enrolled in the IMRT-SIB treatment protocol and two patients without histological identification of adenocarcinoma were excluded from this analysis. Thus, 17 patients completed the IMRT-SIB treatment and were included in this study. There were 15 men and 2 women and the mean postoperative follow-up was 2.5 years (range from 0.22-6.5).

All staging was performed before any treatment began. The mean age at time of diagnosis was 65 years (range, 45-76 years) and 15 patients were men. Of all patients, 88% had a uT3 tumor. A microscopically radical (R0) resection was achieved in 94% of patients. One patient had an R1 resection due to persistent dis-

ease in the gastric cardia. Total pathologic complete response ypT0No is 47%. The node-negative patient after neoadjuvant chemoRT is 82% (Table 2). Table 2 shows the pathologic staging and effects of SIB-based neoadjuvant CRT. The pathologic stages of cancer were T0, T1, T2, T3, and T4 in 8 (47%), 4 (23%), 3 (18%), 2 (12%), and 0 (0%), respectively. A comparison of clinical and pathologic stages revealed that SIB-based neoadjuvant CRT resulted in down-staging of either the T or the N status of 13 (76.5%) patients.

Hematologic toxicity from neoadjuvant chemoradiation with SIB was mild for all patients. A few patients received granulocyte-colony stimulating factor for neutropenia that did not occur during CRT. Among non-hematologic adverse effects, esophagitis ($n = 10$, 58.8%) and pneumonia ($n = 3$, 17%) was more common than pneumonitis ($n = 2$, 11%). Surgical leak was the most common surgical-related complication ($n = 4$, 23.5%) (Table 3). No survivors had symptoms due to late toxicities such as accumulated pleural or cardiac effusions during long-term follow-up. There were no treatment-related deaths.

After a minimum follow-up of 22 mo and a mean survival of 29 mo, the local recurrence rate was 11% ($n = 2$). Most patients had distant failure (35%) or combined local/regional and distant failure (5%). The majority of local recurrences were within 2 years of follow-up. In addition, the anastomosis was the only recurrence site in 5.8% of patients. There was one mediastinal relapse associated with positive nodes that was treated with a full dose of IMRT-SIB. There was no peritoneal carcinomatosis found.

For the 17 patients analyzed, nine were alive and eight had died at the end of follow-up, for a 3-year overall survival rate of 52% (Figure 2). Among the pCR, the 3-year overall survival was 75%, and compared with pathological persistent disease (pPD) the survival was 33% (Figure 2A). The 3-year disease-free survival rate for the 17 patients analyzed was 41.2%. The disease-free survival for the pCR and pPD subgroups were 63.55% and 22.2%, respectively (Figure 2B). There is no statistical significance among the overall survival analysis ($P = 0.0523$) and disease-free survival analysis ($P = 0.0897$). However, there is trend toward improved

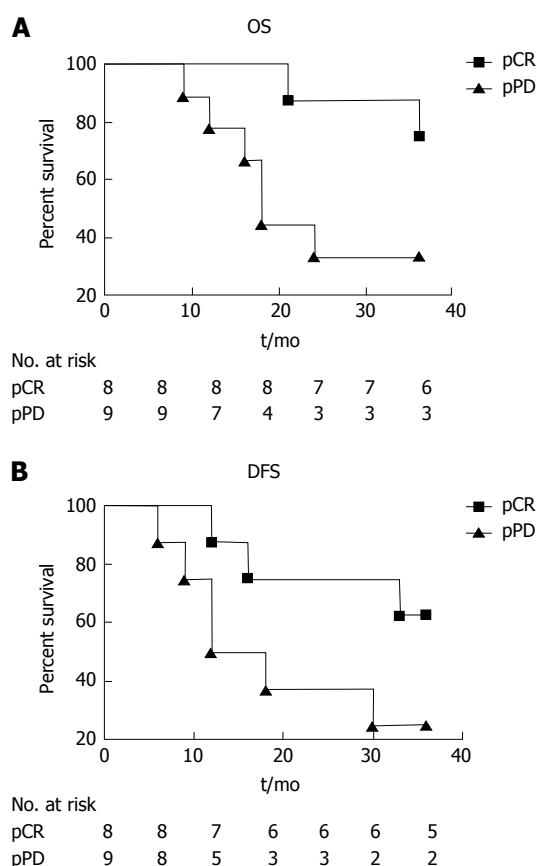


Figure 2 Kaplan-Meier survival curve. A: Overall survival for patients with pCR or without (pPD) ($P = 0.523$; $\chi^2 = 3.767$); B: Disease-free survival ($P = 0.897$; $\chi^2 = 2.879$). The disease-free survival represented from both local regional recurrences at anastomotic site, mediastinum, celiac trunk, or supraclavicular lymph nodes and distant metastases. OS: Overall survival; DFS: Disease free survival; pCR: Pathological completion response; pPD: Pathological persistent disease.

prognosis when comparing pCR vs not complete response, although this did not reach statistical significance.

In univariate survival analysis, among the various factors, post-triple modality node stages were a strong independent favorable predicting factor for survival ($P < 0.01$). Sex, the tumor size, the type of chemotherapy, the tumor location, and tumor configuration were not predicting factors for survival (Table 4).

Patients who had pCR to neoadjuvant treatment had a trend of benefit for the probability of survival compared to patients with pPD after neoadjuvant treatment. The 3-year overall survival rates were 75% vs 33% and the 3-year disease-free survival rates were 62.5% vs 22.2%, respectively. Interestingly, the analysis of histological regression after neoadjuvant showed improved survival rates if no or only rare residual tumor cells were found in the node specimen (Table 4).

DISCUSSION

Many reports show a consistent finding that response to preoperative therapy, particularly the absence of residual disease in the surgical specimen, is a good outcome indicator of better disease-free and overall survival^[8,12-14]. In a comprehensive literature review of 22 studies in

Table 4 Survival by prognostic factor

		Overall survival (%)		P value
		1 yr	3 yr	
Sex				
Female		2	100 (2)	0.017584
Male		15	73 (11)	
Tumor size				
> 3 cm		9	78 (7)	0.34649
< 3 cm		6	83 (5)	
Unknown		2	50 (1)	
Concurrent chemo				
5-FU based		11	82 (9)	0.078225
Cis based		6	83 (5)	
Tumor configuration				
> 180 cm		8	63 (5)	0.382319
< 180 cm		9	89 (8)	
Node status				
Negative		13	85 (11)	0.009262
Positive		4	75 (3)	
Location of primary tumor				
20-35 cm		12	83 (10)	0.024229
> 35 ~		5	80 (4)	
Total		17	82 (14)	

5-FU: 5-fluorouracil.

which patients with esophageal or esophagogastric junction cancer underwent esophagectomy after neoadjuvant CRT, patients with a pCR were two to three times more likely to survive than were those with residual disease in the esophagectomy specimen^[15]. These benefits translate into a 33% to 36% mean absolute survival benefit when a pCR is achieved than when it is not. These assumptions provide the rationale for intensification of preoperative treatment *via* a higher biological dose delivered to the tumor mass, without increasing the surrounding organs' risk of toxicity. The IMRT-SIB approach was based upon better radiographic findings of the biological target through PET scanning prior to CRT. This approach provides a better outline of the biological tumor within a mass, and allows RT dose intensity increase by 10% to the biological activity of tumor, without increasing the overall treatment time and the dose to the surrounding organs at risk^[16].

In the Cross study, although the improvement in overall survival is 14%, the improvement for adenocarcinoma is limited^[7]. The trend of improvement is not statistically significant. The greatest benefit was for squamous cell cancer. Few data were available on how to improve the outcome for esophageal adenocarcinoma and gastroesophageal cancers. Most reports had histology of adenocarcinoma and squamous cell cancer.

We reviewed our single-institute experience on adenocarcinoma using IMRT-SIB dose escalation technique, and found that the toxicity is low and local and that the incidence of distal recurrence is consistent with previous reports^[11,17,18]. Moreover, we found a possible association between that dose escalation with IMRT-SIB and the high pCR rate. The idea behind preoperative CRT in the treatment of esophageal and gastroesophageal junction cancer was to improve survival by reducing

locoregional failure^[13,18]. The high pCR rate through IMRT-SIB dose escalation to the tumor mass itself is a strong favorable prognostic factor for both locoregional and systemic recurrence and maybe even for overall survival^[19]. However, further studies are needed to explore the potential association between SIB-induced pCR and overall survival rate for adenocarcinoma.

The Cross study^[7] and the report by Hoepfner *et al.*^[20] used less than 4500 cGy dose protocol. In the Cross study, the low dose could explain the less favorable results for adenocarcinoma compared with squamous cell cancer histology^[7]. In the Hoepfner *et al.*^[20] report, the low-dose radiation could explain the less favorable results for neoadjuvant chemoradiation compared with perioperative chemotherapy for adenocarcinoma. At least, the suggestion of the low dose of radiation is one of factor contributed to low pCR was reported^[21]. In addition, whether predicting value of the SIB induced pCR and conventional dose resulted pCR are the same? Is there anything else be young dose response relationship between the gross tumor volume and delivered dose? Does dose escalation impact on risk of distant metastasis? More future studies need to explore above questions.

Since all cancers in the present study were in the lower distal esophagus, the radiation field was limited to the lower mediastinum and did not include in supraclavicular regions, and there were no recurrences in the supraclavicular areas. Strict dose constrains to the heart were used and no significant cardiac events were noted. Although some reports suggested that higher treatment morbidity and mortality were associated with neoadjuvant CRT^[20,22], the current cohort's patients had acceptable tolerance.

To our knowledge, ours is the first report of using IMRT-SIB with dose escalation resulting in high pCR in adenocarcinoma of esophagus. However, our study has limitations. First, this was a retrospective review that mostly consisted of the patients who were treated by a dedicated multidisciplinary team at a single institution. Selection biases from the study existed. Second, the sample size was small and only ypN was a positive predictive factor in univariate analysis; a larger size study will provide more reliable conclusions. Moreover, although we have reviewed all esophageal adenocarcinoma cases in our institute from the past 10 years, the IMRT-SIB has been implemented only recently and, as a result, the follow-up for this subgroup of patients was short. In a prospective setting, the patients could be stratified by different prognostic clinical variables in an effort to better elucidate the role of SIB dose escalation in certain patient groups.

In conclusion, the dose escalation through IMRT-SIB in the chemoradiation regimen seems responsible for the down staging of the distal esophageal or gastroesophageal junction tumors. The protocol is well-tolerated, postoperative complications were acceptable, and the complete resection rate is high. This radiation therapy dose escalation strategy warrants further

investigation.

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COMMENTS

Background

Improvement in resection rate is very important for advanced esophageal cancer, balance between toxicity and benefit from neoadjuvant treatment is always the focus of multidisciplinary oncology team. Neoadjuvant concurrent chemoradiation is becoming a standard of care for locally advanced esophageal cancer.

Research frontiers

The authors reported here a protocol that improved resection rate through simultaneous integrated boost (SIB) based dose escalation technique without comprising the toxicities. Few reports using this protocol.

Applications

Intensity modulated radiation therapy with SIB (IMRT-SIB) has much safe toxicity profile and less equipment restraint. This finding needs further large phase III clinical studies to confirm.

Terminology

IMRT-SIB is a novo radiation technique to deliver much higher dose to the biological gross tumor volume defined by positron emission tomographic positive area without increase radiation dose to all other area surrounding to it.

Peer-review

The authors present a retrospective analysis of an intensified regimen in neoadjuvant chemoradiation of advanced distal adenocarcinoma of the esophagus. This work is of interest for the oncology community.

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TOPIC HIGHLIGHT

- 481 Colorectal cancer in the young, many questions, few answers
Deen KI, Silva H, Deen R, Chandrasinghe PC
- 489 On the road to standardization of D2 lymph node dissection in a European population of patients with gastric cancer
Yarema R, de Manzoni G, Fetsych T, Ohorchak M, Pliatsko M, Bencivenga M

REVIEW

- 498 Malignant biliary obstruction: From palliation to treatment
Boulay BR, Birg A
- 509 Genetic risks and familial associations of small bowel carcinoma
Shenoy S

ORIGINAL ARTICLE

Case Control Study

- 520 Genetic polymorphisms of *interleukin 1 β* gene and sporadic pancreatic neuroendocrine tumors susceptibility
Karakaxas D, Sioziou A, Aravantinos G, Coker A, Papanikolaou IS, Liakakos T, Dervenis C, Gazouli M

Contents

World Journal of Gastrointestinal Oncology
Volume 8 Number 6 June 15, 2016

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World Journal of Gastrointestinal Oncology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-85381891
Fax: +86-10-85381893
E-mail: editorialoffice@wjnet.com
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Fax: +1-925-223-8243
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2016 Colorectal Cancer: Global view

Colorectal cancer in the young, many questions, few answers

Kemal I Deen, Hiroshi Silva, Raed Deen, Pramodh C Chandrasinghe

Kemal I Deen, Consultant in Colon and Rectal Surgery, The Asiri Surgical Hospital, Colombo 11600, Sri Lanka

Hiroshi Silva, Pramodh C Chandrasinghe, The University of Kelaniya Medical School, Ragama 11600, Sri Lanka

Raed Deen, The University of Sydney Medical School, Sydney NSW 2006, Australia

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Correspondence to: Kemal I Deen, MD, MS, FRCS, FACRSI, FNat, Ac Sci, Consultant in Colon and Rectal Surgery, The Asiri Surgical Hospital, No.21 Kirimandala Mawatha, Colombo 11600, Sri Lanka. kemaldeen4@gmail.com
Telephone: +94-777-746158

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Abstract

At a time where the incidence of colorectal cancer, a

disease predominantly of developed nations, is showing a decline in those 50 years of age and older, data from the West is showing a rising incidence of this cancer in young individuals. Central to this has been the 75% increase in rectal cancer incidence in the last four decades. Furthermore, predictive data based on mathematical modelling indicates a 124 percent rise in the incidence of rectal cancer by the year 2030 - a statistic that calls for collective global thought and action. While predominance of colorectal cancer (CRC) is likely to be in that part of the large bowel distal to the splenic flexure, which makes flexible sigmoidoscopic examination an ideal screening tool, the cost and benefit of mass screening in young people remain unknown. In countries where the incidence of young CRC is as high as 35% to 50%, the available data do not seem to indicate that the disease in young people is one of high red meat consuming nations only. Improvement in our understanding of genetic pathways in the aetiology of CRC, chiefly of the MSI, CIN and CIMP pathway, supports the notion that up to 30% of CRC is genetic, and may reflect a familial trait or environmentally induced changes. However, a number of other germline and somatic mutations, some of which remain unidentified, may play a role in the genesis of this cancer and stand in the way of a clear understanding of CRC in the young. Clinically, a proportion of young persons with CRC die early after curative surgery, presumably from aggressive tumour biology, compared with the majority in whom survival after operation will remain unchanged for five years or greater. The challenge in the future will be to determine, by genetic fingerprinting or otherwise, those at risk of developing CRC and the determinants of survival in those who develop CRC. Ultimately, prevention and early detection, just like for those over 50 years with CRC, will determine the outcome of CRC in young persons. At present, aside from those with an established familial tendency, there is no consensus on screening young persons who may be at risk. However, increasing awareness of this cancer in the young and the established benefit of prevention in older persons, must be a message that should be communicated with medical students,

primary health care personnel and first contact doctors. The latter constitutes a formidable challenge.

Key words: Colon cancer; Young age; Rectal cancer; Colorectal cancer; Young patients; Survival; Early onset

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Core tip: This review of colorectal cancer in the young focuses on new data that reveal CRC to be more a left sided cancer than previously thought and the predicted rise by the year 2030. The article outlines the genetics of colorectal cancer (CRC) and discusses limitation in current knowledge in establishing a fingerprint for sporadic CRC. Aside from diet in its aetiology, luminal alkalinity and the colonic microbiome may be contributory and require further research. The review discusses the need for increased awareness of CRC in the young and the need for global consensus on screening young people at risk.

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INTRODUCTION

Colorectal cancer (CRC) is now the fourth most common cause of cancer deaths, with 600000 deaths reported worldwide annually - about 8% of all cancer deaths^[1,2]. It is the third most common cancer in men and the second most common cancer in women. The sporadic form, known to affect individuals in their fifth and sixth decades of life^[3], arises from a pre-existing polyp which progresses to cancer through the adenoma-dysplasia-carcinoma sequence; a pathological process which, in general, takes five to ten years^[4], and lends itself to prevention by screening^[5,6]. CRC is a disease of developed nations, and screening by faecal occult blood testing and colonoscopy has stemmed its incidence in those over 50 years^[6]. By contrast, CRC in the young, was a disease prevalent in the developing world^[7-14] compared with Australia, New Zealand and the West, where its prevalence in young individuals was low^[11,15,16]. However, more recently, there has been an increase in the number of reports of CRC in the young from the developed world^[17-19]. This is of concern because the incidence of rectal cancer has risen by 75% in the last 40 years^[20-22], contributing chiefly to the overall rise in cancer prevalence. Furthermore, this disease affects people in the prime of their life, and unlike cancer in older individuals, there is limited knowledge about the aetiology and pathogenesis of CRC in the young. The aim of this review is to present the current status of CRC in the young and to highlight areas for future research.

EPIDEMIOLOGY/PREVALENCE

Historically, CRC in young patients was highest in proportional prevalence from the Asian region. Studies have reported a high young cancer prevalence of 38% in Egypt^[7], 18% in Turkey^[8], 39% in India^[9], 29% in Nepal^[10], 23% from Saudi Arabia^[11], 19.7% from Sri Lanka^[12], 52% from a single institution in Pakistan^[13] and 10.1% from Taiwan^[14]. Most significantly, a recent study from the United States^[19], where the authors evaluated the records of 393241 patients over a 15-years period, revealed an overall decline in CRC by 0.92% - the effect attributed to screening. While this was true for those over 50 years old with CRC, the study observed an alarming increase in CRC in those less than 50 years, specifically, in young patients less than 35 years. Using statistical modelling, the authors predicted an increase in colon cancer by 90% in patients aged 20 to 34 years and 27.7% in those 35 to 49 years old by the year 2030. For rectal cancer, the predicted percentage increase in cancer prevalence for these two age groups was 124.2% and 46% respectively. Gender based analysis of CRC in young patients revealed an equal prevalence in young men and women^[22] contradicting the theory that female hormones are protective of colon and rectal cancer. Furthermore, a 1991 study of young patients in North America showed that the disease occurred in 34% more black men and 45% more black women compared with white Caucasian counterparts^[23]. Most young patients did not report a family history of CRC; O'Connell *et al*^[22] revealed that only 23% of young patients with CRC reported the presence of cancer in a family member.

FAMILY HISTORY

Contrary to previous knowledge, a current estimate of the proportion of CRC likely to have a major hereditary component is between 15% and 30%^[24]. The common heritable syndromes in CRC are either familial adenomatous polyposis (FAP) or hereditary non-polyposis colorectal cancer (HNPCC)^[25-27] known to be found in 2 to 5 percent of all patients with CRC. Familial adenomatous polyposis is defined by phenotype if an individual has multiple colonic polyps, usually over 100, in association with loss of the tumour suppressor gene -the adenomatous polyposis coli-APC gene-located on the long arm of chromosome 5 (5q21)^[25]. Most FAP patients will develop CRC by age 40 years, while in a minority, cancer will manifest in the fifth decade or after, due to the presence of the attenuated FAP gene. In contrast to FAP, HNPCC, first described by Henry Lynch, is characterised by the presence of fewer colonic polyps or cancer that is indistinguishable from sporadic CRC. In both conditions, which are of autosomal dominant inheritance, family history is of prime importance. For HNPCC, an affected member or members of a family should have had either CRC (Lynch type 1-site specific) or other extra-intestinal cancers (Lynch type 2), in association with

an index patient with CRC. In the absence of definitive genetic testing, a detailed family history was essential and formed the core of the Amsterdam and Bethesda criteria to make a diagnosis of HNPCC^[28,29]. Currently, we know that young patients with an underlying genetic syndrome are more likely to have a family history of cancer and present earlier compared with those with no known genetic syndrome, who presented with late stage metastatic disease^[30]. Thus, family history must continue to remain an essential component of clinical evaluation in patients with CRC, while it is essential to note that up to 20 percent of patients with a germline mutation in the study reported by Mork *et al.*^[30] had no family history of CRC.

ANATOMIC DISTRIBUTION

Several studies have reported that CRC in the young is a condition mostly confined to the left colon and rectum; in a retrospective study of young patients, Leff *et al.*^[31] revealed that 65% of cancers were in the rectum and that 83% of all colon and rectal cancers were distal to the splenic flexure. Kumar *et al.*^[32] reported that CRC was confined to the left colon and rectum in 67% of their study population. Furthermore, O'Connell *et al.*^[22] in a structured review of 55 studies comprising 6425 patients with young CRC, reported that cancer of the rectum was most frequent (54%). In the most recent publication of the Surveillance Epidemiology and End Result (SEER) study from the United States, dominance of cancer in the left colon and rectum was again mirrored^[19].

PRESENTATION

Studies have shown that CRC in young patients presents with three cardinal features of rectal bleeding, abdominal pain and alteration in bowel habit - constipation, altered stool diameter, mucoid rectal discharge^[33,34]. In general, CRC diagnosis in young patients was associated with a delay of approximately 6 mo^[33]. Physician related delay in diagnosis was chiefly because of a lack of understanding and suspicion of this disease in the young, where symptoms in young patients were considered due to such benign causes as haemorrhoidal disease by first contact physicians and patients alike. Some other factors that may contribute to delay are patients' preference in seeking non-traditional methods of symptom relief, such as Ayurvedha and Chinese medical treatment, in Asia, and because practitioners of allopathic medicine fail to perform a focused rectal examination at the point of first contact. With current worldwide reports of increasing prevalence of young CRC, it is important that we offer young symptomatic patients flexible sigmoidoscopy early, after comprehensive clinical examination, including focused digital rectal examination.

PATHOLOGY

In young patients, CRC is likely to be found in those

with a heritable syndrome^[28-30] such as FAP and HNPCC. In the Lynch Syndrome, tumours have been known to be predominant in the proximal colon^[35,36], but recent research revealed contradictory data where the most frequent site among early onset CRC patients was the distal colon^[37]. Of these, between 40 and 60 percent were in the rectum^[38,39]. In the WHO classification of tumours^[40], HNPCC and sporadic CRC with microsatellite instability have been classified based on the site and microscopic criteria. These are (1) proximally located mucinous adenocarcinomas which are commonly well circumscribed and are moderate-to-well differentiated; (2) proximally located poorly differentiated adenocarcinomas which show failure of gland formation with malignant epithelium arranged in small clusters, irregular trabeculae or large aggregates in well circumscribed tumours; and (3) adenomas in HNPCC indicating features of high cancer risk including villous and high grade intraepithelial neoplasia which display good circumscription and present as polypoid growths, plaques, bulky masses or ulcers rather than diffuse growths or strictures^[40]. In a single centre study, mucinous and signet-ring histological subtypes and poor to non-differentiated tumours were frequently seen among the young^[38,41,42], and accounted for 41.5% of all tumours^[38]. The incidence of tumour *in situ* (Tis) was lower in young patients compared with older patients and may indicate either failure of early detection or rapid progression from adenoma to carcinoma in the young compared with older patients^[43]. Other features that suggest more aggressive tumour biology in the young compared with older patients are the higher percentages of patients with lymph node metastasis (≥ 4 lymph nodes), distance metastasis and stage IV disease^[41,42].

GENETICS

All colorectal cancers occur from genetic mutations, which are part of a familial syndrome, hereditary syndrome or as sporadic cancer^[44]. Frequent among young patients are either FAP, variants of FAP or HNPCC. Historically, in the sporadic subtype, the origin of CRC was attributed to various common or rare genetic alterations that displayed variable penetrance, and remained largely unidentified^[45]. It is now estimated that up to 30% of CRC may have a hereditary component, with identifiable genetic aberration, especially if cancer occurs in the young^[23,24,30,46]. Next generation sequencing (NGS) is likely to further increase our knowledge of hitherto unidentified chromosome aberrations in association with cancer^[47] resulting in such diagnoses as the Li-Fraumeni syndrome, Cowden's disease, Juvenile polyposis and Peutz-Jegher syndrome^[46].

Different from germline mutations, somatic mutation, that may be spontaneous or follow contact with luminal carcinogens, may result in genetic alteration of a colonocyte in which control of apoptosis is lost in conjunction with a series of chromosomal changes that create microsatellite instability^[43]. In fact, the aetiology and range of hitherto unidentified germline and early onset somatic mutations is likely to be more extensive than

previously understood, which makes our understanding of the pathology in young patients with sporadic cancer even more complex. Essential to our understanding of tumourigenesis is knowledge of preservation of DNA integrity in the intestinal epithelial cell; deep within the base of the intestinal crypt lies the colonocyte stem cell that is covered in a thick layer of mucus. Each stem cell is designed to replicate into a transit amplifier stem cell and an inert stem cell that remains in the protected crypt base, remote from contact with carcinogens that may be present in the lumen of large bowel, thus preserving its DNA intact. In health, upward migration of the amplifier cell will give rise to a functional colonocyte that will shed in 5 to 7 d by genetically determined apoptosis, controlled by the *p53* gene located on chromosome 17 and the mitogen-activated protein kinase pathway (MAPK)^[43]. The MAPK pathway, of which KRAS and BRAF proteins are part, regulates cell proliferation, cell differentiation, cellular aging and apoptosis^[48]. Programmed colonocyte death prevents the propagation of mutagenic change, and constitutes yet another strategy of preserving intestinal cell DNA integrity^[43]. In the adenoma-carcinoma sequence, initialisation of neoplastic change occurs with silencing of the tumour suppressor genes located on chromosome 5 (*APC* gene), followed by serial changes in chromosome 17 (*p53* gene-mutated in colorectal cancer) and chromosome 18 (long arm deletion)^[49]. Furthermore, simultaneous activation of the proto-oncogene K-Ras will lead to uncontrolled cell growth^[49]. Hence, both germline mutations and somatic mutations may drive colorectal cancer in the young.

Currently, the genetic mechanisms that trigger CRC are grounded in three major pathways; chromosomal instability (CIN), microsatellite instability (MSI) and the cytosine-phosphate-guanine island methylator phenotype pathway (CIMP) pathway^[50,51] - mechanisms that create genomic instability, which together with a process that will selectively support mutagenic driver cells, produce colorectal cancer. It is essential in our understanding of this process that none of these pathways is mutually exclusive. However, CIN aberrations, by far, constitute the most common pathway in the development of CRC^[52].

CIN pathway

This describes the classical adenoma-dysplasia-carcinoma sequence in which it is thought that tumour formation is a result of progressive and sequential inactivation of tumour suppressor genes and, correspondingly, activation of tumour promoting oncogenes - mutation in the adenomatous polyposis coli (*APC*) gene being an important initial step in this pathway^[52]. Likewise, it is known that mutation of the KRAS oncogene contributes to CIN-associated sporadic CRC in up to a half of such sporadic cancer^[53]. Since RAS proteins control signaling in cell differentiation and apoptosis, disruption of such pathways will lead to neoplastic transformation. CIN-associated tumours comprise 75% to 80% of all tumours

found in Western populations^[54].

MSI pathway

It is known that formation of new strands of DNA may be interrupted by base pair mismatches, *i.e.*, mutations which may be either deletions or insertions. In health, the role of mismatch repair proteins is to bind, remove and repair the region of the mismatch error. In cells with malfunction of mismatch repair proteins, these mutations will tend to accumulate within areas of DNA coding called microsatellites. Such areas of microsatellite instability are the cause of sporadic CRC^[55].

CIMP pathway

This pathway of CRC differs fundamentally from CIN and MSI, in that, it causes mutation and epigenetic silencing of genes that control the cell cycle outside the APC control system. This pathway is chiefly associated with a group of protein kinases known as BRAF proteins, and usually occurs due to promoter methylation and silencing of the mut-L homologue 1 gene (MLH-1- short arm of chromosome 3), resulting in microsatellite instability. CIMP associated cancer is frequently found in patients of older age, has a slight female preponderance and is associated with right sided colon cancer, similar to the Lynch syndrome. However, it is rare for patients with Lynch syndrome-associated CRC to have BRAF mutations, which helps differentiate Lynch syndrome associated CRC from sporadic CRC^[56]. Thus, it becomes evident that no two colorectal cancers are likely to be the same, and that each will have its own unique characteristic genetic "fingerprint". It is also known that each cancer may have more than one of the aforementioned carcinogenic pathways^[57,58], which makes genetic imprinting of sporadic CRC all that more challenging. Furthermore, since CIN and MSI associated CRC is known to respond differently to chemotherapeutic agents and impact on cancer related survival, to enable tumour specific personalized treatments, future standard pathological tumour work-up may have to include such genetic "fingerprinting".

RISK FACTORS

A historic study of tumour genesis in the colon shed light on the alkaline environment in the lumen of the colon which, combined with secondary bile acids, is a promoter of tumour formation^[59]. N-nitroso compounds and ammonia, produced from bacterial action upon undigested protein products, and secondary bile acids alter the luminal environment, which affect colonocyte function and deplete oxygen levels in the colonic mucosa, thus favouring tumourigenesis. Furthermore, rapid urbanization with environmental pollution, lifestyle alterations such as reduction in physical activity and change in dietary patterns in young individuals^[9,60], may have also contributed to the rising incidence of CRC, although this alone does not explain its disproportionate rise in incidence in previously

low incidence parts of the world^[61].

SURVIVAL

Multiple studies of young patients with CRC from cancer registries have shown that, in young patients, 5-year survival did not differ from older patients despite a greater proportion of locally advanced cancer, regional lymph node involvement and less favourable histological types in the young^[61-63]. Ruiz *et al*^[64] showed an overall survival rate of 69.4% and 67.4% at 5 years for colon and rectal cancer respectively from a cancer registry database in Peru. Likewise, Parc *et al*^[63], reporting survival data from the central South Korean cancer registry, revealed a 5-year survival of 66% for young patients with cancer of the proximal colon, 70% for patients with distal colon cancer and 66% in patients with rectal cancer. However, if young patients with CRC present with concomitant metastasis, or in the case of a small proportion of patients with unfavourable histological features (poorly differentiated cancer, signet ring cancer), survival may be poor^[65]. Chan *et al*^[66] have shown that survival in young patients with a poor prognosis is predictable, and that maximum survival in this group of young patients after surgical intervention is no more than 20 mo.

SCREENING

CRC screening guidelines currently recommend routine screening of individuals from the age of 50 years. The screening tests range from invasive procedures such as flexible sigmoidoscopy and colonoscopy, through imaging investigations such as virtual colonoscopy, to minimally invasive procedures such as faecal occult tests^[67].

Although each test has its own different advantages and limitations, colonoscopy - widely regarded as the gold standard - has shown to decrease the incidence of CRC up to 80%. However, it is essential to note that colonoscopy is not a perfect test - studies have shown a miss rate of 6%-12% of adenomas > 1 cm and 5% for CRC^[67]. Faecal occult tests have shown promise too; an example being, the faecal immunochemical test which has shown high rates of detection of prevalent CRC in an asymptomatic population^[68].

With the rising incidence and mortality of CRC in young patients, effective screening methods must be able to detect these tumours early. Current guidelines suggest that individuals with a family history of CRC or adenomatous polyps, other than FAP, undergo screening earlier than at 50 years. That is, from the age of 40 or 10 years before the youngest cancer affected family member, while those with a family history of FAP undergo screening in adolescence^[68]. Population based early-onset CRC screening has not been justified due to low prevalence, cost and potential adverse procedural outcomes outweighing the benefits^[17]. To detect early onset CRC, suggestions have been to undertake routine screening from 40 years, instead of 50 years - however, decision

analysis models have shown no significant life-year gains for this change^[37].

To combat the rising incidence by screening of potential early onset CRC patients, awareness among physicians, primary healthcare workers and the lay public must increase. For the physician, this should begin at the stage of medical school by integration of preventive medicine and longitudinal cancer prevention modules into medical school curriculums - which have shown positive results^[69], and will improve the future physician's ability to identify young individuals at high risk.

In terms of young patient awareness, it is imperative that young adults are aware of screening for early onset CRC. A study revealed that university students had very poor knowledge of CRC screening, indicating the necessity for early-onset cancer awareness campaigns^[70]. Another feasible plan to improve screening rates is the employment of a well-trained lay cancer-screening navigator; this person's role would involve contacting individuals, discussing the importance of screening for CRC and implementing screening procedures such as faecal tests sent by mail. Although this was a feasible strategy for older patients aged 50 to 74 years^[71], it has yet to be determined how effective this strategy would be in younger individuals.

To avoid low screening rates, patients' screening method preferences require consideration. Studies have shown faecal aversion to be one of the chief hindrances to screening participation, and a survey revealed that 78% of participants would prefer to provide a blood sample instead^[72]. One such blood test to detect CRC, which requires further development, is the assessment of circulating methylated SEPT9 DNA, and although it is able to detect CRC in an asymptomatic individual, improved sensitivity is required for population screening^[73]. A highly sensitive and specific blood test for CRC could very well become the gold standard in the future, and thereby decrease incidence and mortality rates.

CONCLUSION

An epidemic of colorectal cancer in young patients is imminent. Based on better understanding of genetic mechanisms, currently it is estimated that genetic predisposition to colorectal cancer is 30% of all CRC. The figure is likely to be higher in young patients if all young patients with CRC were to have genetic assessment by NGS testing. While the MSI, CIN and CIMP pathways have been isolated and well defined, a number of germline and somatic mutations in CRC are likely to manifest from widespread use of NGS, multiple panel genetic tests. Furthermore, multiple permutations of genetic alterations are likely to show up in individual CRCs, with overlap of previously known syndrome based genetic changes, which will make individual genetic fingerprinting of CRC more complex and perhaps the age of onset of CRC, that is, whether young or older, irrelevant. In lifestyle assessment, populations, such as in Egypt, where consumption of red meat is high seem to have similar proportions of young

patients with CRC compared with predominantly non-meat eating populations, such as is found in India, which further complicates the search for a common lifestyle aetiology. What is common across the world in lifestyle is the growing fast food industry and childhood obesity; more thought and research needs to focus on its contributory role. For the present, the majority of cases of CRC remains sporadic and of multifactorial origin: Diet and nutrition, obesity, the colonic microbiome, smoking, alcohol consumption and hitherto unknown germline or somatic mutation. The role of screening for CRC in young patients is not likely to follow a "one test fits all" policy until we have worldwide genetic data in this group of patients. At present, mass screening by flexible sigmoidoscopy is expensive and may yield low productive rates. However, better education of medical students, primary healthcare personnel and first contact doctors, about the benefit of prevention and early detection of CRC in the young is likely to improve early detection rates in young persons. Whether early detection influences lead-time in such young patients with cancer remains unresolved, as some studies have shown a clear cut-off in survival at around 2 years. It is a formidable challenge to fight the rising incidence and mortality in early onset CRC patients, an effort that will require global co-operation and consensus.

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2016 Gastric Cancer: Global view

On the road to standardization of D2 lymph node dissection in a European population of patients with gastric cancer

Roman Yarema, Giovanni de Manzoni, Taras Fetsych, Myron Ohorchak, Mykhailo Pliatsko, Maria Bencivenga

Roman Yarema, Taras Fetsych, Department of Oncology and Medical Radiology, Lviv National Medical University named after Danylo Halytskyi, 79010 Lviv, Ukraine

Giovanni de Manzoni, Maria Bencivenga, Department of Surgery, General and Upper G.I. Surgery Division, University of Verona, 37126 Verona, Italy

Myron Ohorchak, Department of Abdominal Surgery, Lviv Regional State Cancer Diagnostic and Therapeutic Center, 79000 Lviv, Ukraine

Mykhailo Pliatsko, Department of Endoscopic, Lviv Regional Clinical Diagnostic Center, 79010 Lviv, Ukraine

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Correspondence to: Roman Yarema, MD, PhD, Department of Oncology and Medical Radiology, Lviv National Medical University named after Danylo Halytskyi, Pekarska str., 69, 79010 Lviv, Ukraine. yaremarom@rambler.ru
Telephone: +38-67-9406933
Fax: +38-32-2757632

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Abstract

The amount of lymph node dissection (LD) required during surgical treatment of gastric cancer surgery has been quite controversial. In the 1970s and 1980s, Japanese surgeons developed a doctrine of aggressive preventive gastric cancer surgery that was based on extended (D2) LD volumes. The West has relatively lower incidence rates of gastric cancer, and in Europe and the United States the most common LD volume was D0-1. This eventually caused a scientific conflict between the Eastern and Western schools of surgical thought: Japanese surgeons determinedly used D2 LD in surgical practice, whereas European surgeons insisted on repetitive clinical trials in the European patient population. Today, however, one can observe the results of this complex evolution of views. The D2 LD is regarded as an unambiguous standard of gastric cancer surgical treatment in specialized European centers. Such a consensus of the Eastern and Western surgical schools became possible due to the longstanding scientific and practical search for methods that would help improve the results of gastric cancer surgeries using evidence-based medicine. Today, we can claim that D2 LD could improve the prognosis in European populations of patients with gastric cancer, but only when the surgical quality of LD execution is adequate.

Key words: Gastric cancer; D2 lymph node dissection; Evidence-based medicine; European patients; Regional lymph nodes

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Core tip: The amount of lymph node dissection required

during surgical treatment of gastric cancer has been quite controversial. We can now claim that D2 lymph node dissection improves the prognosis in European populations with gastric cancer, but only when the surgical quality of the lymph node dissection execution is adequate.

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INTRODUCTION

Radical surgery for malignant tumors traditionally includes mandatory one-piece removal of regional lymph nodes (LNs). This approach was introduced over 100 years ago by an American surgeon, W.S. Halsted, and has been used to determine the extent of surgery in basic sites of neoplasia including tumors in the gastrointestinal tract. Despite its high clinical effectiveness and use as a standard treatment in Asia, extensive D2/D3 lymph node dissection (LD) has not been widely used in gastric cancer (GC) surgery in Europe and the Americas until recently.

Indeed until recently, European clinical recommendations for cancer treatment did not suggest D2 LD as a surgical standard of care^[1]. The relevance of this issue is also evident when considering the surgical standard of Western randomized trials on multimodal treatment for GC. The MAGIC trial set the standard for combined treatment of GC in the European Union, and D2 LD was performed in only 42.5% of patients^[2]. The US standard multimodal treatment for GC is based on the INT 0116 trial^[3] in which an extended LD was performed in only 10% of patients. In a large-scale clinical trial on perioperative chemoradiotherapy effectiveness (the CRITICS trial; ongoing in Europe), the planned extension of LD is more limited than D2^[4]. Thus, the issue of standardization in lymphadenectomy extension for GC in Western countries remains relevant.

DEFINITION AND LEVELS OF LYMPHNODAL DISSECTION IN GASTRIC CANCER

Lymphatic efflux from the stomach travels through a complex multidirectional network^[5]. Lymph from different sections of the stomach is drained into the para-aortal LN collector through one of four routes: (1) left subdiaphragmatic *via* the LN in the circulation of the left lower diaphragmatic artery; (2) abdominal *via* the LN along the left gastric, splenic, and common hepatic arteries and the celiac trunk; (3) upper mesenteric that receives lymph from the subpyloric LNs and runs along

the upper mesenteric artery; and (4) retropancreatic, which is associated with LNs of the hepatoduodenal ligament, upper mesenteric vessels and common hepatic artery. Both the left subdiaphragmatic and abdominal routes drain lymph from the upper third of the stomach. The lymphatic efflux from the gastric body drains primarily through the abdominal route, and lymph efflux from the distal stomach drains through abdominal, upper mesenteric and retropancreatic routes^[6].

Metastases to regional LNs are diagnosed in 37%-65% of patients with tumors in the gastric corpus, in 44%-80% of patients with tumors in the proximal stomach, and in 50%-59% of patients with tumors in the distal stomach^[7,8]. The involvement of regional LNs depends directly on the depth of primary tumor invasion. In intra- and sub-epithelial tumors, regional lymphogenous metastases are diagnosed in 0%-5.5% and 19%-31% of patients, respectively^[7,9]. In muscle or subserosal layer invasions, regional LN involvement increases to 30%-62%; in serous membrane tumors, regional LN metastases are found in 74% of patients, and 90%-91% in cases with infiltration of adjacent organs^[7].

The first one-piece tissue dissection of regional lymphogenous metastasis during the course of GC surgery was carried out in 1962 by Jinnai *et al*^[10]. Since then, the concept of extended radical LD has become an essential stage in the strategy of GC surgical treatment in Japan. Research in the field of lymph node (LN) topography and extended clinical efficiency formed the basis of the first edition of "General Rules for the Gastric Cancer Study", which was published in the early 1960s under the auspices of the Japanese Research Society for Gastric Cancer^[11]. The first English edition of these guidelines was published in Europe in 1995. Subsequently, research performed by the Japanese Gastric Cancer Association (JGCA) formed the basis for a second English edition based on the Japanese classification of gastric cancer by the JGCA^[12] as well as Japanese gastric cancer treatment guidelines^[13]. These guidelines describe the following groups of stomach LNs (Table 1, Figure 1).

According to the classification of gastric cancer by the JGCA (1998)^[12], the stomach lymphatic system consists of three LN compartments. Each of these is a temporary barrier that prevents tumor cells from entering the lymphatic system. Grouping stomach lymph collectors into compartments created the basis for determining the gradation of category "N" at staging and a theoretical basis for the extension of LD according to tumor site as reported in the following table (Table 2)^[12]. The LN groups 12b, p and above are classified as N3 - in the given classification-this is equivalent to distant metastases.

Of note, in the last version of tumor-node-metastasis (TNM) classification introduced by the Union for International Cancer Control (UICC)^[14], category "N" is determined not by the topography but rather by the number of affected regional LNs. Accordingly, in the last version of JGCA guidelines (2011)^[13], the extension of nodal dissection is defined according to the extension of

Table 1 The lymphatic system of the stomach^[12]

LN groups	LN topography
Nº1	Right paracardiac LNs
Nº2	Left paracardiac LNs
Nº3	LNs along the lesser curvature
Nº4sa	LNs along the short gastric vessels
Nº4sb	LNs along the left gastroepiploic vessels
Nº4d	LNs along the right gastroepiploic vessels
Nº5	Suprapyloric LNs
Nº6	Infrapyloric LNs
Nº7	LNs along the left gastric artery
Nº8a	LNs along the common hepatic artery (anterosuperior group)
Nº9	LNs at the celiac trunk
Nº10	LNs at the splenic hilum
Nº11p	LNs along the proximal splenic artery
Nº11d	LNs along the distal splenic artery
Nº12a	LNs in the hepatoduodenal ligament (along the hepatic artery)
Nº12b	LNs in the hepatoduodenal ligament (along the bile duct)
Nº12p	LNs in the hepatoduodenal ligament (behind the portal vein)
Nº13	Retro-pancreaticoduodenal LNs
Nº14a	LNs along the superior mesenteric artery
Nº14v	LNs along the superior mesenteric vein
Nº15	LNs along the middle colic vessels
Nº16	Para-aortic LNs
Nº17	LNs on the anterior surface of the pancreatic head
Nº18	LNs along the inferior margin of the pancreas
Nº19	Infradiaphragmatic LNs
Nº20	LNs in the esophageal hiatus of the diaphragm

LNs: Lymph nodes.

gastric resection as reported in the following figures.

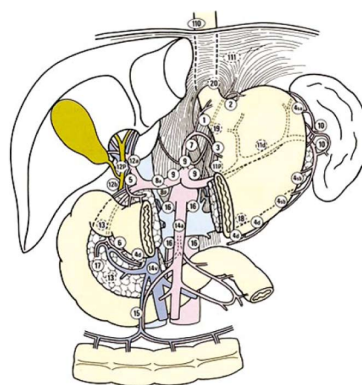
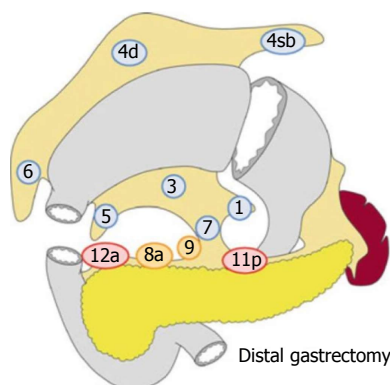
During distal subtotal gastrectomy, the lymph node dissection levels are as follows: (1) D0: LD in a volume less than D1; (2) D1: Nº1, 3, 4sb, 4d, 5, 6, 7; (3) D1 +: D1 plus Nº8a, 9; and (4) D2: D1 plus Nº8a, 9, 11p, 12a (Figure 2).

In gastrectomy, the LD levels are as follows: (1) D0: LD in a volume less than D1; (2) D1: Nº1-7; (3) D1 +: D1 plus Nº8a, 9, 11p; and (4) D2: D1 plus Nº8a, 9, 10, 11p, 11d, 12a (Figure 3).

Levels of LD in proximal subtotal gastrectomy: (1) D0: LD in a volume less than D1; (2) D1: Nº1, 2, 3a, 4sa, 4sb, 7; and (3) D1 +: D1 plus Nº8a, 9, 11p (Figure 4).

LD extended beyond these definitions are classified as D2 +. Their effectiveness remains controversial; therefore, they are currently not recommended for routine use in clinical practice^[13].

Gastric cancer classification by JGCA (1998) has demonstrated its high efficiency in several clinical studies^[5,15,16]. LN staging based on topography laid the grounds for JGCA's classification. These are considered anatomical in contrast to the rather mechanistic quantitative approach of the UICC classification. This allows for consideration of disease propagation and for more accurate prognosis. In support of this thesis, the correlated survival of patients with lesions of various LN groups has been studied patients with the same number of regional lymphogenous metastases, survival

**Figure 1** Topography of stomach lymph node groups^[12].**Figure 2** Lymph node dissection levels in distal subtotal gastrectomy^[13].

differed depending on the LN collectors in which lesions were located^[17]. Thus, localization as well as the quantity of metastatically-affected regional LNs has a probable prognostic value. According to Y. Noguchi^[18], in N0, LN lesion groups 1-6 (N1 according to JGCA), LN lesion groups 7-12 (N2), and LN groups 13-16 (N3), the 5-year survival rate was 85%, 60%, 25% and 11%, respectively.

A significant advantage of the second JGCA gastric cancer classification in terms of practical application is its direct link with the volume of LD based on the staging principle of lymphogenous metastasis. Of note, the Japanese classification uses the term "regional lymph node". This is defined not only by the lymph node topography, but also by the site of the primary tumor in the stomach; the UICC classification does not provide this differentiation.

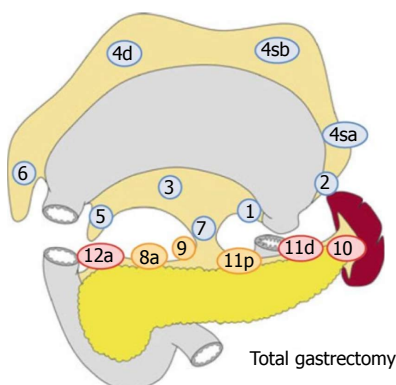
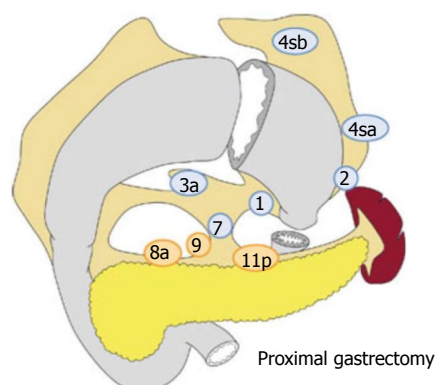
Another obvious advantage of the classification offered by JGCA^[12] lies in the possibility of extrapolating data about the regional LN condition into the UICC classification. The reverse conversion is not possible; therefore, it is not possible to conduct a comparative analysis of retrospective studies in a different series.

Western pathologists and surgeons criticize the Japanese GC classification mainly because of its complexity and also because precision mapping is laborious in practice. However, the Eastern and Western GC classifications are finally approaching each other. This tendency can be observed in the latest edition of the

Table 2 Lymph node groups (compartments 1-3) by location of tumor

Location lymph node station	LMU/MUL	MLU/UML	LD/L	LM/M/ML	MU/UM	U	E+
No. 1 rt paracardial		1	2	1	1	1	
No. 2 lt paracardial		1	M	3	1	1	
No. 3 lesser curvature		1	1	1	1	1	
No. 4sa short gastric		1	M	3	1	1	
No. 4sb lt gastroepiploic		1	3	1	1	1	
No. 4d rt gastroepiploic		1	1	1	1	2	
No. 5 suprapyloric		1	1	1	1	3	
No. 6 infrapyloric		1	1	1	1	3	
No. 7 lt gastric artery		2	2	2	2	2	
No. 8a ant comm hepatic		2	2	2	2	2	
No. 8b post comm hepatic		3	3	3	3	3	
No. 9 celiac artery		2	2	2	2	2	
No. 10 splenic hilum		2	M	3	2	2	
No. 11p proximal splenic		2	2	2	2	2	
No. 11d distal splenic		2	M	3	2	2	
No. 12a lt hepatoduodenal		2	2	2	2	3	
No. 12b,p post hepatoduod		3	3	3	3	3	
No. 13 retropancreatic		3	3	3	M	M	
No. 14v sup mesenteric v.		2	2	3	3	M	
No. 14a sup mesenteric a.	M	M	M	M	M	M	
No. 15 middle colic	M	M	M	M	M	M	
No. 16a1 aortic hiatus	M	M	M	M	M	M	
No. 16a2,b1 paraaortic, middle	3	3	3	3	3	3	
No. 16b2 paraaortic, caudal	M	M	M	M	M	M	
No. 17 ant pancreatic	M	M	M	M	M	M	
No. 18 inf pancreatic	M	M	M	M	M	M	
No. 19 infradiaphragmatic	3	M	M	M	3	3	2
No. 20 esophageal hiatus	3	M	M	M	3	3	1
No. 110 lower paraesophag	M	M	M	M	M	M	3
No. 111 supradiaphragmatic	M	M	M	M	M	M	3
No. 112 post mediastinal	M	M	M	M	M	M	3

M: Lymph nodes regarded as distant metastasis.

**Figure 3** Lymph node dissection levels in gastrectomy^[13].**Figure 4** Lymph node dissection levels in proximal subtotal gastrectomy^[13].

TNM UICC classification and the latest editions of the JCGA gastric cancer treatment guidelines^[13,14].

DEBATE ON THE EXTENT OF LYMPHNODAL DISSECTION: EASTERN VS WESTERN POSITION

Results of a retrospective analysis of LD D2 were first published in Japan in 1970 by Mine *et al*^[19]. The authors reported a slight increase in the survival rate among

patients with pN0 and a probable increase in the 5-year survival rate from 10% to 21% in the group pN+. Similar results were reported in a study by Kodama *et al*^[20], who indicated an increase in the 5-year survival rate from 33% to 58% in the entire group of patients.

In the 1970s and 1980s, Japanese surgeons developed a doctrine of aggressive preventive GC surgery based on the extended (D2) and super-extended (D3) LD volumes^[21]. Concurrently, in Europe and the United States, the most common LD volume was D0-1. Due to the relatively lower GC incidence rates in the West,

European and American surgeons continued to reframe the ideology and master the techniques of extended interventions in GC cases until the end of the 1990s. This eventually caused a scientific conflict between the Eastern and Western schools of surgical thought. Japanese surgeons used D2 LD in surgical practice, whereas European surgeons insisted on repetitive clinical trials in the European patient population. They reasoned that certain biological differences in GC were present in the "Eastern" type^[22].

One of the most significant publications from that time was a study of a European population of patients with GC by Pacelli *et al.*^[23]. The authors reported a probable increase in the 5-year survival rate from 30% (D1, LD) to 49% (D2, 3 LD) for patients with stage III GC and from 50% to 65% in the entire group of patients.

Similar results were obtained by a group of German surgeons supervised by Siewert *et al.*^[24] during the course of a prospective multicentric trial of nearly 2500 patients. A probable increase in the survival rate was reported in patients with stages II - IIIA GC. However, in patients with pN2 (TNM UICC) or with extensive tumor invasion of the gastric serosa, D2 LD was not associated with increased survival.

Over time, researchers increasingly noted the low credibility of non-randomized studies. The results of the first randomized trials published by Dent *et al.*^[25] and Robertson *et al.*^[26] featured high rates of postoperative complications and mortality. However, the results did not provide high levels of credibility because of the small numbers of patients enrolled. The first large-scale randomized multicentric study of the efficacy of D2 LD in a population of European patients with GC was carried out in the 1990s.

This study, known as the Dutch trial^[27], involved 1078 randomized patients and was organized by the Dutch Gastric Cancer Group. At the same time, the British MRS (Medical Research Society) carried out its own trial^[28] with 400 randomized patients. The first results of these studies were preliminarily published in 1997 at the Second International Gastric Cancer Congress (IGCC) in Munich. However, the necessity of compliance with the full volume of D2 LD dramatically increased the frequency of splenectomies (up to 37% in the Dutch study and up to 65% in the British) and resections of the pancreas (30% in the Dutch study and 56% in the British) in all groups. These studies showed a dramatic increase in the number of postoperative complications after D2 LD (from 25% after performing D0-1 in the control group up to 43% in the Dutch trial and from 28% to 46% in the British trial). They also showed an increase in the postoperative mortality rate (from 4% to 10% in the Dutch trial and from 6.5% to 13% in the British trial)^[27,28]. In the Eastern Asian series however, the rate of postoperative complications was 17%-21%^[29,30]. The postoperative mortality rate after D2 LD in Eastern clinics was also significantly lower than in Europe-less than 2% in the Japanese nationwide registry^[31] and less than 1%^[30] or even zero^[29] in specialized centers.

After a 5-year follow-up of European randomized studies, the expected increase in survival of D2 LD group was not achieved; the 5-year survival in the Dutch trial was 45% in group D1 LD and 47% in group D2 LD. In the British trial, it was 35% in group D1 LD and 33% in group D2 LD^[32,33] (Figure 5).

Thus, the European oncology society preliminarily concluded that the extended LD volumes used in European GC patients were ineffective. This was based on evidence-based medicine and relied on the results of the two major Western randomized trials. However, a detailed analysis of this study and all potential reasons for the lack of a positive result were shown at the 1999 IGCC in Seoul. The summary of this analysis was later published in the *New England Journal of Medicine*^[34]. Despite a good design and detailed statistical analysis, the study had some serious shortcomings that made the results ambiguous. These included:

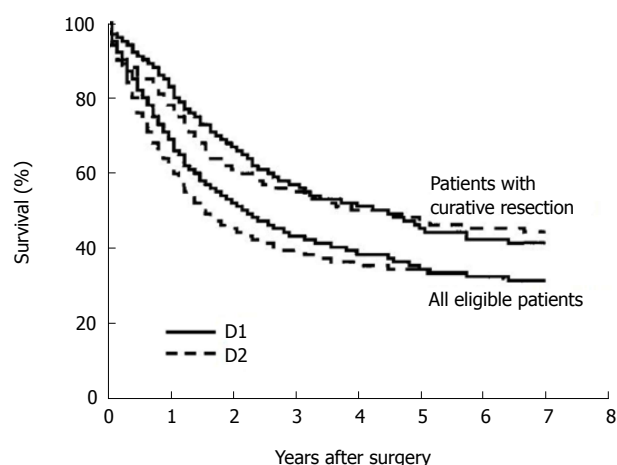
The large number of participating surgical centers (about 80 clinics), which resulted in surgeons obtaining an insufficient amount of practical experience in the surgical procedures required for the study. For instance, some surgeons performed fewer than 5 D2 LD surgeries per year. This not only potentially affected the level of postoperative complications and mortality, but also led to a reduction in LN removal in the course of D2 LD and consequently to a reduction in radical surgeries^[34].

There was a lack of surgery standardization (there were no clear criteria for splenectomy or spleen-saving dissection of the 10th LN group, instrumental or manual anastomosis, etc.).

Conversely, surgeons participating in the randomized trial in Taiwan performed a minimum of 80 D2 LD surgeries before the study began. The results of that study revealed a possible increase in survival rates when extended volumes of LD were performed^[35].

The median number of LNs removed is an important indicator of LD quality. Significant geographic fluctuations of this indicator in the performance of D2 LD have now been established. There are diametrically polar indicators in European randomized trials. In the British study, the median number of removed LNs was 17^[28]; in the Dutch study, the number was 30^[32]. There were 25-26 LNs removed in the Western retrospective studies^[36,37] and 54 LNs removed in Japanese specialized centers^[30]. The minimum adequate number of LNs to be removed in gastric cancer surgeries-according to the requirements of TNM UICC (2009)^[14]-is 15. This level of LD was provided in 86%^[36] to 95%^[37] of patients in the Western retrospective studies and in 100% of patients in the Japanese studies^[30]. According to Siewert *et al.*^[24], the efficiency of LD execution can meet the standards of D2 only when a minimum of 26 LNs are removed.

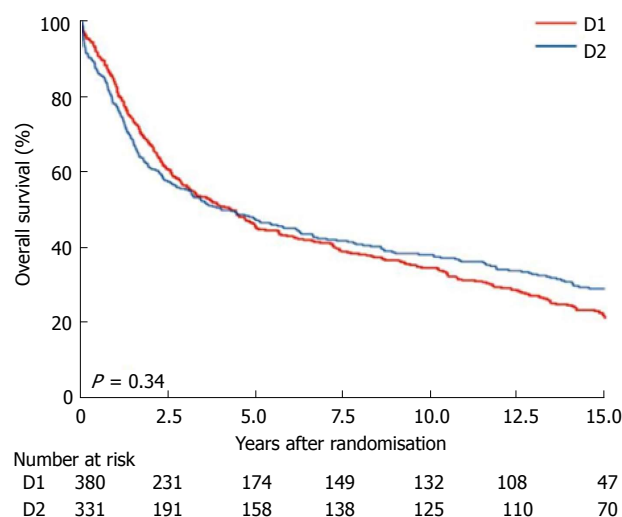
The average frequency of metastatic lesions in LNs of group N°10th (LNs of the splenic hilum) in various tumor sites in the stomach is 8.8%. Metastatic lesions in these LNs are likely to worsen the prognosis^[38]. The application of splenectomy on principle including for LN dissection of the 10th group was not effective in patients

Figure 5 Patient survival in the Dutch trial^[32].

with GC until recently. A small study conducted in Korea by Yu *et al.*^[39] demonstrated a tendency toward increased survival after splenectomy; however, this result was not statistically significant. A meta-analysis conducted in 2009 by Yang *et al.*^[40] also confirmed an increase in the 5-year survival rate of patients with GC after splenectomy. According to other authors^[38], unless the tumor has invaded the spleen, splenectomy is necessary only in case of LN lesions in group N^o4sa. Therefore, despite the fact that LN dissection of the 10th group is regulated by the JGCA guidelines (2011)^[13], the role of splenectomy as a standard stage of D2 LD remains controversial. The answer to this question will likely be clarified soon after the publication of the results of a large randomized trial investigating the efficacy of splenectomy in Japanese patients with cancer of the upper third of the stomach (JCOG 0110 that began in Japan in 2002)^[41].

Despite the previous pessimistic results, Hartgrink *et al.*^[42] conducted a second analysis of the "Dutch material" in 2001. They found a significant increase in survival in group D2 LD, especially in patients with metastases in LNs of the first stage of metastasis (N1 by JGCA). After 15 years of observation of patients during the Dutch trial, no significant difference in survival between groups under observation has not been noted. However, when the most controversial group of patients with splenectomies and resection of the pancreatic gland was excluded from the analysis, the 15-year survival rate increased dramatically from 22% in D1 LD to 35% in D2 LD ($P = 0.006$)^[43] (Figure 6).

In 2013, the results of meta-analysis obtained by 12 randomized controlled major European trials on LD D2 effectiveness were published. These clearly proved the thesis concerning an increased risk of postoperative complications with D2 LD and the possible increase in survival only in the group that did not have splenectomy and resection of the pancreatic gland^[44]. Therefore, in the latest European oncology guidelines, D2 LD is the standard surgical procedure but only in highly

Figure 6 Survival of patients in the Dutch trial after a 15-year observation^[43].

specialized centers with extensive experience in such surgeries as well as postoperative care^[45].

According to the Japanese guidelines on the gastric cancer treatment issued by JGCA (2011)^[13], the algorithm of surgical treatment in patients with GC is as follows (Figure 7).

The amount of LD required during surgical treatment of gastric cancer surgery has been quite controversial. Today, however, in light of evidence-based medicine, one can observe the results of this complex evolution of views: D2 LD is considered an unambiguous standard of GC surgical treatment in specialized centers according to national recommendations in Germany^[46], the United Kingdom^[47] and Italy^[48] as well as mutual recommendations of the European Society of Medical Oncologists, Surgical Oncologists and Radiation Therapists (ESMO-ESSO-ESTRO)^[45]. Such a consensus of the Eastern and Western surgical schools became possible due to the longstanding scientific and practical search for methods that would help improve the results of GC surgeries using evidence-based medicine^[49]. In Western surgical terminology, D2 LD is now called a standard volume of intervention, whereas D2 + LD is an extended operation.

This debate into the effectiveness of extended (D2 + LD) interventions in GC cases remains open. A well-known clinical study conducted by Sasako *et al.*^[34] did not demonstrate an increase in survival after D2 + para-aortic LD for patients with resectable GC. However, many recent studies have demonstrated the possibility of increased survival after the application of extended LD in a selected group of patients with a high risk of metastasis in LNs of the N^o16 station^[50,51].

Furthermore, the effectiveness of laparoscopic D2 LD in GC cases remains undetermined. Today, clinical research is underway in the KLASS-2 trial, which aims to determine the effectiveness of such interventions. The impact of interventions with D1 +, D2 and D2 + LD on the risk of intraperitoneal progression of GC after

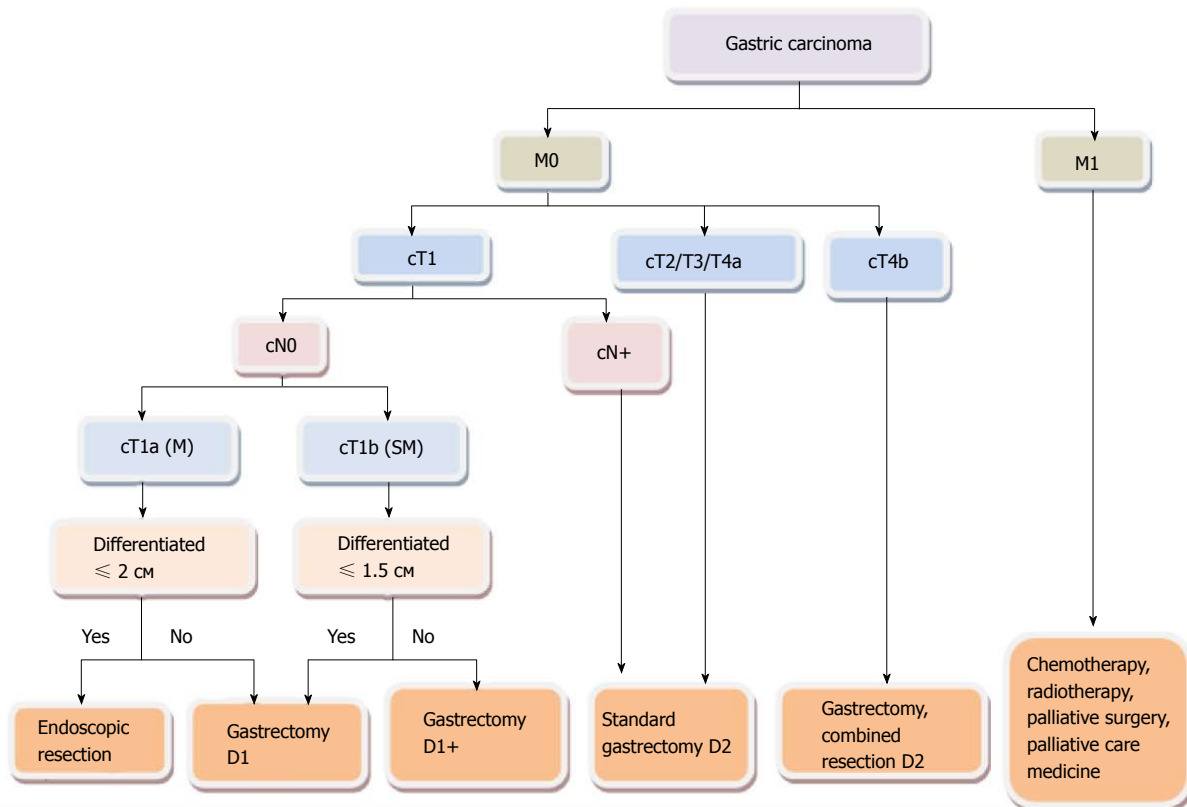


Figure 7 Algorithm of surgical treatment of patients with gastric cancer according to the guidelines provided by Japanese Gastric Cancer Association (2011)^[13].

surgery^[6] remains unknown.

CONCLUSION

The data show that D2 LD can improve the prognosis in European GC patients, but only when the surgical quality of LD execution is adequate. As part of the 10th IGCC in 2013 in Verona, Italy, the former president of the *European Society of Surgical Oncology*, Professor C. van de Velde, noted in his expert lecture that “the only way to improve the efficiency of surgical treatment of gastric cancer in Europe is to place patients in specialized surgical centers, provide training so that individual surgeons could specialize on the issue of LD D2 and an objective and permanent audit on quality of lymphadenectomy in each surgical center”.

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Malignant biliary obstruction: From palliation to treatment

Brian R Boulay, Aleksandr Birg

Brian R Boulay, Division of Gastroenterology and Hepatology, Department of Medicine, University of Illinois Hospital and Health Sciences System, Chicago, IL 60612, United States

Aleksandr Birg, Department of Medicine, University of Illinois Hospital and Health Sciences System, Chicago, IL 60612, United States

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Correspondence to: Brian R Boulay, MD, MPH, Division of Gastroenterology and Hepatology, Department of Medicine, University of Illinois Hospital and Health Sciences System, 840 S Wood Street, MC 716, Chicago, IL 60612, United States. bboulay@uic.edu
Telephone: +1-312-4131999
Fax: +1-312-4133798

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Abstract

Malignant obstruction of the bile duct from cholan-

giocarcinoma, pancreatic adenocarcinoma, or other tumors is a common problem which may cause debilitating symptoms and increase the risk of subsequent surgery. The optimal treatment - including the decision whether to treat prior to resection - depends on the type of malignancy, as well as the stage of disease. Preoperative biliary drainage is generally discouraged due to the risk of infectious complications, though some situations may benefit. Patients who require neoadjuvant therapy will require decompression for the prolonged period until attempted surgical cure. For pancreatic cancer patients, self-expanding metallic stents are superior to plastic stents for achieving lasting decompression without stent occlusion. For cholangiocarcinoma patients, treatment with percutaneous methods or nasobiliary drainage may be superior to endoscopic stent placement, with less risk of infectious complications or failure. For patients of either malignancy who have advanced disease with palliative goals only, the choice of stent for endoscopic decompression depends on estimated survival, with plastic stents favored for survival of < 4 mo. New endoscopic techniques may actually extend stent patency and patient survival for these patients by achieving local control of the obstructing tumor. Both photodynamic therapy and radiofrequency ablation may play a role in extending survival of patients with malignant biliary obstruction.

Key words: Pancreatic neoplasms; Cholangiocarcinoma; Extrahepatic cholestasis; Stents; Catheter ablation

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Core tip: Treatment of malignant biliary obstruction from cholangiocarcinoma or pancreatic cancer can be performed *via* endoscopic, percutaneous, or surgical means. The decision of when or how to achieve biliary decompression depends on the patient's condition, location of stricture, and stage of malignancy. Not all patients require biliary decompression, particularly with resectable tumors. Self-expanding metallic stents or plastic stents may be used for distal malignancy, depending on stage and prognosis. Stents, nasobiliary drainage, or percutaneous drains may

be used for hilar strictures. Endoscopic catheter-based therapies such as photodynamic therapy or radiofrequency ablation may prolong patient survival by achieving local tumor control.

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INTRODUCTION

Obstruction of the extrahepatic bile ducts from a malignant process presents both a diagnostic and therapeutic challenge. It is a common problem, with as many of 70% of pancreatic cancer patients presenting with obstruction upon diagnosis^[1]. Obstruction may serve as the initial sign of disease - such as in the classic presentation of painless jaundice in pancreatic ductal adenocarcinoma - or may occur during progression of malignancy once the diagnosis is established. The two most common malignant neoplasms known to occlude the bile ducts are pancreatic ductal adenocarcinoma and primary bile duct cancer (cholangiocarcinoma). Other causes of malignant biliary obstruction can include ampullary carcinoma, primary duodenal adenocarcinoma, pancreatic neuroendocrine tumors, or occlusion of the hepatic hilum due to lymphadenopathy at the porta hepatis (as seen in metastatic colon cancer or lymphoma). Of note, some premalignant lesions such as biliary papillomatosis may cause an obstructive picture similar to malignancy. Benign conditions such as autoimmune cholangiopathy must also be ruled out, so obtaining tissue *via* endoscopic retrograde cholangiography (ERCP) with brush biopsy or core biopsy, or endoscopic ultrasound with fine needle aspiration (FNA) is paramount^[2]. Only once a firm diagnosis of malignancy is secured can the final choice of treatment be made.

Occlusion of the bile ducts may cause debilitating symptoms such as pruritus and malaise, and thus treatment is often recommended on that basis alone. This may come in the form of surgical resection if the patient presents with resectable disease. However, both pancreatic cancer and cholangiocarcinoma are notorious for presenting at an advanced stage in which immediate surgery is contraindicated. Treatment goals for these patients include downstaging of the tumor with chemoradiotherapy, or strictly palliative measures. Relief of biliary obstruction is recommended in either setting. Treatment of distal malignant biliary obstruction from pancreatic cancer is typically managed by an endoscopically placed single biliary prosthesis, whereas hilar strictures can be more challenging to manage due to the need to access the left and right systems of the biliary tree.

Within the past decade endoscopic techniques have been developed to treat tumor ingrowth into the bile duct with photodynamic therapy or radiofrequency ablation,

and recent studies show promise in expanding the role of endoscopic treatment. While the primary role at this time is to provide biliary decompression and relieve jaundice, the ability to provide therapy for these tumors represents a major shift in the role of the endoscopist. This review will consider the options for management based on the location of obstruction, as well as the stage of the underlying malignancy.

THE EFFECT OF JAUNDICE

The decision whether to decompress obstructed bile ducts in a patient with resectable disease has traditionally been quite controversial. Jaundice has long been recognized as an important preoperative risk factor in the setting of malignancy^[3,4]. Several mechanisms have been described through which jaundice exerts its negative effects. Jaundice is thought to impair cellular immunity, allowing tumor growth and metastatic progression if left untreated^[5]. In addition, obstruction to flow of bile decreases its availability in the enteric system for the absorption of lipid-soluble vitamins, including vitamin K, leading to coagulopathy and increased surgical bleeding risk. Even additional administration of oral vitamin K may be inadequate to reach appropriate levels of coagulation in obstructive cases^[6], which may further complicate any planned surgery. Bacterial and endotoxin translocation through the intestinal mucosa has also been demonstrated in jaundiced patients, making SIRS and sepsis a serious complication that can develop even prior to surgery^[6]. Jaundice has also been shown to increase the risk of infection if not treated before surgery^[7], as well as complications after surgery^[8]. Thus, there is a theoretical benefit to biliary drainage for relief of jaundice prior to surgical resection of these tumors.

It is thought the benefit of preoperative biliary drainage (PBD) may vary depending on the level of obstruction and planned surgery: Distal biliary obstruction from pancreatic cancer or distal cholangiocarcinoma may be treated surgically without the need for preoperative decompression, while hilar obstruction may require decompression to improve surgical outcomes. The differences in strategy likely stem from the need for partial hepatic resection in the treatment of hilar tumors, which may benefit from preoperative decompression.

MALIGNANT DISTAL BILIARY OBSTRUCTION

As many as 70% of patients with newly diagnosed pancreatic cancer have some degree of biliary tract obstruction at the time of diagnosis. Decompression *via* endoscopic stent placement can palliate jaundice and pruritus for symptomatic relief^[9]. Stent placement may also speed allow the patient to begin chemotherapy regimens by reducing the risk of chemotoxicity in a cholestatic liver^[10]. Endoscopic stent placement into the common bile duct is a fairly routine procedure (technically successful in over 90% of cases) and

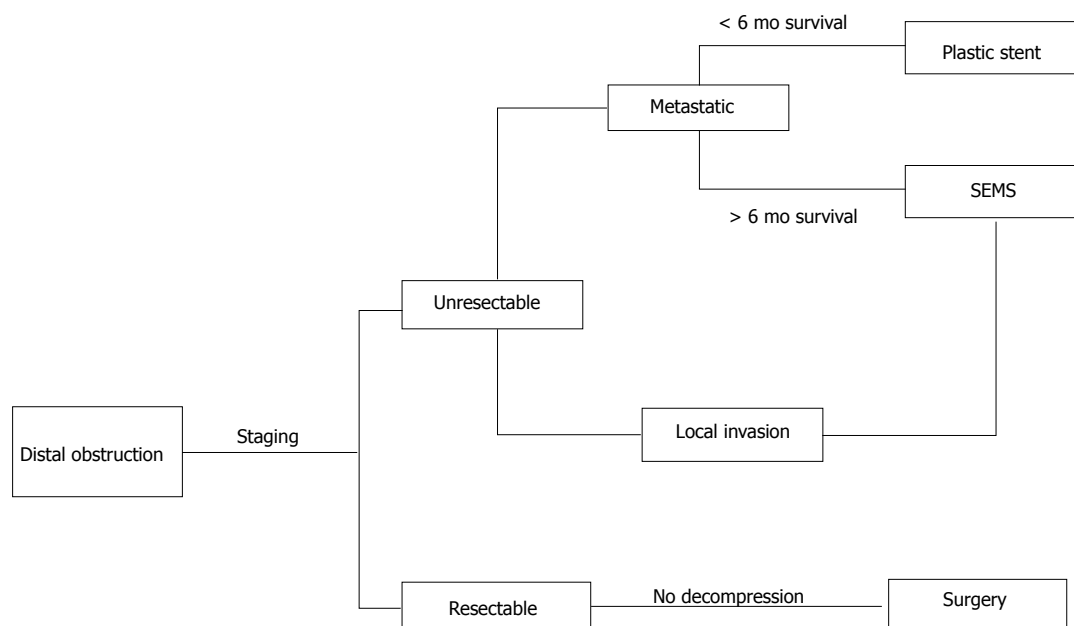


Figure 1 Algorithm for treatment of distal malignant biliary obstruction based on disease stage. Patients who are not candidates for ERCP with stent placement may undergo EUS-BD or percutaneous drainage. Adapted from Boulay BR, Parepally M. *World J Gastroenterol* 2014; **20**: 9345-9353. SEMS: Self-expanding metallic stent; ERCP: Endoscopic retrograde cholangiography; EUS-BD: Endoscopic ultrasound-guided biliary drainage.

has thus become the most common method of achieving biliary decompression^[11]. The choice of plastic or metallic stents depends on factors such as cost-effectiveness, expected length of survival, and diagnostic certainty. Over the past decade the use of self-expanding metal stents (SEMS) has become more common for treatment of both benign and malignant biliary strictures. While surgeons initially discouraged use of SEMS for pancreatic cancer due to concerns of increasing the difficulty of resection, SEMS do not interfere with planned pancreaticoduodenectomy as long as the stent does not involve the hilum^[12]. Thus the role for SEMS in treatment of obstruction from pancreatic cancer has grown in recent years.

Distal cholangiocarcinoma has a similar presentation, pattern of spread, and poor prognosis when compared to pancreatic ductal adenocarcinoma. At times the two diseases may be indistinguishable from each other upon initial presentation. Distal cholangiocarcinoma tends to infiltrate the adjacent pancreas, duodenum, and vasculature as well as nearby lymphatics, leading to locally advanced disease and eventually metastases. Thus, the same staging evaluation can be performed to assess for local invasion, with the goal of curative surgical resection when possible^[13]. Once the stage is known, similar principles of biliary decompression are applied as in pancreatic ductal adenocarcinoma. The management is based on disease stage as depicted in Figure 1.

RESECTABLE DISEASE

Surgical resection is the definitive treatment for patients who present with early-stage pancreatic cancer^[14]. PBD in resectable pancreatic cancer is not automatically recommended. Despite the beneficial effects of relieving

jaundice, PBD has been associated with increased complications including various types of infections as well as pancreatic fistulas^[15-17]. In 2010, van der Gaag *et al.*^[18] reported a randomized trial of 202 patients demonstrating that preoperative biliary drainage with stents was linked to increased complications compared to surgery alone in resectable pancreatic cancer. In this seminal study, rates of serious complications were 39% in the early-surgery group and 74% in the preoperative drainage group. Of note, the preoperative biliary drainage group waited 4 to 6 wk for surgery and were treated with plastic stents, both of which may have contributed to the poor performance of the biliary decompression group. However, based largely upon the experience noted by van der Gaag *et al.*^[18], preoperative biliary decompression for distal biliary obstruction is not recommended except to treat cholangitis or intractable pruritus.

LOCALLY ADVANCED DISEASE AND NEOADJUVANT THERAPY

Even those patients who undergo surgical resection of pancreatic cancer have poor long-term survival rates, so there is growing interest in the use of neoadjuvant therapy to boost outcomes, even for resectable pancreatic tumors^[19]. Neoadjuvant chemoradiotherapy has also been used for locally advanced tumors to downstage them and permit eventual surgical resection. Biliary stents are placed prior to neoadjuvant chemoradiotherapy, with the expectation of remaining patent until the time of surgery. Unfortunately, this expectation has not always been met when plastic stents are used during the preoperative period.

Numerous studies have now shown that SEMs are preferable to plastic stents in patients undergoing neoadjuvant therapy^[20-24]. Retrospective reviews of plastic stent performance during neoadjuvant therapy have demonstrated poor performance with the frequent need for unplanned stent exchange due to stent occlusion or cholangitis^[20,25]. Adams *et al*^[22] described a complication rate nearly 7 times higher with plastic stents, with a 3 times higher rate of hospitalization among a 52 patient cohort. SEMs are clearly more expensive than plastic stents, but their lower occlusion rates (and thus fewer unplanned stent exchanges) make them a more cost effective choice for patients undergoing neoadjuvant chemoradiotherapy with planned surgical resection^[26].

There are several choices of SEM type for use in patients with malignant distal biliary obstruction undergoing neoadjuvant therapy or in palliative cases. When considering uncovered (USEMSs) or covered stents (CSEMSs), the difference in design leads to a trade-off in adverse events between tissue ingrowth in USEMS and stent migration in CSEMS. One recent meta-analysis concluded that CSEMSs afforded an average of 61 d longer patency than USEMSs in palliative cases, with the cost of an increased incidence in migration (RR 8.11)^[27]. In contrast, a retrospective cohort study showed no difference in overall obstruction (CSEMSs 35% vs USEMSs 38%) among 749 patients, merely that the mechanisms of obstruction varied by stent design (tumor ingrowth vs debris)^[28]. Partially covered SEMs appear to have similar performance characteristics to fully covered SEMs and USEMS^[27,29].

Novel stent designs may further improve the performance of SEMs. A modified CSEMS with low axial force and uncovered flare ends has been developed with the goal of reducing stent migration, and when compared to USEMS had significantly longer patency (mean 219.3 d vs 166.9 d) and fewer unplanned procedures (23% vs 37%) compared to USEMSs^[30]. Drug eluting stents have been designed in an attempt to improve SEMs prevent tumor ingrowth and stent occlusion^[31]. An early multicenter prospective study using a paclitaxel-eluting stent did not show improved performance compared to conventional USEMS, though other stents are currently in development^[32]. Anti-reflux stents have been developed to limit duodenal contents into the bile ducts and limit stent occlusion^[23]. Initial experience with anti-reflux SEMs has yielded conflicting results, with one study showing long-term patency possibly exceeding conventional SEMs^[33] while a smaller study showed a disappointing rate of early occlusion^[34]. Further studies are needed to demonstrate the efficacy of anti-reflux and drug-eluting SEMs to determine their role in maintaining long-term stent patency.

Despite the wealth of data demonstrating the superiority of SEMs over plastic stents for malignant biliary obstruction, endoscopists may prefer to place a removable plastic stent if the diagnosis of malignancy is uncertain at that time of ERCP (such as in facilities where endoscopic

ultrasound with FNA and rapid on-site evaluation by cytopathologists is not available). In this situation, benign conditions such as chronic pancreatitis or autoimmune cholangiopathy may be suspected as the etiology of the biliary stricture, and a removable stent is preferable. The use of a fully covered SEMs is ideal when suspicion of malignancy is high and life expectancy exceeds 4 mo.

PALLIATIVE DECOMPRESSION IN DISTAL OBSTRUCTION WITH METASTATIC DISEASE

In the setting of incurable pancreatic cancer, patients may often present with advanced disease and limited life expectancy. SEMs may not be cost-effective for these patients, since their main advantage of durable patency is not applicable. SEMs cost 15-40 times more than most plastic stents, and are only cost effective if the patient survives > 4 mo^[26]. Some authors have suggested that SEMs should be used only in patients without distant metastases^[35]. The presence of liver metastases has been shown to predict mortality in pancreatic cancer, though the data is sparse and generally used only to determine operative risk^[36-38]. Although determination of life expectancy can be difficult and more research is needed to identify prognostic factors, endoscopists should be aware of the cost savings with plastic stents in patients with poor functional status and limited expected survival.

NON-ENDOSCOPIC BILIARY DRAINAGE IN MALIGNANT DISTAL OBSTRUCTION

In cases where ERCP fails or cannot be performed, percutaneous transhepatic biliary drainage (PTBD) has traditionally been used to create a tract for internal and external drainage. External drains have the potential downside of requiring emptying and flushing of the drain as well as routine drain exchange^[39]. Recent trials of percutaneous SEMs placement have also demonstrated good safety and effectiveness^[40-42]. An alternate second-line approach is endoscopic ultrasound-guided biliary drainage (EUS-BD), which has been increasingly shown to be both safe and effective when standard ERCP approaches fail. This technique can be used to achieve decompression *via* EUS-guided rendezvous procedure, EUS-guided choledochoduodenostomy, and EUS-guided hepatic gastrostomy^[43]. Complications can include bile leak, bleeding, or pneumoperitoneum. EUS-BD remains technically complex and limited to high-volume expert centers^[44].

Surgical biliary bypass remains an option, particularly when life expectancy exceeds 6 mo. Trials comparing surgical bypass to endoscopic therapy have shown similar mortality and fewer incidents of recurrent biliary obstruction when surgery was performed, though these

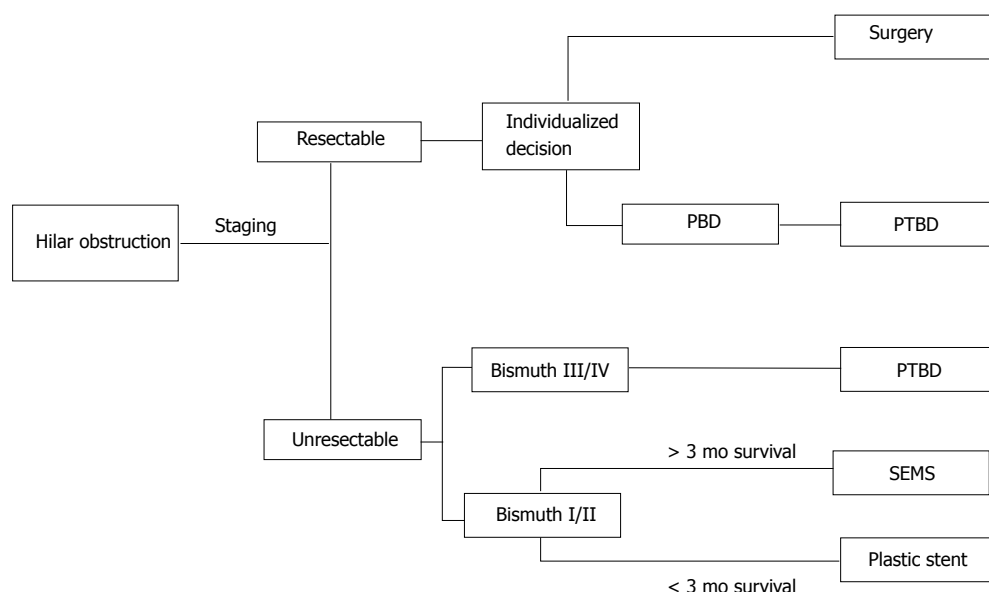


Figure 2 Algorithm for treatment of hilar malignant biliary obstruction. PBD is an individualized decision based on local expertise. PTBD: Percutaneous transhepatic biliary drainage; SEMS: Self-expanding metallic stent; PBD: Preoperative biliary drainage.

trials preceded the widespread use of SEMS and do not reflect current endoscopic practice^[45,46]. Surgical bypass can also relieve biliary and gastric outlet obstruction at the same time *via* creation of a surgical gastrojejunostomy and biliary bypass. However, biliary and gastroduodenal obstruction can also be relieved endoscopically during the same procedure, with placement of a duodenal SEMS followed by an endoscopic approach to the papilla for ERCP guided biliary stent placement or even EUS-BD^[47-50]. Thus, while ERCP is the preferred method for management of malignant distal biliary obstruction, a multidisciplinary team including interventional radiologists and surgeons is ideal for management of unusually difficult strictures when ERCP fails.

HILAR CHOLANGIOCARCINOMA: SURGICALLY TREATABLE DISEASE

Malignant obstruction of the extrahepatic bile ducts at the liver hilum can be much more difficult to treat, given the possible involvement of the left and right hepatic ducts and the need to decompress the left and right lobes individually. Complete resection is the only curative treatment for cholangiocarcinoma. Unfortunately, most patients will present with obstructive symptoms or frank jaundice later in the disease course^[51]. Resection modalities vary depending on the location of the malignancy: Intrahepatic tumors are treated with hepatic resection while extrahepatic tumors can be classified as hilar cholangiocarcinoma (Klatskin tumor) or distal cholangiocarcinoma. The level of the cystic duct demarcates hilar vs distal tumors. Hilar cholangiocarcinoma may still require partial hepatic resection as part of definitive management^[52]. In patients with Bismuth class 4 strictures involving the left and right hepatic duct, or vascular involvement of the hepatic

artery or portal vein, surgical resection is contraindicated and neoadjuvant or palliative techniques are indicated^[53]. Figure 2 depicts the overall management of hilar obstruction based on disease stage.

Several different imaging modalities can be used to determine the degree of proximal tumor extension. This is of critical importance in deciding on resectability and the optimal means of achieving biliary decompression. Computed tomography (CT) is the most commonly used type of imaging at the time of diagnosis, and multidetector CT (MDCT) has an accuracy of 86% in evaluating the ductal extent of hilar cholangiocarcinoma^[54]. Magnetic resonance imaging (MRI) with magnetic resonance cholangiopancreatography (MRCP) provides detailed images of the biliary tree, though the presence of biliary stents can reduce the accuracy of staging^[55]. The accuracy of MRI in determining hilar involvement is estimated at 89%, similar to that of MDCT. Both types of imaging also provide information regarding lymph nodes and direct invasion of nearby structures. In contrast, direct cholangiography by either ERCP or percutaneous means may not allow imaging of the entire biliary tree due to complete obstruction of some segments by tumor. Direct cholangiography is also invasive and presents risks of bleeding or infection which are avoidable with cross-sectional imaging. Thus, MDCT or MRCP are both acceptable initial imaging strategies for treatment planning, though the presence of stents may favor the use of MDCT.

When a patient with hilar cholangiocarcinoma presents with resectable disease, the treatment team must consider whether to pursue PBD. As with pancreatic cancer, preoperative biliary drainage has long been a debated topic, though most authorities advocate against it in the absence of cholangitis^[56,57]. Drainage is recommended in

special situations such as cholangitis as well as patients with symptomatic jaundice (e.g., pruritus) or renal failure^[10]. In the absence of these factors, preoperative drainage has been previously discouraged on the basis that it confers no mortality benefit but may cause adverse events. Meta-analyses and systematic reviews of randomized controlled trials of preoperative biliary drainage have found increased complications and morbidity when PBD was performed, though overall mortality does not differ between the two groups^[58,59].

Nonetheless, it is standard practice to perform preoperative biliary drainage in countries such as Japan and South Korea, and there is reason to believe it can be helpful in optimizing surgical outcomes with proper patient selection. One reason for this enthusiasm has been increased recognition of the flaws in earlier studies which argued against preoperative drainage. A large number of studies from 1980s to early 2000s did not have standardized timing to clear obstruction and jaundice, with some prolonged periods that contributed to stent occlusions and associated complications^[60]. Even now, the optimal bilirubin level for surgery and optimal duration of PBD have not been established. The risk of drain occlusion or inflammatory change within the bile duct with prolonged drainage must be weighed against the risk of progressive obstruction on the outcome of liver resection during curative surgery. Thus preoperative biliary drainage is indicated when there will be a delay for surgery, such as in patients who undergo selective portal vein embolization in the setting of inadequate future liver remnant. In addition, the high-quality cholangiograms obtained in PBD can assist with treatment planning by delineating the extent of tumor involvement within the segmental bile ducts (though staging with MDCT or MRCP can provide adequate staging information without the risks of decompression).

It is possible that the benefit of PBD depends on the location of biliary obstruction within the hilum. Farges *et al.*^[61] performed a multicenter retrospective analysis of 366 patients undergoing extended right or left hepatectomy for hilar cholangiocarcinoma. PBD was not found to improve mortality overall, though a subgroup analysis revealed improved mortality in patients undergoing right hepatectomy (through reduced incidence of post-operative liver failure) while mortality was worse with left hepatectomy (through increased risk of sepsis)^[61]. This intriguing data will require confirmation with additional studies but may help guide the controversial decision whether to perform PBD in the future.

HILAR CHOLANGIOCARCINOMA: TECHNIQUES FOR PREOPERATIVE BILIARY DRAINAGE

Currently three techniques are available for preoperative biliary drainage of hilar malignancy: PTBD, endoscopic nasobiliary drainage (ENBD), and endoscopic retrograde biliary drainage (ERBD) with stent placement. No randomized controlled trial has been performed to compare these techniques. For hilar tumors, ERBD can be technically

challenging with the need to place multiple stents to drain obstructed biliary segments. The rate of complications is high, with reports showing morbidity of 25%-50% and mortality rates of 3%-5% with hilar tumors^[62]. The complications are mainly due to cholangitis with stent failure or inadequate drainage^[3,8].

ENBD has the advantage of providing drainage while allowing repeat cholangiography as needed prior to surgery with easy access to the drained intrahepatic segments^[7]. Nasobiliary drainage is also a safe technique with fewer complications than PTBD^[8]. However, nasobiliary tubes can be easily dislodged and may be poorly tolerated due to patient discomfort^[63]. The use of multiple nasobiliary tubes to provide bilateral drainage for Bismuth IV hilar tumors has been performed but is technically demanding.

Percutaneous transhepatic biliary drainage has become the preferred method for preoperative biliary decompression in some centers. This technique is attractive due to its low complication rate when compared to endoscopic stent placement. Kloek *et al.*^[3] reviewed 101 patients who had undergone PBD and found 48% infection rate with ERBD, while PTBD was associated with only 9% infection rate. The chief problem with PTBD is the risk of tumor seeding along the drain tract, which is estimated at 5%-20%^[7,8]. External transhepatic drains may cause patient discomfort and sometimes require additional oral intake of bile acid supplements, representing an uncomfortable nuisance to the patient^[8]. Despite these drawbacks, the safety and comparative ease of drain placement (compared to ENBD) make PTBD a reasonable option depending on the local expertise of the treatment team.

HILAR CHOLANGIOCARCINOMA: PALLIATIVE THERAPY FOR MALIGNANT BILIARY OBSTRUCTION

While preoperative treatment tends to employ ENBD or PTBD, these therapies are less practical in the setting of unresectable disease. For patients with Bismuth I or II tumors, ERBD has similar performance to PTBD while being less invasive. However, patients with more advanced hilar obstruction (Bismuth III or IV) are more difficult to palliate with biliary stents. A retrospective review of 126 patients with Bismuth III-IV obstruction demonstrated higher success rates with PTBD over SEMS (93% vs 77%), though median survival was similar between the two groups^[64]. Thus, PTBD is generally favored over endoscopic therapy even for palliation in advanced hilar obstruction.

The goal for palliative drainage is to relieve jaundice by draining an adequate liver volume (50% or more). This can be achieved with a single stent in Bismuth I tumors, though the strategy with more advanced hilar disease is more complex. Drainage of > 50% of the liver volume may require more than one catheter or stent, though the right lobe of the liver takes up 50%-60% of the liver volume and successful drainage of the right lobe with a single stent may be sufficient to relieve

jaundice. The question of whether to place a single or multiple drains can be answered by volume assessment of the liver (volumetry) using cross-sectional imaging by CT or MRI. MRI imaging can be used to guide the placement of a single stent to access the dominant lobe and provide adequate drainage with a single stent^[65].

Much like the treatment of distal obstruction with pancreatic malignancies, the past decade has seen increasing use of SEMS over plastic stents. There have been numerous studies that show significant advantages to metal stents vs plastic polyethylene stents^[56]. The length of patency for SEMS compared to plastic is much higher (as much as 12 mo vs 3 mo), owing to the 8-10 mm diameter of SEMS compared to the narrower 7, 8.5 or 10 French plastic stents. Complications from occlusion or migration are less common, though SEMS are notably much more expensive than plastic stents^[66]. Of course, patients with low performance status or advanced illness may not be expected to benefit from the prolonged duration of patency in SEMS; patients with life expectancy of < 3 mo would achieve the same benefit of palliation with lower costs using plastic stents. Cost analysis has demonstrated superiority of SEMS when patient survival is expected to exceed 3 mo^[56,66-68]. As with pancreatic cancer patients, the treating endoscopist must consider expected length of survival when choosing an appropriate and cost-effective stent for palliation.

When bilateral or multisegmental stents are required for adequate biliary drainage, various techniques are available to place SEMS and take advantage of the prolonged duration of patency relative to plastic stents. Not all patients will require multiple SEMS; a meta-analysis by Sawas *et al*^[69] shows no statistical difference in rate of failures or cholangitis between unilateral and bilateral SEMS placement for hilar tumors. Other retrospective data has favored the use of bilateral stents over unilateral stenting, with superior length of patency using multiple stents^[70]. The two main techniques for use of multiple stents for hilar tumors are the "side by side" method, in which both stents are placed in parallel, or the "stent-in-stent" or "Y" method, in which the second stent is placed through the mesh interstices of the first stent with their distal ends overlapping^[71]. There are no large studies indicating which method is superior in terms of technical or clinical success. Additional studies have looked at a 3-branch stent-in-stent to allow for better patency of stenting, with promising results but high degree of challenge for the endoscopist^[72,73]. It is generally recommended that endoscopic therapy of advanced hilar strictures be performed by experienced endoscopists in a tertiary center with available backup by interventional radiologists and surgeons.

ENDOSCOPIC ADJUVANT TREATMENT OF BILIARY OBSTRUCTION: MOVING BEYOND STENTING

Patients with cholangiocarcinoma and pancreatic cancer

continue to have notoriously poor prognoses when surgical cure is not an option. Meta-analyses have not shown significant improvement with standard chemoradiation regimens for biliary malignancies^[74], likely due to late presentation and aggressive nature on presentation. However, two endoscopic therapies aimed at providing local control of malignant biliary obstruction have shown some promise in early studies.

Over the past decade the use of photodynamic therapy (PDT) has been studied for palliation of unresectable cholangiocarcinoma. This technique employs a photosensitizing molecule such as porfimer sodium which accumulates in tissue with rapid turnover such as malignant cells. After 48 h laser irradiation is then used to treat the tumor, leading to selective apoptosis within the tumor mass *via* generation of oxidative radicals. The application of oxygen and light can be performed through a cholangioscope for precise phototherapy administration to limit damage on normal tissue^[75]. The first randomized controlled trial of PDT when compared to biliary stenting alone showed a dramatic increase in survival time from 98 d to 493 d^[76]. Another RCT also showed median survival increased from 210 to 630 d^[77]. Retrospective data also contributes to the body of information supporting increased survival and quality of life when PDT is used in addition to biliary stents as well as chemotherapy^[78,79]. Side effects from phototherapy are mainly related to photosensitivity, requiring patients to avoid direct sunlight for 4-6 wk. In addition, the high cost of PDT may be a factor preventing its widespread use for local control of unresectable cholangiocarcinoma.

Radiofrequency ablation, previously used for colonic or esophageal malignancies as well as hepatocellular carcinoma, has also been increasingly studied for local treatment of biliary obstructive malignancies. Compared to PDT, it offers low cost and is technically simple to perform. RFA induces ablative necrosis and can be used to palliate known biliary malignancies by using a bipolar probe placed at the site of obstruction^[75]. RFA can be performed percutaneously or *via* a catheter inserted *via* ERCP. Ablation uses 7-10 W bursts to create coagulative necrosis of the intraductal tumor mass and when performed *via* ERCP is followed by biliary stent placement^[51,75]. Plastic stents are applied when future ablations are planned, while SEMS may be used when a single session is planned. The risk of adverse events is low but includes hemobilia and biliary fistulas. The body of literature supporting RFA for biliary malignancies is not as robust as that for PDT, consisting mostly of retrospective series^[80]. A retrospective comparison by Strand *et al*^[81] compared results in 48 patients (16 RFA, 32 PDT) which demonstrated similar median survival (9.6 mo in RFA, 7.5 mo in PDT). Future studies will be required to determine the optimal techniques for RFA, as well as the patient populations who are most likely to benefit. European studies have also investigated the use of RFA therapy to treat occlusion of SEMS without the need for additional stent placement^[82].

The role of RFA in distal malignant biliary obstruction has

not been defined, though early experience is encouraging. In a retrospective study of 20 patients undergoing RFA of biliary strictures, 8 patients had distal obstruction due to pancreatic adenocarcinoma or intraductal papillary mucinous neoplasm. The study showed a median increase of 3.5 mm in bile duct diameter following RFA treatment, with maintenance of stent patency at 30 d^[83]. Similarly, a registry of 69 patients who underwent RFA for malignant biliary obstruction included 19 patients with pancreatic cancer. Again, the median diameter of the bile duct improved following RFA treatment. Interestingly, the pancreatic cancer patients responded better to RFA than cholangiocarcinoma patients, with RR 1.8 for stricture improvement^[84]. Other outcomes such as length of survival have not yet been studied in these patients. While further data is needed, including high-quality prospective data, the ability to achieve local control and prolong survival in these diseases using endoscopic therapies is an exciting prospect.

CONCLUSION

The management of malignant biliary obstruction requires consideration of several factors prior to the act of decompression *via* endoscopic or percutaneous means. The location of the stricture and underlying malignancy will affect the approach, as hilar stricture may be much more difficult to treat compared to simple strictures of the distal common bile duct. The stage of underlying malignancy also plays a role, as resectable disease may not typically require preoperative biliary drainage. For most cases, preoperative biliary drainage is discouraged due to the high incidence of infectious complications. In patients undergoing neoadjuvant therapy for locally advanced disease, SEMS appear to be the optimal approach for distal strictures while most data for hilar strictures appears to favor PTBD or ENBD. For palliation in advanced malignancy, SEMS are generally favored if life expectancy exceeds 3-4 mo, while plastic stents are favored in patients with particularly poor prognosis. The use of photodynamic therapy and radiofrequency ablation to achieve local control of these malignancies is an important step in prolonging survival, and presents an opportunity for endoscopists to have an increased role beyond stent placement in improving the quality and quantity of life for patients with cancer and biliary obstruction.

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Genetic risks and familial associations of small bowel carcinoma

Santosh Shenoy

Santosh Shenoy, Department of Surgery, KCVA, University of Missouri at Kansas City, Kansas, MO 64128, United States

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Correspondence to: Santosh Shenoy, MD, FACS, Department of Surgery, KCVA, University of Missouri at Kansas City, 4801 E Linwood Blvd, Kansas, MO 64128, United States. shenoy2009@hotmail.com
Telephone: +1-816-8614700-55431
Fax: +1-816-9224609

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Abstract

Adenocarcinoma of small intestines (SBA) is a relatively rare malignancy with poor outcomes due to delayed diagnosis. Fifty percent of patients have metastases on presentation and therefore early detection and treatment offers the best long term outcomes. Certain genetic polyposis syndromes and familial diseases are associated

with increased risks for SBA. These include familial adenomatous polyposis (FAP), Lynch syndromes (LS), Juvenile polyposis syndrome, Peutz-Jeghers syndrome, Crohn's disease (CD) and celiac disease. Mutations in *APC* gene, Mismatch repair genes, *STK11* gene, and *SMAD4* gene have been implicated for the genetic diseases respectively. While there are no specific inherited genetic mutations for CD, genome-wide association studies have established over 140 loci associated with CD. CpG island mutations with defects in mismatch repair genes have been identified in celiac disease. Significant diagnostic advances have occurred in the past decade and intuitively, it would seem beneficial to use these advanced modalities for surveillance of these patients. At present it is debatable and no clear data exists to support this approach except for established guidelines to diagnose duodenal polyps in FAP, and LS. Here we discuss the genetic alterations, cancer risks, signaling mechanisms and briefly touch the surveillance modalities available for these genetic and clinical syndromes. English language articles from PubMed/Medline and Embase was searched were collected using the phrases "small-bowel adenocarcinoma, genetics, surveillance, familial adenomatous polyposis, lynch syndromes, Peutz-Jeghers syndrome, juvenile polyposis syndrome, CD and celiac disease". Figures, tables and schematic diagram to illustrate pathways are included in the review.

Key words: Small intestinal adenocarcinoma; Genetic risks; Mutations; Signaling pathways; Surveillance

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Core tip: Adenocarcinoma of small intestine (SBA) is a relatively rare malignancy with poor outcomes due to delayed diagnosis. Certain genetic and familial diseases are associated with increased risks for SBA. These include Familial adenomatous polyposis, lynch syndromes, juvenile polyposis syndrome, Peutz-Jeghers syndrome, Crohn's disease and celiac disease. We discuss the clinical implications of

this aggressive cancer focusing on the genetic and familial associations, signaling mechanisms and available diagnostic modalities for surveillance.

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INTRODUCTION

Small intestine comprises majority of the anatomical length and absorptive surface of gastrointestinal (GI) tract but accounts for less than five percent of GI tract malignancies^[1]. According to the seer's database an estimated 9410 new small bowel adenocarcinoma (SBA) cases and 1260 deaths may have occurred in the United States in 2015^[2].

Certain genetic syndromes and familial diseases are associated with SBA (Table 1). These are a heterogeneous group of familial polyposis and non-polyposis syndromes, inflammatory bowel diseases, and autoimmune diseases with distinct epidemiology, genetics, clinical presentation, treatment strategies, surveillance and outcomes.

These groups with inherent risk for both small bowel and colorectal cancers (CRC) have established surveillance recommendations for CRC but there are no clear guidelines for surveillance of small bowel cancers. Significant diagnostic advances have occurred in the past decade and patients may benefit for small bowel surveillance using these diagnostic modalities.

FAMILIAL ADENOMATOUS POLYPOSIS

Familial adenomatous polyposis (FAP) is an autosomal dominant genetic disorder affecting approximately 1:10000 newborns caused by mutation of the APC gene on the long arm of chromosome 5. Multiple polyps of the colon and rectum are pathognomic of FAP. Polyps could be sessile or pedunculated and histology's may vary from tubular to villous adenoma. Most patients develop polyps by second decade and if untreated colon malignancy by the fourth decade (15% of gene carriers by age 10 years, 75% by 20 years, and 90% by 30 years)^[3].

The incidence of small intestinal cancers in FAP is not clear however the adenoma-carcinoma sequence for development of cancer is well established^[3-6]. In addition these patients are predisposed to multiple small bowel adenomatous polyps usually in the duodenum and periampullary region^[7,8].

The pathogenesis of these polyps is due to dysregulation of the canonical Wnt/ β -catenin pathway. The APC protein is a tumor suppressor, involved in cell adhesion, transduction and transcription, cell cycle control, maintenance of fidelity of chromosomal segregation and apoptosis. As part of a

scaffolding protein complex, it is a negative regulator of Wnt signaling pathway (Figure 1).

In the absence of Wnt signaling, cytosolic β -catenin that is not bound by cell-cell adherens junction is transferred to the degradation complex consisting of the proteins APC, axin, casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3). CK1 and GSK3 phosphorylate and prime the unbound β -catenin targeting it for ubiquitination, and leads it to proteasome to be digested. This prevents translocation and accumulation of β -catenin into the nucleus.

Normally nuclear translocation of β -catenin leads to the expression of genes such as c-Myc and Wnt target genes: Promoting cell growth, division, proliferation and differentiation. It also regulates cell-cell adhesion and is important for tissue formation^[4,5].

More than 700 mutations of APC gene have been identified with the classic and attenuated types of FAP. APC gene mutation leads to production of truncated, nonfunctional version of this protein. This truncated APC protein fails to suppress the canonical Wnt/ β -catenin pathways even in the absence of Wnt signaling, results in unopposed translocation of β -catenin in the nucleus and stimulates transcription of c-Myc and other Wnt target genes that leads to the formation of polyps and predispose to cancers^[4,5]. In addition APC also interacts with microtubules, loss of APC may lead to mitotic spindle defects, leading to chromosome abnormalities when cells divide.

While colorectal polyps and cancer remains the primary tumors in FAP and advanced surgical techniques have reduced mortality from colorectal carcinoma, the leading second primary malignancy in these individuals is duodenal and small bowel carcinoma. The prevalence of duodenal adenomas is 50%-90% and these patients carry a relative risk of 330 for adenocarcinoma or up to 5% lifetime risk. The risk is highest in periampullary adenomas^[9]. The adenoma formation is not restricted to the duodenum but also noted in jejunum and ileum in 50%-75% of the patients. Studies using video capsule endoscopy (VCE) and balloon-assisted enteroscopy (BAE) confirm the presence of jejunal and ileal polyps frequently in FAP, especially with extensive duodenal polyposis^[10-12].

The increased risk for SBA appears to correlate between the severity of duodenal polyposis and presence of jejunal polyps^[10-13]. Scattered case series, report an association of marked duodenal polyposis, with higher stages of the disease on diagnosis and worse prognosis^[7]. Spigelman in 1989 developed an endoscopic scoring system (stage 1-4) to describe the severity of duodenal polyps in FAP. Predictors include the number, size, histology and the degree of dysplasia^[8]. The risk of progression to adenocarcinoma is associated with the size and histology of these polyps: 8.3% risk for sub centimeter polyps to 30% for polyps greater than 2 cm. Tubular adenoma carries a risk of (14%), increases to (23%) for tubulo-villous adenoma, and (36%) for villous

Table 1 Genetic risks and familial associations of small bowel carcinoma

Syndrome	Mode of inheritance	Mutated/associated gene	Relative risk (95%CI)	Lifetime risk for SBA	Polyps/pathway
FAP ^[7,29]	Autosomal dominant (AD)	APC	330 (132-681)	3%-5%	Adenoma-carcinoma
HNPCC/ LS ^[13,14,29]	AD	MMR (MSH2, MSH6, MLH1, PMS2)	291 (71-681)	1%-4%	Adenoma-carcinoma
PJS ^[31,36,37]	AD	STK11	500 (220-1306)	1.7%-13%	Hamartoma, adenoma-Ca
JPS ^[38,41]	AD	BMPRIA, SMAD4	Unknown	Unknown	Hamartoma, adenoma-Ca
Crohn's disease	Unknown (genome wide studies have associated 140 loci)	Unknown	30-60 ^[44-49] (15-609)	2.2% after/25 yr	Dysplasia-carcinoma
Celiac disease	Association with HLA-DQ2,HLA-DQ8	Unknown	60-80 ^[61-63] (7-240)	< 1%	Adenoma-carcinoma

FAP: Familial adenomatous polyposis; APC: Adenomatous polyposis coli; HNPCC: Hereditary nonpolyposis colorectal cancer; MMR: Mismatch repair gene; LS: Lynch syndrome; PJS: Peutz-Jeghers syndrome; STK11: Serine threonine kinase; JPS: Juvenile polyposis syndrome; BMPRIA: Bone morphogenetic protein receptor, type IA; SMAD4: Mothers against decapentaplegic homolog; HLA: Human leukocyte antigen complex.

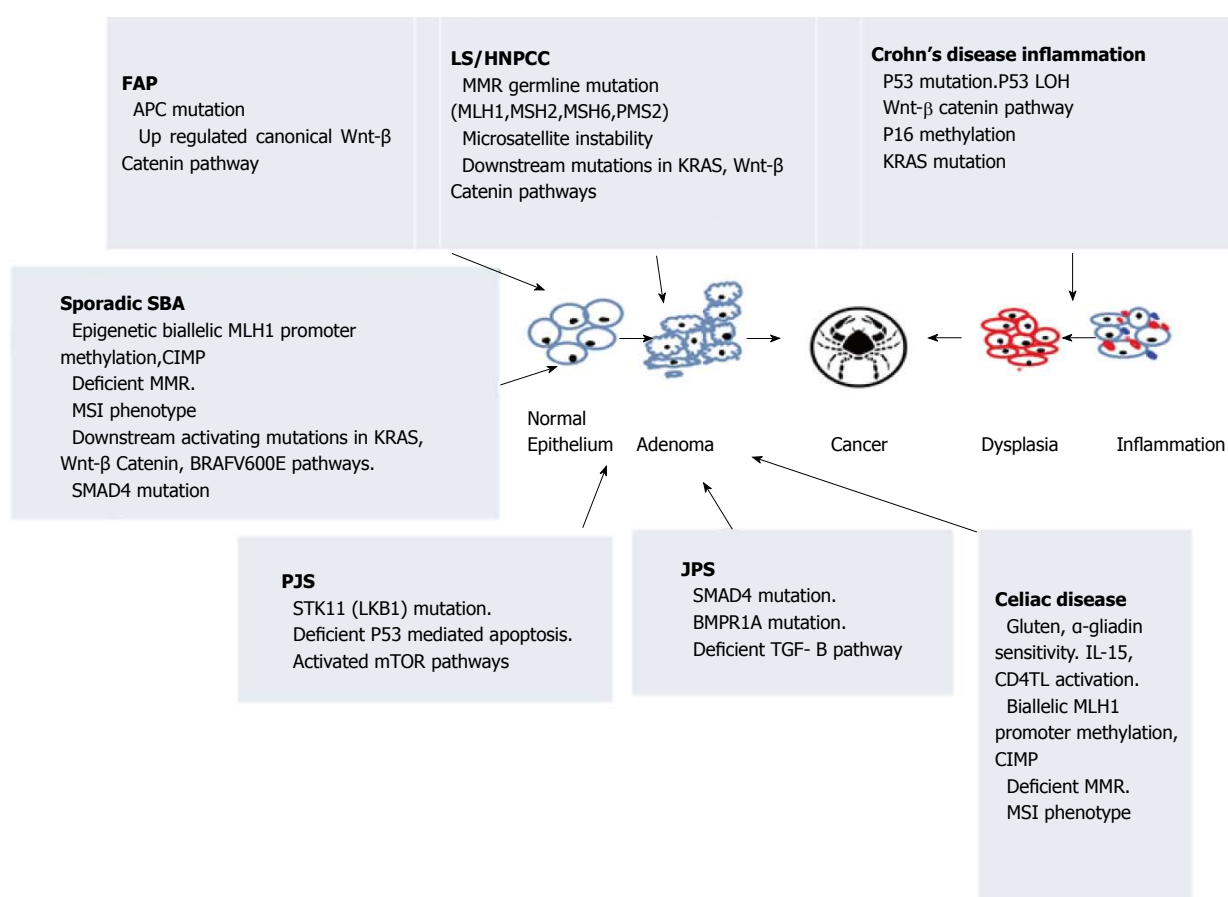


Figure 1 Schematic drawing of genetic and molecular pathways predisposing to small bowel carcinoma. Wnt: Wingless-type MMTV integration site family; KRAS: Kirsten rat sarcoma viral oncogene homolog; LOH: Loss of heterozygosity; CIMP: CpG island methylator phenotype; MSI: Microsatellite instability; BRAFV600E: V-raf murine sarcoma viral oncogenes homolog B; mTOR: Mammalian target of rapamycin; TGF-β: Transforming growth factor β; IL: Interleukin; CD4TL: CD4 T-lymphocytes.

adenomas^[1,14,15]. The significance of small bowel polyps beyond the duodenum is not defined given the fact that up to 44% of patients with FAP develop extensive (stage 4) duodenal polyps with aging but overall incidence of cancer is less than 5%^[13,16].

Due to this reason gastroduodenal surveillance with

endoscopy is generally limited for duodenal polyps^[7]. The exact age and interval to begin surveillance upper endoscopy is still debatable, some authors recommend annual endoscopy starting after colonic polyps are diagnosed or as early as 15 years of age^[17] while other authors suggest starting at age 25 years and interval



Figure 2 65-year-old male with familial adenomatous polyposis, previous total proctocolectomy 35 years ago with ileostomy adenocarcinoma: Polypoid growth at the ileostomy orifice.

based on severity as suggested by Spigelman grading system^[13,18].

Based on the existing data there are no recommendations or guidelines for surveillance of small bowel beyond the duodenum in FAP. Further research is required to identify what patients with FAP are at an increased risk for small bowel carcinoma^[13,19].

One unique subset of patients is FAP with ileostomy and ileoanal pouch carcinoma. Currently prophylactic restorative proctocolectomy with ileoanal pouch anastomosis (IPAA) is the preferred operation in FAP. Previously total proctocolectomy with end ileostomy was the operation most often performed for FAP^[20,21]. These patients with functioning ileostomies have an inherent risk for development of ileostomy adenocarcinoma (Figure 2). Adenomas frequently form in 35% of ileoanal pouches, examined in FAP who underwent restorative proctocolectomy^[22]. The risk of developing adenomas increases with the longevity of these functioning ileostomies. The estimated risks at 5, 10 and 15 years were 7%, 35%, 75% respectively. This predilection to form adenomas, may progress to adenocarcinoma. Positive immunostaining of β -catenin, p53 and frequent occurrence of KRAS mutations suggests adenoma-carcinoma sequence similar to colorectal cancers^[23]. The current recommendations for these patients is periodic clinical and endoscopic examination of their stomas and pouches with biopsies of any suspicious lesions^[21,24].

LYNCH SYNDROME

Lynch syndromes (LS) are an autosomal dominant genetic disorder with germline mutations of mismatch repair genes (MMR): MLH1, MSH2, MSH6 and PMS2. MLH1 and MSH2 mutation variants represent about 90% of families with LS; MSH6 variants in another 7%-10% and PMS2 mutation in less than 5%. Germline deletions in EPCAM (epithelial cell adhesion molecule) inactivate MSH2 in a small subset (< 1%) of patients with LS^[17].

Affected individuals carry the risk for colorectal, endometrial and ovary, genitourinary tract, stomach, hepatobiliary, pancreas and small bowel cancers (Figure 3).

The pathogenesis of these tumors involves microsatellites, which are short stretches of DNA with repetitive sequences of nucleotides and are susceptible to acquiring errors when MMR gene function is impaired. MMR genes present on different chromosomes coordinate the activities of other proteins such as DNA polymerase that maintain the fidelity of DNA replication and genomic integrity. MMR system encode for proteins that form DNA MMR complexes. These correct small insertions or deletions that may occur during somatic division. Thus MMR system proofreads and repairs defects that were overlooked by DNA polymerase.

Cancerous cells with defective MMR gene function exhibit microsatellite instability. This refers to an inconsistent number of microsatellite nucleotide repeats when compared to normal tissue. This phenotype with a markedly high rate of mutations involving cell-cycle regulation increases the risk of malignancy (Figure 1)^[17].

Immunohistochemistry of the tumor samples are used to detect the absence of the protein products of mismatch repair genes. These gene products function as dimers: MSH2 protein may complex with MSH6 or MSH3 protein, and MLH1 protein complexes with PMS2 or PMS1 protein. MSH6 and PMS2 proteins are unstable when unpaired. A pathogenic variant in MSH2 typically results in loss of expression of the proteins MSH2/MSH6 and a germline pathogenic variant in MLH1 results in loss of expression of the proteins MLH1/PMS2. Germline pathogenic variants in MSH6 and PMS2 typically do not result in loss of MSH2 or MLH1 expression because these proteins are still present in other pairings.

LS accounts for 3% to 5% of all CRC^[25] and it is the commonest inherited colon cancer syndrome. The average age of malignancy in LS is 44 years, vs 64 years in sporadic CRC^[3,17].

The risk factors for SBA in LS patient's increase with age, beginning at 40 years and a tenfold rise by the age of 60^[26]. Compared to sporadic SBA in general population, patients with LS present a decade earlier. About 10% of patients develop cancers before the age of 30^[27]. The lifetime risk for SBA is estimated as 1%-4% and is greater than 100 fold risk compared to general population^[13,14,28].

In 30%-70% patients with LS small bowel cancer may be the primary malignancy to manifest^[29]. The incidence appears higher in MLH1, and MSH2 carriers compared to MSH6^[13,29]. Further regional variations between various registries have been noted for the incidence of small bowel cancer. For instance Finnish and French (HNPCC/LS) patients have lesser incidence of small bowel cancer compared to Dutch (HNPCC/LS) patients^[28,29].

Most data from series of patients point to adenoma-carcinoma sequence comparable to colorectal neoplasia. Molecular data as described earlier indicate accumulation of mutations as an inciting event in the development of small bowel cancers similar to colorectal cancers. Some authors recommend that patients presenting with SBA routinely undergo analysis of the MMR phenotype

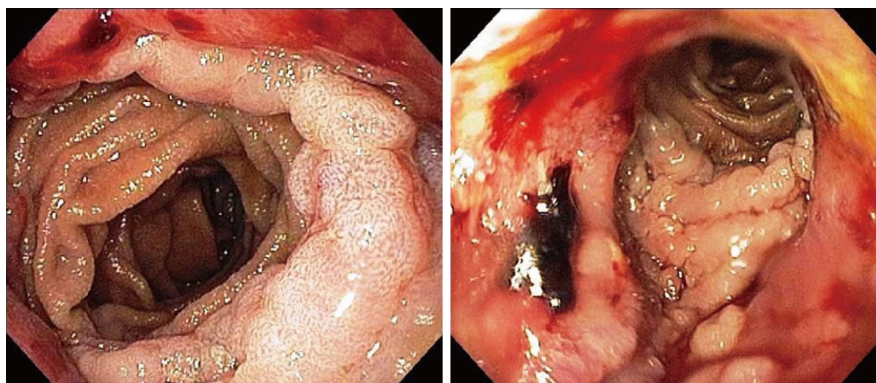


Figure 3 Sixty-nine-year-old male with family history of Lynch syndrome, jejunal adenocarcinoma, viewed on small bowel enteroscopy.

and screened for LS^[13,28,29]. This is especially true for histological findings of mucinous tumors infiltrated with lymphocytes and pushing tumor border suggestive of MSI phenotype in 75% patients^[29]. There are implications in choosing adjuvant chemotherapy regimen in this phenotype, as cancers deficient in MMR proteins may be resistant to 5-FU based chemotherapy^[30].

Upper endoscopic surveillance is recommended over the age of 30 years for gastric and duodenal polyps however at present there are no guidelines for small bowel cancer surveillance in LS^[13,14,17,26,28].

PEUTZ-JEGHERS SYNDROME

Peutz-Jeghers syndrome (PJS) is an autosomal dominant condition with mutation in the serine threonine kinase 11 (*STK11*) genes on the short arm of chromosome 19. The incidence of PJS is reported to be 1 in 50000 to 1 in 200000 live births. PJS is characterized by melanin spots on the buccal mucosa and predilection to form multiple gastrointestinal hamartomas and polyps. These are scattered throughout the small bowel, predominantly in the jejunum and ileum.

The *STK11* gene (also called *LKB1*) encodes for enzyme serine/threonine kinase 11^[31]. *STK11* is a tumor suppressor gene and associates with TP53 to regulate TP53-dependent apoptosis pathways^[32]. It also has a role in cell polarity, cell metabolism and energy homeostasis^[33]. Inactivation of *STK11* is an early event in the development of hamartoma and adenocarcinoma. In addition to loss of *STK11* function and altered TP53 expression, adenocarcinomas in PJS also demonstrate loss of heterozygosity (LOH) in 17p and 18q. These deletions are associated with an increased tendency of disease dissemination in colorectal cancer. *STK11* also exerts its inhibitory effects by phosphorylating and activating 14 protein kinases, all related to the AMP-activated protein kinases (AMPK)^[33]. AMPK is an evolutionally conserved serine threonine kinase and its activation by *STK11* leads to upregulation of signaling through the TSC (Tuberous sclerosis) complex. This in turn negatively regulates mTOR pathways. Loss of *STK11* activity leads to increased mTOR activity and characterized by an increased risk of malignancy (Figure 1)^[31,33,34].

Hamartomatous and adenoma polyps are scattered

throughout the small bowel, predominantly in the jejunum and ileum. Patients with PJS are predisposed to multiple GI tract and non GI tract malignancies which include breast, ovaries, testicular, pancreas, esophagus, stomach and non-small cell lung cancers^[34,35].

SBA has been known to occur in PJS. Meta-analysis of SBA in PJS compared to general population indicates a relative risk of 520^[36]. The life time incidence for adenocarcinoma is 1.7%-13% and rises rapidly in elderly^[36,37]. Adenocarcinoma originates from both adenomas and hamartomas. Intraepithelial neoplasia is observed in the hamartoma lesions^[29,36,37]. Due to the rarity of this condition, current surveillance protocols are not evidence-based. Endoscopies are performed more often to detect polyps which may pose a risk for intussusception, obstruction rather than cancers. Routine screening is recommended, beginning at age 18 with every 2-3 year interval^[31,35,36]. Recent study suggests surveillance with VCE beginning at the age 8 years and performed every three years if polyps are detected at initial examination. With a negative initial exam, surveillance should recommence at 18 years^[31].

JUVENILE POLYPOSIS SYNDROME

Juvenile polyposis syndrome (JPS) is an autosomal dominant disorder which is characterized by multiple hamartomatous polyps of the gastrointestinal tract and is the most common hamartomatous polyp syndromes with prevalence estimated to be between 1 in 16000 to 1 in 100000^[3,38]. Juvenile refers to the sporadic inflammatory hamartomatous polyps of childhood, rather than the age of onset. Most affected individuals have some polyps by age 20 years^[3]. Most are benign polyps, but malignant transformation may occur resulting in increased lifetime risk for colon (10%-40%) and stomach (21%) cancers and less commonly involving the small bowel and pancreas. The lifetime risk of SBA has been difficult to estimate due to the rarity of the disease and is also reduced by screening polypectomies. Malignant transformation occurs through traditional adenoma to cancer transformation sequence. Multiple genetic alterations similar to colorectal neoplasia also play a role in neoplastic transformation of juvenile polyps^[39].

Two genes, SMAD4, BMPR1A, have been implicated in

the pathogenesis of polyps in JPS. They encode proteins for either, transforming growth factor- β (TGF- β) or bone morphogenetic protein (BMP) signaling pathways. The *SMAD* gene on chromosome 18q21.1, adjacent to *DCC* (deleted in colon cancer) is a part of the TGF- β signal transduction pathway. SMAD4 proteins transmit TGF- β related growth-suppressing signals from cell membranes to nucleus mediating growth inhibition and apoptosis. The SMAD4 protein serves both as a transcription factor and as a tumor suppressor^[3]. More than 60 mutations in the *SMAD4* gene have been implicated in JPS. This results in the production of a truncated, nonfunctional protein thereby preventing transmission of TGF- β growth suppressing signals from the cell surface to the nucleus (Figure 1) leading to unregulated cell growth and susceptibility to polyp formation in JPS.

Mutations in *BMPR1A* on chromosome 10 are found in 20% to 25% of individuals with JPS^[40]. *BMPR1A* is a serine-threonine kinase (STK) type I receptor of the TGF- β superfamily, which when activated leads to phosphorylation of SMAD4 proteins. Mutations result in abnormal *BMPR1A* protein which cannot bind to ligands in the TGF- β pathway and interferes with the activation of the SMAD protein complex^[41].

Given the rarity of this disease there is no data on the incidence, relative risks, or life time risks of SBA and at present no guidelines exist for surveillance. Some authors do recommend upper endoscopy every 3-5 years from age 15, and repeated annually if polyps are diagnosed^[42].

CROHN'S DISEASE

Crohn's disease (CD) is an autoimmune inflammatory bowel disease affecting the GI tract with predilection for small intestine. The prevalence in North America ranges from 26.0 to 198.5 cases per 100000 persons. The incidence rates range from 3.1 to 14.6 cases per 100000 people per year^[43]. CD is characterized by transmural granulomatous inflammation of the small bowel in a discontinuous fashion and a tendency to form stenosis, strictures and fistulae. Adenocarcinoma of small intestines is a rare complication of CD with meta-analysis showing relative risks reported to be between 30 as 60 (95%CI: 15.9-60.9) compared to the general population^[44-48] and cumulative risk of 2.2% after 25 years of regional ileitis^[48,49]. The risk increases with chronicity of the disease, young age of onset, male sex, distal small bowel disease with strictures and fistulae.

CD results from abnormal mucosal immune response to environmental factors in genetically susceptible hosts. The granulomatous inflammation comprises of aggregates of macrophages, lymphocytes, plasma cells, and multinucleated giant cells that are formed in response to the release of inflammatory cytokines such as tumor necrosis factor^[15,50]. Etiologies in the pathogenesis of inflammatory bowel disease include genetic susceptibility, environmental, microbial factors and their interaction with intestinal epithelial cells and components of innate and adaptive immune system. Genetic susceptibility is confirmed with

higher prevalence in monozygotic twins and the familial clustering of the disease. A meta-analysis of six twin studies with a combined set of 112 MZ and 196 DZ twin pairs reported concordance rates of 30.3% and 3.6% respectively^[51]. Since 2006, genome-wide association studies have established over 140 loci associated with CD risk, however the significance and the contribution to the disease risk remains to be defined^[52].

The GI tract is continuously exposed to commensal internal flora and also pathogenic organisms and other environmental antigens. The integrity of the mucosal barrier is maintained by tight junctions occurring between adjacent epithelial cells and the relative impermeability of the apical villous epithelium which serves as an important function in the innate immune system. Complementing these are other cells such as Paneth cells which secrete antimicrobial substances such as, lysozymes, cysteine-rich defensins, and IgA and goblet cells which secrete mucus^[15,53]. These and other intrinsic defense mechanisms in the intestinal mucosa dilute, limit the adherence and invasion of commensal and pathogenic microorganisms and antigens. Alteration of this barrier leads to abnormal immune response by the effector lymphocytes and other proinflammatory cytokines leading to a state of chronic intestinal inflammation and its sequelae. Inflammatory cytokines produced by the immune system includes interleukins, chemokines, growth factors, and extracellular proteases. They interact with cell surface receptors and subsequently target genes which influence clonal neoplastic proliferation, angiogenesis and invasion through the basement membrane. In addition, excessive formation of reactive oxygen and nitrogen free radicals are potentially damaging to DNA and the integrity of cell surface membranes^[15].

Adenocarcinoma in CD is seen in the effected segments of the bowel which suggests inflammation-dysplasia-carcinoma sequence^[45,48,54,55]. Genetic alterations occur, which transform dysplastic mucosa to carcinoma. The prevalence of *MSI*, *APC*, *DCC* gene mutations are low, one study however showed 43% of patients with adenocarcinoma in CD carry K-RAS mutations, and overexpression of *p53* gene product in 71% of Crohn's associated carcinoma^[54]. Overexpression of *p53* is helpful to elucidate transformation from inflammation to dysplasia as inflammation does not overexpress *p53*^[55]. A mutational analyses of multiple areas of intestine from ten patients with CD and intestinal cancer, mutations in *KRAS*, *CDKN2A* (p16), and *TP53* that were observed in tumor cells was also present in non-tumor, and both nondysplastic and dysplastic epithelium suggestive of a field defect in CD^[56].

Another study on 41 patients with CD and small bowel cancer showed dysplasia association in 50% of the patients suggesting an inflammation-dysplasia-adenocarcinoma sequence in CD-related SBA, similar to what is observed in chronic colitis-related colorectal cancer (Figure 1)^[55,56]. The rarity of adenocarcinoma in CD makes mutation studies difficult. Perhaps analysis in multinational pooled data may reveal more information.

Symptoms highly suspicious for adenocarcinoma are development of a new small bowel stricture refractory to steroids or maximal medical management or a long standing quiescent disease with newly diagnosed small bowel obstruction. These warrant attention without delay. Compared to adenocarcinoma arising *de novo*, adenocarcinoma in CD present at a median age of 48 years, is more common in males, ileum as most common site and mucinous signet ring cell is more frequently seen^[57]. Early diagnosis and small bowel resection offers the best success for long term survival. Unfortunately majority of adenocarcinoma are diagnosed on post-operative specimens of resected bowel with metastatic nodal disease noted in 50% and distant metastases in 40% of patients. At present however there are no surveillance guidelines to detect SBA in patients with CD however study investigating the benefit of endoscopic surveillance of the small bowel lesions greater than 10 years duration is in progress^[55].

CELIAC DISEASE

Celiac disease is a chronic inflammatory autoimmune small intestinal disorder due to gluten sensitivity, an antigen in wheat, barley, rye and malt. It occurs in adults and children and affects 1% of the population. Celiac disease is associated with both human leukocyte antigen (HLA) and non-HLA genes and with other immune disorders, notably juvenile diabetes and thyroid disease. It is genetically associated with individuals positive for human leukocyte antigen-DQ2 or DQ 8. Familial aggregation is noted with 70% concordance in monozygotic twins^[58]. α -gliadin; a component of gluten is a 33 amino acid peptide sequence and is resistant to degradation by the proteases in the human intestines. Immune response to gliadin promotes inflammatory reaction in the small bowel. Infiltration of the lamina propria and the epithelium with chronic inflammatory cells (predominantly CD4 lymphocytes) triggers a cascade releasing cytokines, interferon- γ , interleukin-15 and metalloproteinases resulting in destruction of enterocytes, crypt hyperplasia and villous atrophy^[59,60].

Patients with celiac disease have an increased risk for enteropathy associated lymphomas as well as adenocarcinoma of the small intestine compared to the general population^[59,61]. Given the rarity of celiac disease and adenocarcinoma the true incidence is difficult to ascertain, however the reported relative risk is increased between 60-80 compared to the general population^[61-63]. Most commonly seen in jejunum, the natural history seems to follow the adenoma-carcinoma sequence as seen in colorectal neoplasms. Small bowel mucosa in celiac disease does not show any premalignant field defect or dysplasia in mucosa adjacent to the adenocarcinoma. However the mechanism for formation of adenomas in celiac disease has not yet been elucidated^[64].

Recent molecular studies have shown that celiac disease associated adenocarcinomas in the elderly are characterized by high level of CpG island methylation

(CIMP), MLH1 inactivation, microsatellite instability (MSI) and defect in the MMR pathways (Figure 1)^[65-67]. Methylation of CpG sites within the promoters of genes can lead to their silencing. This feature is found in a number of human cancers. Similar to LS as described earlier, celiac disease should be considered in the differential diagnosis in patients presenting with sporadic SBA, in the elderly, especially with MSI positivity^[65-67]. These sporadic and celiac associated tumors however show CIMP (CpG island methylator phenotype) and BRAFV600E hotspot mutations that serve to distinguish them from LS cases.

The risk for adenocarcinoma rises in longstanding, untreated celiac disease. Symptoms of celiac disease diagnosed in children and treated with gluten free diet often improve. This may create a false notion of having overcome the disease, with resurgence later in life. Development of new symptoms of weight loss, abdominal pain, anemia, blood loss, and fever in patients who were on a gluten free diet should raise suspicion of neoplastic transformation and should be thoroughly evaluated^[59]. At present there are no guidelines for small bowel surveillance for adenocarcinoma or lymphoma in asymptomatic patients with celiac disease.

SURVEILLANCE MODALITIES FOR SMALL BOWEL

The manifestations of small bowel malignancy are generally nonspecific and often diagnosed in advanced stages. Fifty percent of patients have metastases at diagnosis. Mean duration of symptoms before diagnosis is 10 mo^[68]. Diagnosis is often made with a combination of diagnostic tests which includes both endoscopy and radiography. Considerable advances have occurred in endoscopic techniques with introduction of capsule endoscopy and balloon assisted endoscopy. Also advances in both computed tomography (CT) and magnetic resonance imaging (MRI) enterography and enteroclysis are playing an increasing role in evaluation of small bowel diseases.

Endoscopy

Esophagogastroduodenal (EGD) endoscopy with front and side viewing camera is the standard diagnostic procedure and is accurate in identifying, biopsy of lesions proximal to the ligament of Trietz. Push enteroscopy can visualize the duodenum, proximal jejunum while balloon assisted enteroscopy (BAE) can visualize the entire small bowel (Figure 3). However the latter techniques are time consuming, technically challenging and often requires deep sedation or general anesthesia^[69]. BAE encompasses both single and double balloon techniques and can be performed through the oral or anal route. A complete small bowel examination can be accomplished in up to 80% of the patients. It carries the advantage of ability to perform endoscopic interventions such as biopsy, polypectomy and marking the lesion^[69-71]. A fewer studies

utilizing BAE techniques have confirmed the presence of small bowel polyps in patients with FAP^[10,71,72].

Video capsule endoscopy

Video capsule endoscopy (VCE) has become one of the most important investigational tools for small bowel mucosal evaluation. Due to ease of the procedure it has become a first line tool to detect small bowel abnormalities in non-obstructed patients for evaluation of small intestinal diseases such as occult GI bleeding, suspected CD, celiac disease, small bowel tumors, and motility disorders^[73]. Most VCE studies show the presence of small bowel polyps ranging 50%-87% in patients with FAP^[11,12] and there are a few case series suggesting the role of VCE in LS^[74,75]. A study comparing VCE to MRI showed the advantage of VCE to detect smaller polyps. Polyps larger than 15 mm were detected equally in both groups, whereas smaller polyps were seen much more often with capsule endoscopy. Polyps that were smaller than 5 mm were exclusively seen with capsule endoscopy. However, location of the detected polyps and determination of their exact sizes was more accurate by MRI^[76,77].

Drawback for VCU include capsule retention, missed polyps < 1 cm, especially duodenal polyps (due to rapid transit)^[73,78]. Using combination of VCE and subsequent BAE for endoscopic intervention offers an ideal method of surveillance and treatment in these polyposis syndromes, avoiding a laparotomy. The value of such approach is yet to be demonstrated^[13].

CT and MRI enterography and enteroclysis

Advances in temporal and spatial resolution offered by CT scan and MRI scan with newer enteric agents used to distend the small bowel have replaced barium radiography as the preferred diagnostic tests. Both CT and MRI scan provide details of the bowel wall and the mesentery and the surrounding viscera. Enterography entails using oral contrast while for enteroclysis a nasojejunal tube need to be inserted to deliver the contrast. Enteroclysis provides better bowel distension offers improved mucosal details. MRI enteroclysis has been shown to be a more dynamic and sensitive than CT enteroclysis for mucosal details. These are due to better soft tissue contrast that is achieved with MRI^[79,80]. A study on 150 patients with MRI enteroclysis showed sensitivity, specificity of 86% and 98% respectively^[81]. A recent study compared VCE to MRI enteroclysis with results showing higher specificity of MRI images in detecting small bowel lesions^[82]. The authors attributed this to the distension of the small bowel with enteroclysis and a three dimensional views compared to a uni-directional view of the VCE. Secondly MRI enteroclysis may be beneficial in stenosis or strictures in small bowel disease as the risk of capsule retention are eliminated.

CONCLUSION

Certain genetic and familial diseases are associated

with increased risks for SBA. The pathogenesis and molecular mechanisms for some of these syndromes are described and the risk varies according to the types of polyps and polyposis syndromes. Although the overall incidence of SBA is low the prognosis remains dismal due to nonspecific symptoms and often a delay in diagnosis. Intuitively it would seem that use of surveillance modalities may benefit these patients at higher risk for SBA. At present it is debatable and there is no data to support this approach except for established guidelines to diagnose duodenal polyps in FAP, and LS. Further research, perhaps multi-institutional study is warranted focusing on identifying patients who are at risk for small intestinal adenocarcinoma and on optimal surveillance strategies.

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Case Control Study

Genetic polymorphisms of *interleukin 1 β* gene and sporadic pancreatic neuroendocrine tumors susceptibility

Dimitrios Karakaxas, Anna Sioziou, Gerasimos Aravantinos, Ahmet Coker, Ioannis S Papanikolaou, Theodoros Liakakos, Christos Dervenis, Maria Gazouli

Dimitrios Karakaxas, Christos Dervenis, Department of General Surgery, Agia Olga Hospital, 14233 Athens, Greece

Anna Sioziou, Maria Gazouli, Department of Basic Medical Sciences, Laboratory of Biology, Medical School, National and Kapodistrian University of Athens, 11527 Athens, Greece

Gerasimos Aravantinos, Second Department of Medical Oncology, Agioi Anargiroi Cancer Hospital, Kifisia, 14564 Athens, Greece

Ahmet Coker, Department of General Surgery, Ege University School of Medicine, 35040 Izmir, Turkey

Ioannis S Papanikolaou, Hepatogastroenterology Unit, 2nd Department of Internal Medicine and Research Unit, Attikon University General Hospital, Medical School, National and Kapodistrian University of Athens, 12462 Athens, Greece

Theodoros Liakakos, 1st Department of Surgery, Laiko Athens General Hospital, Medical School, National and Kapodistrian University of Athens, 11527 Athens, Greece

Author contributions: Karakaxas D, Aravantinos G, Coker A, Papanikolaou IS, Liakakos T and Dervenis C collected the samples and the patients data; Karakaxas D, Sioziou A and Gazouli M performed the experiments; Liakakos T, Dervenis C and Gazouli M designed the study, wrote and corrected the manuscript.

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Correspondence to: Maria Gazouli, PhD, Assistant Professor of Molecular Biology, Department of Basic Medical Sciences, Laboratory of Biology, Medical School, National and Kapodistrian University of Athens, Michalakopoulou 176, 11527 Athens, Greece. mgazouli@med.uoa.gr
Telephone: +30-210-7462231
Fax: +30-210-7462231

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Abstract

AIM: To evaluate the association between the interleukin 1 β (IL-1 β) polymorphisms and the pancreatic neuroendocrine tumor (pNET) development.

METHODS: A case-control study was conducted analyzing IL-1 β polymorphisms using germline DNA collected in a population-based case-control study of pancreatic cancer (51 pNET cases, 85 pancreatic ductal adenocarcinoma cases, 19 intraductal papillary mucinous neoplasm and 98 healthy controls).

RESULTS: The distribution of genotypes for the -511

C/T polymorphism in the pNET patient groups showed significant difference compared to the control group. It is known that the carriers of the IL-1 β -511T allele have increased concentrations of IL-1 β . The -511 CT and TT high-expression genotypes were over-represented in pNET patients.

CONCLUSION: The findings of this study suggested a possible role of IL-1 β -511 C/T genotypes in the pathogenesis of pNETs since the presence of the IL-1 β -511 CT and TT genotypes and the T allele was associated with an increased risk of pNET only.

Key words: Interleukin 1 β ; Neuroendocrine tumors; Pancreas

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Core tip: Pancreatic neuroendocrine tumors (pNETs) are a heterogeneous group of rare neoplasms derived from pancreatic endocrine cells and have significantly different tumor biology and present better prognosis compared with tumors of the exocrine pancreas, like pancreatic adenocarcinomas. It is widely accepted that chronic inflammation contributes to pathogenesis of many pancreatic diseases, including pancreatic carcinogenesis. Interleukin 1 β (IL-1 β) is a highly active pro-inflammatory cytokine with multiple biological effects, such as directing cancer cells to either neuroendocrine differentiation or to development of adenocarcinoma. The purpose of the study was to evaluate the association between the IL-1 β polymorphisms and the pNET development.

Karakaxas D, Sioziou A, Aravantinos G, Coker A, Papanikolaou IS, Liakakos T, Dervenis C, Gazouli M. Genetic polymorphisms of interleukin 1 β gene and sporadic pancreatic neuroendocrine tumors susceptibility. *World J Gastrointest Oncol* 2016; 8(6): 520-525 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v8/i6/520.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v8.i6.520>

INTRODUCTION

Pancreatic neuroendocrine tumors (pNETs) are a heterogeneous group of rare neoplasms derived from pancreatic endocrine cells^[1-5]. The annual incidence of pNETs is estimated to be approximately 3.65 per 100000 individuals in the United States and occur sporadically or may be associated with genetic syndromes such as multiple endocrine neoplasia type 1 (MEN-1), von Hippel-Lindau syndrome (VHL), von Recklinghausen disease (neurofibromatosis NF-1), and tuberous sclerosis complex (TSC)^[6-8].

pNETs are mainly considered functionally inactive tumors, but when related with hormone or peptide over-production, such as insulin, gastrin, glucagon, vasoactive intestinal polypeptide (VIP) and somatostatin they are responsible for many characteristic clinical syndromes, with insulinoma being the most common pNETs are usually

asymptomatic^[9,10], have significantly different tumor biology, and present better prognosis compared with tumors of the exocrine pancreas, like pancreatic adenocarcinomas (PDACs)^[11].

The molecular basis of pNETs pathogenesis is poorly characterized but several recent reports have been conducted in order to clarify their etiology^[12].

It is widely accepted that chronic inflammation contributes to pathogenesis of many pancreatic diseases, including pancreatic carcinogenesis^[13,14]. However, the exact mechanism by which chronic inflammation promotes carcinogenesis is still unknown. During carcinogenesis the host-mediated anti-tumor activity is suppressed, whereas pro-inflammatory events support tumor growth, angiogenesis, invasion and metastasis^[15]. The inflammatory response is mediated by cytokines, which are glycoproteins or soluble proteins and their role in cancer immunity and carcinogenesis has been well established^[16-18].

Neuroendocrine tumors express various cytokines and growth-factors. Several pro-inflammatory cytokines have been found in pNETs tissue suggesting their involvement in pNET development^[19-21]. Additionally, numerous studies suggested that gastroenteropancreatic-NETs occur more frequently in the environment of chronic inflammation^[22-24]. Thus, cytokines such as interleukin 1 (IL-1) poses an important role in neuroendocrine tumors since direct cancer cells to either neuroendocrine differentiation or to development of adenocarcinoma, while exogenously added IL-1 results in a decrease of chromogranin A (CgA) and simultaneous increase in carcinoembryonic antigen (CEA) secretion^[25].

IL-1 β is a highly active pro-inflammatory cytokine with multiple biological effects^[26]. IL-1 β protein levels are related to the intensity of the inflammatory response, and regarding to pancreas, IL-1 β is implicated in cancer progression, especially tumor invasiveness, metastasis and angiogenesis^[27,28].

The IL-1 β gene is located in the IL1 cluster on chromosome 2q and several single nucleotide polymorphisms (SNPs) of this gene influence the regulation of its expression and function have been studied^[29-32]. There are two SNPs in the proximal promoter region of the IL-1 β gene, -511 C/T and +3954 T/C, which both have been correlated with gastrointestinal cancers, such as gastric, hepatocellular cancer (HCC) and pancreatic cancer^[33-36]. Recently, Cigrovski Berković *et al.*^[37] reported that the IL-1 β -511 SNP contributes to the pNET susceptibility.

We conducted a case-control study to analyze IL-1 β polymorphisms as risk factors for pNETs using germline DNA collected in a population-based case-control study of pancreatic cancer [51 pNET cases, 85 PDAC cases, 19 intraductal papillary mucinous neoplasm (IPMN) and 98 healthy controls] conducted in the Athens, Greece and Izmir, Turkey areas.

MATERIALS AND METHODS

Patients

The case-control study included 51 pNET cases (22

Table 1 Characteristics of the patients and controls *n* (%)

Characteristic	PDAC	pNET	IPMN	Controls
Total number	85	51	19	98
Mean age (yr)	59.12	56.31	57.91	58.9
Gender				
Male	51 (60)	20 (39.2)	11 (57.9)	74 (75.5)
Female	34 (40)	31 (60.8)	8 (42.1)	24 (24.5)
Tumor stage				
I	13 (15.3)			
II	36 (42.4)			
III	33 (38.8)			
IV	3 (3.5)			
G stage				
G1		35 (68.6)		
G2		14 (27.5)		
G3		2 (3.9)		
Tumor location				
Head	64 (75.3)	19 (37.3)	7 (36.8)	
Body and tail	21 (24.7)	32 (62.7)	12 (63.2)	
Differentiation status				
Well	10 (11.8)			
Moderate	39 (45.9)			
Poor	36 (42.3)			

pNET: Pancreatic neuroendocrine tumor; PDAC: Pancreatic adenocarcinoma; IPMN: Intraductal papillary mucinous neoplasm.

nonfunctional and 29 functional), 85 PDAC cases, 19 IPMN and 98 healthy controls (Table 1). None of the cases had a history of chronic pancreatitis. For subsequent analysis, we excluded cases and controls with known genetic syndromes (*e.g.*, MEN1, MEN2, VHL or TSC). Controls were healthy blood donors with no evidence of inflammation. The diagnosis in all cases was established by standard procedures and confirmed histopathologically either from operatively resected tumors or biopsy tissues, in cases of unresectable tumors. Before commencement of the study, the Ethical committee at the participating centers approved the recruitment protocols. All participants were informed regarding the study, and their written consent was provided.

Genotyping

Genomic DNAs were isolated from peripheral ethylenediaminetetraacetic acid-treated blood of patients and healthy controls using the NucleoSpin Blood Kit (Macherey-Nagel, Germany). The *IL-1 β* -511 C/T (rs16944) polymorphism was detected by PCR-RFLP using the set of primers: 5'-TGGCATTGATCTGGTTCATC-3' and 5'-GTTTAGGAATCTTCCCACTT-3'. The 35 cycles of PCR were carried out at 94 °C for 5 min, 94 °C for 1 min, 58 °C for 40 s and 72 °C for 1 min and the final cycle of 72 °C for 5 min. Amplified PCR products were digested with *Ava*I for 2 h at 37 °C. The fragments of 189- and 116-bp revealed homozygosity for the C allele, and 305-bp indicated homozygosity for the T allele. The +3954 C/T (rs 1143634) polymorphism was detected with the 5'-TCAGGTGTCCTCGAAGAAATCAAA-3' and 5'-GGTTTTTGTCTGTGAGTCCC-3' set of primers and the cycling parameters for that was 94 °C for 5 min, 94 °C for 45 s, 56 °C for 45 s and 72 °C for 45 s and the final cycle

of 72 °C for 5 min. After 35 cycles the PCR product were digested for 2 h at 65 °C with *Taq*I. The fragments of 97- and 85-bp revealed homozygosity for the C allele and on the other hand 182-bp fragments showed homozygosity for the T allele.

Statistical analysis

Genotype frequencies were compared with the χ^2 with Yate's correction using S-Plus (v. 6.2, Insightful, Seattle, WA). Odds ratios (ORs) and 95% CIs were obtained with GraphPad (v. 3.00, GraphPad Software, San Diego, CA). The *P* values are all two-sided, and *P* values of < 0.05 were considered to be significant. Hardy-Weinberg equilibrium was verified by calculating the expected frequencies and numbers and was tested separately in patients and in controls using the goodness-of-fit χ^2 test. Haplotype analysis was performed using the <http://bioinfo.iconcologia.net/SNPstats> software.

RESULTS

The clinicopathological characteristics of the studied population are summarized in Table 1. The genotype frequencies of the *IL-1 β* -511 C/T and +3954 C/T polymorphisms between PDAC, pNET, IPMN patients and controls are given in Table 2. All genotype distributions were in Hardy-Weinberg equilibrium. The distribution of genotypes for the -511 C/T polymorphism in the pNET patient groups only showed significant difference compared to the control group. It is known that the carriers of the *IL-1 β* -511T allele have increased concentrations of IL-1 β ^[38]. The -511 CT and TT high-expression genotypes were over-represented in pNET patients (Table 2). However, the presence of the +3954T

Table 2 Genotype and allele frequencies of the interleukin 1 β -511 C/T and +3954 C/T polymorphisms in pancreatic adenocarcinoma, pancreatic neuroendocrine tumor and intraductal papillary mucinous neoplasm patients and controls

	Controls (n = 98)	PDAC (n = 85)	P; OR (95%CI)	pNET (n = 51)	P; OR (95%CI)	IPMN (n = 19)	P; OR (95%CI)
-511 C/T							
CC	44	35	1	13	1	6	1
CT	47	44	0.64; 1.18 (0.64-2.16)	31	0.04; 2.23 (1.04-4.81)	10	0.59; 1.56 (0.52-4.65)
TT	7	6	1; 1.08 (0.33-3.49)	7	0.04; 3.95 (1.13-13.84)	3	0.16; 3.14 (0.64-15.56)
CT + TT	54	50	0.37; 1.36 (0.75-2.44)	38	0.02; 2.38 (1.13-5.02)	13	0.32; 1.76 (0.62-5.03)
C allele	135	114	1	57	1	22	1
T allele	61	56	0.74; 1.09 (0.7-1.69)	45	0.03; 1.75 (1.07-2.86)	16	0.19; 1.61 (0.79-3.28)
+3954 C/T							
CC	45	50	1	33	1	8	1
CT	44	28	0.08; 0.57 (0.31-1.07)	16	0.07; 0.49 (0.24-1.03)	10	0.79; 1.28 (0.46-3.54)
TT	9	7	0.59; 0.7 (0.24-2.04)	2	0.19; 0.3 (0.06-1.49)	1	1; 0.62 (0.07-5.64)
CT + TT	53	35	0.1; 0.59 (0.33-1.07)	18	0.04; 0.46 (0.23-0.93)	11	0.81; 1.18 (0.43-3.15)
C allele	134	128	1	82	1	26	1
T allele	62	42	0.16; 0.71 (0.45-1.12)	20	0.03; 0.53 (0.29-0.94)	12	0.85; 1.07 (0.51-2.25)

pNET: Pancreatic neuroendocrine tumor; PDAC: Pancreatic adenocarcinoma; IPMN: Intraductal papillary mucinous neoplasm.

allele seems to have a protective role in the pNET development since it is found to be over-represented in healthy controls. The haplotype analysis did not reveal any significant association. No significant association was found between genotypes, haplotypes, and clinico-pathological data of the patients.

DISCUSSION

PNETs are a rare, heterogeneous group of neuroendocrine tumors. They usually have a better prognosis than the PDACs. The cause of these tumors is not fully understood, but differential expression of proinflammatory cytokines were found in pNET tissues^[19-21]. The findings of this study suggested a possible role of IL-1 β -511 C/T genotypes in the pathogenesis of pNETs since the presence of the IL-1 β -511 CT and TT genotypes and the T allele was associated with an increased risk of pNET only. None significant correlation was found with PDAC and IPMN cases. Although Barber *et al.*^[36], reported that the +3954 C/T polymorphism of the IL-1 β gene predisposes to pancreatic cancer; our findings did not reveal any significant association. Additionally, they are partly in agreement with the findings of Cigrovski Berkovic *et al.*^[37], which suggest that there is an association between the IL-1 β -511 C/T genotype and the susceptibility to pNET, especially functional pNETs. In our study we did not find any haplotype combination to be statistically associated with the susceptibility to pNETs, neither PDAC nor IPMN cases, but we observed that the +3954T allele is over-represented among healthy controls compared to pNET cases suggesting that this allele might have a protective role in pNET development.

Carcinogenesis in the gastrointestinal tract and pancreas is often associated with chronic inflammation^[39-42]. It is known that the carriers of the -511T allele associated with high IL-1 β serum levels^[38], and in different type of cancers IL-1 β levels correlate with inflammation, worse prognosis and carcinoembryonal antigen (CEA) levels, a well-known

biomarker of tumor exocrine differentiation^[25,43].

Our previous results suggested that TNF- α -1031 polymorphism is associated with the development of pNET and IPMN^[41], and several studies supported that pro-inflammatory cytokines were detected in pNET tissues signifying their etiological involvement^[19,44]. Taken these into consideration future studies in larger populations are needed to elucidate the role of cytokines and inflammatory pathway in the sporadic pNET development.

COMMENTS

Background

Carcinogenesis in the gastrointestinal tract and pancreas is often associated with chronic inflammation. The study provides evidence of a role of interleukin 1 β (IL-1 β) -511 C/T genotypes in the pathogenesis of pancreatic neuroendocrine tumors (pNETs).

Research frontiers

PNETs are a rare, heterogeneous group of neuroendocrine tumors. They usually have a better prognosis than the pancreatic adenocarcinomas. The cause of these tumors is not fully understood, but differential expression of proinflammatory cytokines were found in pNET tissues. Identifying genetic factors associated basically with pNET incidence may help in the primary prevention of pNET across the globe.

Innovations and breakthroughs

The study suggested a possible role of IL-1 β -511 C/T genotypes in the pathogenesis of pNETs since the presence of the IL-1 β -511 CT and TT genotypes and the T allele was associated with an increased risk of pNET only.

Applications

The study contributes to elucidate the role of cytokines and inflammatory pathway in the sporadic pNET development.

Terminology

PNETs: Pancreatic neuroendocrine tumors; PDACs: Pancreatic adenocarcinomas; IPMN: Intraductal papillary mucinous neoplasm.

Peer-review

This is an interesting study that looks at IL-1 β as a potential inflammatory

cytokine stimulus for tumour formation in pNETs. While chronic inflammation is known to contribute to carcinogenesis, in the pancreas, this is peculiar to PDAC where association with chronic pancreatitis is not uncommon.

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**MINIREVIEWS**

- 526 Pancreatic injury in patients with septic shock: A literature review

Chaari A, Abdel Hakim K, Bousselmi K, Etman M, El Bahr M, El Saka A, Hamza E, Ismail M, Khalil EM, Kauts V, Casey WF

ORIGINAL ARTICLE**Basic Study**

- 532 MicroRNA-320 family is downregulated in colorectal adenoma and affects tumor proliferation by targeting CDK6

Tadano T, Kakuta Y, Hamada S, Shimodaira Y, Kuroha M, Kawakami Y, Kimura T, Shiga H, Endo K, Masamune A, Takahashi S, Kinouchi Y, Shimosegawa T

Retrospective Cohort Study

- 543 Opioid-sparing effect of selective cyclooxygenase-2 inhibitors on surgical outcomes after open colorectal surgery within an enhanced recovery after surgery protocol

Lohsiriwat V

Retrospective Study

- 550 Management of colorectal neoplasia during pregnancy and in the postpartum period

Aytac E, Ozuner G, Isik O, Gorgun E, Stocchi L

- 555 Prognostic value of inflammation-based markers in patients with pancreatic cancer administered gemcitabine and erlotinib

Lee JM, Lee HS, Hyun JJ, Choi HS, Kim ES, Keum B, Seo YS, Jeon YT, Chun HJ, Um SH, Kim CD

Observational Study

- 563 Undernutrition, risk of malnutrition and obesity in gastroenterological patients: A multicenter study

Rizzi M, Mazzuoli S, Regano N, Inguaggiato R, Bianco M, Leandro G, Bugianesi E, Noè D, Orzes N, Pallini P, Petroni ML, Testino G, Guglielmi FW

SYSTEMATIC REVIEWS

- 573 Systematic review of laparoscopic vs open surgery for colorectal cancer in elderly patients

Fujii S, Tsukamoto M, Fukushima Y, Shimada R, Okamoto K, Tsuchiya T, Nozawa K, Matsuda K, Hashiguchi Y

Contents

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Editorial Board Member of *World Journal of Gastrointestinal Oncology*, Han-Xiang An, MD, Doctor, Department of Radiation Oncology, University Hospital Marburg, Marburg D-35043, Germany

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WJGO covers topics concerning carcinogenesis, tumorigenesis, metastasis, diagnosis, prevention, prognosis, clinical manifestations, nutritional support, molecular mechanisms, and therapy of benign and malignant tumors of the digestive tract. The current columns of *WJGO* include editorial, frontier, diagnostic advances, therapeutics advances, field of vision, mini-reviews, review, topic highlight, medical ethics, original articles, case report, clinical case conference (Clinicopathological conference), and autobiography. Priority publication will be given to articles concerning diagnosis and treatment of gastrointestinal oncology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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World Journal of Gastrointestinal Oncology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-85381891
Fax: +86-10-85381893
E-mail: editorialoffice@wjnet.com
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Pancreatic injury in patients with septic shock: A literature review

Anis Chaari, Karim Abdel Hakim, Kamel Bousselmi, Mahmoud Etman, Mohamed El Bahr, Ahmed El Saka, Eman Hamza, Mohamed Ismail, Elsayed Mahmoud Khalil, Vipin Kauts, William Francis Casey

Anis Chaari, Karim Abdel Hakim, Kamel Bousselmi, Mahmoud Etman, Mohamed El Bahr, Ahmed El Saka, Eman Hamza, Mohamed Ismail, Elsayed Mahmoud Khalil, Vipin Kauts, William Francis Casey, Department of Intensive Care, King Hamed University Hospital, Al Muharaq 24343, Bahrain

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Correspondence to: Anis Chaari, MD, Department of Intensive Care, King Hamed University Hospital, Building 234, Road 2835, Block 228, Bussaiten, Al Muharaq 24343, Bahrain. anischaari2004@yahoo.fr
Telephone: +973-38073955
Fax: +973-17766428

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Abstract

Sepsis and septic shock are life threatening condition associated with high mortality rate in critically-ill patients. This high mortality is mainly related to the inadequacy between oxygen delivery and cellular demand leading to the onset of multiorgan dysfunction. Whether this multiorgan failure affect the pancreas is not fully investigated. In fact, pancreatic injury may occur because of ischemia, overwhelming inflammatory response, oxidative stress, cellular apoptosis and/or metabolic derangement. Increased serum amylase and/or lipase levels are common in patients with septic shock. However, imaging test rarely reveal significant pancreatic damage. Whether pancreatic dysfunction does affect the prognosis of patients with septic shock or not is still a matter of debate. In fact, only few studies with limited sample size assessed the clinical relevance of the pancreatic injury in this group of patients. In this review, we aimed to describe the epidemiology and the physiopathology of pancreatic injury in septic shock patients, to clarify whether it requires specific management and to assess its prognostic value. Our main finding is that pancreatic injury does not significantly affect the outcome in septic shock patients. Hence, increased serum pancreatic enzymes without clinical features of acute pancreatitis do not require further imaging investigations and specific therapeutic intervention.

Key words: Septic shock; Pancreas; Lipase; Amylase; Prognosis

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Core tip: Pancreatic injury is common in septic shock patients. Tissue hypoperfusion is the main leading cause of pancreatic insult. Other factors such as oxidative stress

and cellular apoptosis have been reported to enhance the pancreatic damage. The clinical relevance of increased level of pancreatic enzymes is not well established. In fact, hyperamylasemia and/or hyperlipasemia are not associated with higher mortality. Moreover, most of the imaging investigations do not show significant morphological changes of the pancreas. Hence, disturbed serum pancreatic enzymes without clinical evidence of acute pancreatitis should not trigger any specific therapy.

Chaari A, Abdel Hakim K, Bousselmi K, Etman M, El Bahr M, El Saka A, Hamza E, Ismail M, Khalil EM, Kauts V, Casey WF. Pancreatic injury in patients with septic shock: A literature review. *World J Gastrointest Oncol* 2016; 8(7): 526-531 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v8/i7/526.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v8.i7.526>

INTRODUCTION

Severe sepsis and septic shock are common life-threatening conditions in critically-ill patients^[1-3]. Despite recent therapeutic advances and the establishment of internationally accepted guidelines regarding the management of patients suffering from septic shock, the overall mortality in these patients ranges from 30% to 60%^[2,4,5]. This high mortality is usually associated with the onset of multiple organ dysfunction. In fact, a few studies have reported that the worsening of organ function as well as the increase in the number of the failing organs is significantly associated with poor outcome in both adult and pediatric patients^[6,7]. Accordingly, it has been reported that the onset of acute kidney injury is associated with a significant rise in the intensive care unit (ICU) mortality up to 50%-70% and that the highest mortality has been in patients with a high score on the severity of illness scale and/or in those who require renal replacement therapy^[2,8-10]. Similarly, hypoxic liver injury in patients with septic shock has been reported to be associated with a mortality as high as 50%^[11,12]. Experimental and clinical studies also suggest that gut ischemia is one of the hallmarks of septic shock^[13-15]. However, whether pancreatic exocrine function is also impaired in septic shock patients has not been fully investigated. Moreover, there is still debate regarding the optimum modality for management of pancreatic insult as well as its prognostic value.

The aim of this review is to describe the epidemiology and the physiopathology of pancreatic injury in septic shock patients, to clarify whether it requires specific management and to assess its prognostic value.

RESEARCH

A systematic literature search was conducted through Pubmed by using the following Medical Subheadings terms: Septic shock, sepsis, lipase, amylases and acute pancreatitis. Different Boolean operator combinations

(AND/OR) were attempted. Overall, 97 articles were selected for this review. We didn't proceed to any language restriction and only the studies published between 1996 and 2016 were considered.

EPIDEMIOLOGY OF PANCREATIC INJURY IN SEPTIC SHOCK

The incidence of pancreatic injury in critically-ill patients is extremely variable according to the used definition. High levels of amylase levels have been reported in 32% to 79% of patients admitted in medical or surgical ICUs^[16-19]. However, most of these studies have concluded that this elevation is not always due to pancreatic insults^[16,18]. In fact, the proportion of non-pancreatic isoamylase in patients with hyperamylasemia has been reported to range from 30% to 74% of the total serum amylase^[16,18]. Hence, other markers have been used to assess the exocrine pancreatic dysfunction in critically-ill patients. Lipase is one such marker which is more specific for the diagnosis of pancreatitis^[20]. Similar to hyperamylasemia, increased lipase serum level is also common in critically-ill patients. In fact, Manjuck *et al.*^[21] reported that hyperlipasemia is found in 40% of the patients requiring ICU admission. Similarly, Denz *et al.*^[19] reported increased serum lipase levels in 57% of critically-ill patients. Recent guidelines have highlighted that the rise of one or both of these two enzymes should be higher than three times the upper limit of normal range to be considered as a useful criterion for acute pancreatitis^[22]. However, only a limited number of patients admitted to the ICU with a diagnosis other than pancreatitis fulfill this definition^[23] and/or have significant morphological changes in pancreatic anatomy on imaging tests^[19,21]. Only a few studies have focused on the exocrine pancreatic dysfunction in the subgroup of critically-ill patients with septic shock^[23-25]. Hence, epidemiological data regarding the pancreatic function impairment in this group of patient is lacking.

PHYSIOPATHOLOGY OF PANCREATIC INJURY IN SEPTIC SHOCK

The pathophysiology of pancreatic injury in septic shock patients is not fully understood. The most commonly accepted hypothesis is pancreatic ischemia^[26,27]. However, few experimental and human studies have suggested that other mechanisms might also be involved such as cell apoptosis^[28,29], increased release of nitric oxide by the endothelial cells^[30], platelets activation^[31], ischemia - reperfusion phenomenon^[32], elevated triglyceride levels and the development of biliary sludge^[33].

Pancreatic ischemia

Severe hypotension and tissue hypoperfusion are the main hallmarks of septic shock^[34,35]. Experimental studies have shown that gut perfusion is severely impaired in the early stages of septic shock^[14,36]. In a porcine model of septic shock caused by fecal peritonitis, Ijungdahl *et al.*^[14]

have reported that the oxygen consumption of the gut, including that of pancreas, is markedly increased in this condition. This is accompanied by a significant decrease in the gut intramucosal pH which occurs even before the lactate rises in the arterial blood. The pancreas is particularly sensitive to hypotension. In fact, a temporary ischemia for 40 min has been shown to be sufficient to cause significant pancreatic injury on histological examination, presenting mainly as peripheral necrosis of the lobules^[37]. Several studies have suggested that the impairment of pancreatic perfusion is more pronounced in septic shock. In fact, in an experimental animal model study, Raper *et al.*^[26] reported that the cardiac output is increased during the hyper dynamic phase of septic shock. Concomitantly, the systemic blood flow is increased in the gallbladder and the colon whereas it is markedly decreased in the pancreas. This demonstrates that the oxygen delivery to the pancreatic cells is significantly decreased despite the considerable increase of their oxygen requirement^[26].

Beside these macro-circulatory abnormalities, pancreatic injury related to septic shock can also be explained by micro-circulatory and cellular dysfunctions^[38]. In fact, severe sepsis and septic shock are commonly associated with coagulation abnormalities, usually manifested as disseminated intravascular coagulation^[39,40]. Several forensic studies have reported ischemic and necrotic changes in various organs. These include occlusion and fibrin deposition in small and mid-size vessels, observed in patients who die from septic shock^[41]. These abnormalities are triggered mainly by an overwhelming inflammatory reaction which is orchestrated by the immune host defense in response to the endotoxin aggression^[34,39]. The expression of the tissue factor by the mononuclear, polymorphonuclear and endothelial cells activates the coagulation cascade^[42,43]. Activation of platelets, down-regulation of anticoagulant pathways and reduced fibrin removal due to inhibition of fibrinolysis enhances microvascular thrombosis^[39]. Experimental studies have shown that the pancreatic microcirculation is deeply disturbed in septic shock. In fact, in a model of fecal peritonitis, Hildebrand *et al.*^[27] reported that the microcirculatory flow is reduced by 50% in various splanchnic organs within 240 min. The flow normalizes after fluid resuscitation in all the organs, except in the pancreas.

Although the most widely accepted hypothesis used to explain pancreatic dysfunction in patients with septic shock is pancreatic ischemia, significant pancreatic injury has also been reported in normotensive sepsis model. This suggests that other mechanisms may also be responsible for causing pancreatic ischemia^[44].

Cellular apoptosis

Delayed and inappropriate management of septic shock is associated with a worse outcome due to multiple organ dysfunction syndrome (MODS)^[45-48]. The main cause of MODS in this condition is the uncontrolled inflammatory storm caused by overwhelming host

immune response^[49]. Beside the deleterious effect of this reaction on the macrocirculation and microcirculation, as described above, the pro-inflammatory cytokines—mainly interleukine (IL)-1 β , IL-6 and tumor necrosis factor (TNF)- α - also activate the NF- κ B pathway. This causes cellular self-destruction and apoptosis^[50]. This has been demonstrated in the hepatocytes and the immune cells with severe Gram-negative bacterial infection^[50,51]. Experimental studies have shown that the exposure of pancreatic cells to lipopolysaccharide is associated with apoptosis and increased release of TNF- α , IL-1 β and IL-8. Damage to the Acinar cells consists of nuclear fragmentation, abnormal cytoplasmic vacuoles and cellular swelling^[28,29,52,53]. Unlike these experimental studies, there is no evidence to suggest that apoptosis is a major cause of exocrine pancreatic dysfunction in patients suffering from septic shock. In fact, histological studies performed in patients who died from septic shock and multiorgan failure have shown that the apoptosis of acinar cells is seen only in a scattered manner^[54].

Other mechanisms

Other hypothesis that may explain pancreatic injury in patients suffering from septic shock.

The oxidative stress: Oxidative stress has been demonstrated in patients suffering from septic shock patients^[55]. The main causes of mitochondrial dysfunction and increased release of reactive oxygen species are ischemia/reperfusion phenomenon and inflammation^[55,56]. Other factors, such as the activation of the phagocytic cells and the production of nitric oxide by the endothelial cells, have been shown to aggravate the oxidative stress^[57]. The cellular damage in sepsis is enhanced by the depletion of antioxidants and scavenger enzymes such as glutathione and thiamine in the plasma^[56,58]. Several studies have suggested that the oxidative stress can induce pancreatic damage in septic shock^[32,59].

Triglycerides: Serum triglyceride level has been reported to be significantly increased in septic shock^[60,61]. Moreover, compared to those patients who survived septic shock, patients who died from it have been found to have a higher serum triglyceride level over the first 7 d of their illness^[62]. Whether the high level of serum triglyceride seen in patients with septic shock is enough to induce pancreatic cell damage need to be investigated.

CLINICAL RELEVANCE OF PANCREATIC INJURY

The clinical relevance of increased amylase and/or lipase in patient with septic shock has been poorly investigated. Whether pancreatic injury is only a satellite phenomenon or a major condition affecting the prognosis of this group of patients is still a matter of debate. In fact, only a few studies, most of them with a small number of patients, have investigated pancreatic dysfunction in critically-

ill patients^[19,21,23-25,44]. Pezzilli *et al.*^[23] have reported that amylase and lipase levels are significantly increased in patients with septic shock in comparison to a control group. However, none of the included patients met the criteria of acute pancreatitis and no significant correlation was found with mortality. These findings have been corroborated by post-mortem pancreatic tissue sample examination which has not shown significant morphological changes^[24].

Available data suggest that imaging tests should not be requested for all critically-ill patients with deranged pancreatic enzymes as long as the clinical assessment does not suggest acute pancreatitis. In fact, Denz *et al.*^[19] reported that contrast enhanced computed tomography performed for all patients with a serum lipase level higher than 450 U/L was positive only in 35% of the patients. None of these patients had severe necrotizing pancreatitis which required specific management. However, the authors have reported that imaging abnormalities are more common in patients with increased blood levels of pancreatitis-associated protein. This raises the question: Which marker can be considered as a reliable test to assess the pancreatic dysfunction?

Even though the available data shows that the increase in the levels of pancreatic enzymes does not affect the mortality in critically-ill patients, the pancreatic dysfunction may cause malnutrition in patient with prolonged stay in intensive care units. In fact, the pancreatic secretory function is important for the digestion and absorption of fats, protein and carbohydrates^[63]. In a prospective cross-sectional study of 563 critically-ill patients, Wang *et al.*^[64] reported that the prevalence of exocrine pancreatic insufficiency in these patients is 52.2% although only 34.9% of these patients had increased serum lipase levels and only 30.2% had increased serum amylase levels. The definition of exocrine pancreatic insufficiency in their study was based on decreased fecal elastase-1 concentration (< 200 mcg/g). The authors have also found that both shock and sepsis are independent factors which predict exocrine pancreatic insufficiency. Tribi *et al.*^[25] have reported similar results as they found that the concentration of amylase and chymotrypsin in the duodenal juice is significantly lower in patients with sepsis or septic shock than in healthy volunteers. Moreover, the concentration of trypsin is significantly lower in septic shock patients than sepsis patients without shock. The therapeutic implications of these findings need to be investigated by further studies.

CONCLUSION

Pancreatic injury is common in patients suffering from septic shock. Increase in levels of pancreatic enzymes does not significantly affect the outcome. Only those patients who show clinical features of acute pancreatitis need to undergo further radiological investigations. However, pancreatic dysfunction may affect the nutritional state of patients receiving enteral feeding and requiring prolonged ICU stay. Whether specific treatment should be considered to avoid malnutrition in these patients need to

be investigated further.

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Basic Study

MicroRNA-320 family is downregulated in colorectal adenoma and affects tumor proliferation by targeting CDK6

Toshihiro Tadano, Yoichi Kakuta, Shin Hamada, Yosuke Shimodaira, Masatake Kuroha, Yoko Kawakami, Tomoya Kimura, Hisashi Shiga, Katsuya Endo, Atsushi Masamune, Seiichi Takahashi, Yoshitaka Kinouchi, Tooru Shimosegawa

Toshihiro Tadano, Yoichi Kakuta, Shin Hamada, Yosuke Shimodaira, Masatake Kuroha, Yoko Kawakami, Tomoya Kimura, Hisashi Shiga, Katsuya Endo, Atsushi Masamune, Tooru Shimosegawa, Division of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai 980-8574, Japan

Seiichi Takahashi, Community Gastroenterology (Iwaki Kyoritsu Hospital), Tohoku University Graduate School of Medicine, Sendai 980-8574, Japan

Yoshitaka Kinouchi, Health Administration Center, Center for the Advancement of Higher Education, Tohoku University, Sendai 980-8574, Japan

Author contributions: Takahashi S, Kinouchi Y and Shimosegawa T designed the research; Tadano T, Shimodaira Y, Kuroha M, Kawakami Y, Kimura T, Shiga H and Endo K performed the experiments; Kakuta Y, Hamada S and Masamune A analyzed data; Tadano T and Kakuta Y wrote the paper.

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Correspondence to: Toshihiro Tadano, MD, Division of Gastroenterology, Tohoku University Graduate School of Medicine, 1-1

Seiryō, Aoba, Sendai, 980-8574, Japan. md465719@med.tohoku.ac.jp
Telephone: +81-22-7177171
Fax: +81-22-7177177

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Abstract

AIM: To investigate the microRNA (miRNA) expression during histological progression from colorectal normal mucosa through adenoma to carcinoma within a lesion.

METHODS: Using microarray, the sequential changes in miRNA expression profiles were compared in colonic lesions from matched samples; histologically, non-neoplastic mucosa, adenoma, and submucosal invasive carcinoma were microdissected from a tissue sample. Cell proliferation assay was performed to observe the effect of miRNA, and its target genes were predicted using bioinformatics approaches and the expression profile of SW480 transfected with the miRNA mimics. mRNA and protein levels of the target gene in colon cancer cell lines with a mimic control or miRNA mimics were measured using qRT-PCR and Western blotting. The expression levels of miRNA and target gene in colorectal tissue samples were also measured.

RESULTS: Microarray analysis identified that the miR-320 family, including miR-320a, miR-320b, miR-320c, miR-320d and miR-320e, were differentially expressed in adenoma

and submucosal invasive carcinoma. The miR-320 family, which inhibits cell proliferation, is frequently downregulated in colorectal adenoma and submucosal invasive carcinoma tissues. Seven genes including CDK6 were identified to be common in the results of gene expression array and bioinformatics analyses performed to find the target gene of the miR-320 family. We confirmed that mRNA and protein levels of CDK6 were significantly suppressed in colon cancer cell lines with miR-320 family mimics. CDK6 expression was found to increase from non-neoplastic mucosa through adenoma to submucosal invasive carcinoma tissues and showed an inverse correlation with miR-320 family expression.

CONCLUSION: MiR-320 family affects colorectal tumor proliferation by targeting CDK6, plays important role in its growth, and is considered to be a biomarker for its early detection.

Key words: CDK6; Colorectal cancer; MiR-320 family; Colorectal adenoma; Laterally spreading type

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Core tip: We investigated for the first time the sequential changes of miRNA expression profiles in colonic lesions from matched samples; histologically, non-neoplastic mucosa, adenoma, and submucosal invasive carcinoma were microdissected from a tissue sample. We have shown that the miR-320a, miR-320b, miR-320c, miR-320d are downregulated from colorectal adenoma and miR-320e is downregulated from colorectal submucosal carcinoma tissue and the miR-320 family suppresses tumor proliferation by targeting CDK6. The miR-320 family may play an important role in the growth of colorectal tumors and can be considered as a biomarker for the early detection of colorectal tumors.

Tadano T, Kakuta Y, Hamada S, Shimodaira Y, Kuroha M, Kawakami Y, Kimura T, Shiga H, Endo K, Masamune A, Takahashi S, Kinouchi Y, Shimosegawa T. MicroRNA-320 family is downregulated in colorectal adenoma and affects tumor proliferation by targeting CDK6. *World J Gastrointest Oncol* 2016; 8(7): 532-542 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v8/i7/532.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v8.i7.532>

INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies resulting in cancer-related deaths in the world^[1]. The adenoma-carcinoma sequence, which involves a series of changes from normal colorectal epithelium through an adenoma to an invasive and metastatic tumor, has been widely recognized as an important developmental mechanism in CRC^[2]. According to this theory, the colorectal adenoma (CRA) is considered

to be a precursor lesion of CRC; the removal of CRAs by polypectomy has been shown to reduce the incidence and mortality due to CRCs^[3]. Endoscopic mucosal resection and endoscopic submucosal dissection (ESD) enable resection of almost all intramucosal neoplasia with submucosal invasion of less than 1000 μ m in the colon^[4]. Thus, detection of colorectal tumors in the early stages has become increasingly important in treatment and prognosis.

The adenoma-carcinoma sequence is accompanied by several genetic and epigenetic alterations, such as mutations of cancer-associated genes and epigenetic modifications, including changes in DNA methylation, histone modifications, and microRNAs (miRNAs)^[5,6]. However, the involvement of miRNAs in the mechanism of this sequence remains undetermined. miRNAs are small (19-23 nucleotide) endogenous non-coding RNAs that regulate gene expression by targeting the 3' untranslated region (UTR) of mRNA. miRNAs play fundamental roles in various biological processes^[7]. Accumulating evidence indicates that miRNAs are frequently dysregulated in human cancers^[8], and alterations of miRNA expression in colorectal tumors have been well documented. For example, miRNA profiles of CRC compared with those of normal mucosa^[9] and adenoma^[6,10] have been reported. However, limited reports exist on the sequential changes in miRNA expression during histological progression from normal colonic mucosa through colorectal adenoma to early carcinoma in a lesion from a patient.

Furthermore, according to the Paris and Japanese classification^[11,12], a flat colorectal lesion exceeding 10 mm in diameter is classified as a "laterally spreading type (LST)" and subclassified into a granular (G) or a non-granular (NG) type. The percentage of gene mutation in the protruded tumor is hypothesized to be different from that of the LST^[13]. When we focused on miRNA, expression changes of some miRNAs in exophytic and flat elevated tumors were reported^[14]; however, there is no analysis comparing miRNA expressions of the LST with those of the protruded tumor.

Therefore, we analyzed miRNA expressions of both LSTs and protruded tumors as a specific feature of the stepwise progression from adjacent non-neoplastic mucosa to adenoma and submucosal invasive carcinoma using matched samples to compare accurate miRNA expression in each phase.

MATERIALS AND METHODS

Tissue samples

Formalin-fixed, paraffin-embedded (FFPE) colorectal tissue samples that included carcinoma, adenoma, and adjacent non-neoplastic mucosa were obtained from patients who underwent ESD at Tohoku University between January 2011 and December 2014 and provided written informed consent for study participation. All the cancers were limited to submucosal carcinomas, which were classified as T1MON0 (American Joint Committee on Cancer Staging

Manual) in this study. The 18 colorectal samples were LST (15 carcinoma with adenoma and 3 carcinoma without adenoma) and the 3 colorectal samples were protruded-type carcinoma with adenoma.

A senior pathologist reviewed the histopathological grades and types of all cases. Regions of colorectal carcinoma, pre-existing adenoma, and adjacent non-neoplastic mucosa in a resection specimen were microdissected using a laser microdissection (LMD) system (Leica Microsystems, Wetzlar, Germany). This study was reviewed and approved by the ethics committee and the institutional review board at the Tohoku University Hospital.

Cell lines and transfection

Human colon cancer cell lines HT29 and SW480 were obtained from the American Type Culture Collection (Manassas, VA) and then grown in Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO₂. The human intestinal epithelial cells (InEpC) were obtained from Lonza, Japan.

We used mirVana™ miRNA 320a, b, c, d, and e mimic; mimic control; inhibitor; and inhibitor control (Life Technologies, Carlsbad, CA). These RNA oligonucleotides were transiently transfected into SW480 and HT29 cells using the Lipofectamine RNAiMAX Reagent (Invitrogen, United States) according to the manufacturer's instructions.

RNA isolation

Total RNA from microdissected FFPE samples was purified using a miRNAeasy FFPE kit (QIAGEN, Hilden, Germany). Total RNA isolated from cultured cells using TRIzol reagent (Invitrogen) was purified with an RNeasy Mini kit (QIAGEN).

miRNA microarray analysis

The RNA from FFPE samples was labeled with a miRNA complete labeling and Hyb kit (Agilent Technologies, Santa Clara, CA, United States), and the labeled probes were hybridized onto Agilent Human miRNA Microarray Rel 19.0 according to the manufacturer's instructions. The arrays were scanned and the data were extracted and analyzed using the Agilent Feature Extraction software and Agilent GeneSpring software.

Quantitative real-time polymerase chain reaction

RNA samples were reverse transcribed into cDNA using QuantiTect Reverse Transcription Kit and miScript II RT Kit (QIAGEN). Each cDNA sample was analyzed in triplicate using the QuantiFast SYBR Green PCR kit (QIAGEN) with the StepOnePlus real-time PCR system (Life Technologies). A comparative $\Delta\Delta C_t$ method was used for the quantification of the miR-320 family and CDK6 expression; expression levels of the hsa-miR-320 family and CDK6 were normalized by those of U6 and GAPDH, respectively. A miScript Primer Assay (QIAGEN) was used for the miR-320 family and U6. The following primer sets were used for other quantitative reverse transcription (qRT)-PCR assays:

CDK6 Forward: 5'-GGATAAAGTTCCAGAGCCTGGAG-3';
CDK6 Reverse: 5'-GCGATGCACTACTCGGTGTGAA-3';
and GAPDH Forward: 5'-ATCAGCAATGCCTCCTGCAC-3';
Reverse: 5'-ATGGCATGGACTGTGGTCAT-3'.

Cell proliferation assay

Human colon cancer cell lines SW480 were seeded in 96-well plates, and cell proliferation was measured 24, 48, 72, 96, and 120 h later using the CellTiter96 Aqueous One Solution Cell Proliferation Assay (MTS assay; Promega, Madison, WI, United States) according to manufacturer's instructions.

Gene expression analysis and miRNA target prediction

Gene expression profiling of SW480 transfected with a mimic control or miR-320a mimics was performed using the SurePrint G 3 Human Gene Expression 8x60K v2 Microarray Kit (Agilent Technologies) according to the manufacturer's instructions. Candidates of miRNA target genes were selected according to the results of these mRNA expression analysis and two different bioinformatics algorithms-TargetScan (<http://www.targetscan.org>) and Pic tar (<http://pictar.mdc-berlin.de/>).

Protein extraction and Western blot

Total cell lysates were prepared using a mammalian cell extraction kit (BioVision, Mountain View, CA, United States). Protein concentrations in the lysates were measured using the BCA Protein Assay kit (Pierce Chemical Co., Rockford, IL, United States). Equal amounts of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. After incubation with Tris-buffered saline and Tween-20 containing an ECL blocking agent, the membranes were incubated with primary antibodies against CDK6 (Cell Signaling Technology, Inc., Danvers, MA, United States) or α -tubulin (B512, Sigma) at 4 °C overnight and further incubated with secondary antibodies for 1 h at room temperature. Reactive bands were detected using the ECL Prime Western Blotting Detection Reagent (GE Healthcare, Bucks, United Kingdom).

Statistical analysis

Data from at least three independent experiments were analyzed. Statistical analysis was conducted using Excel (Microsoft). The difference between two groups was analyzed using the paired *t*-test for miRNA array data and the Student's *t*-test for qRT-PCR data. The statistical significance of correlations between the expressions of the miR-320 family and CDK6 mRNA was evaluated using Pearson's correlation analysis. *P* < 0.05 was considered statistically significant.

RESULTS

Most miRNAs associated with the adenoma-carcinoma sequence were common to LSTs and protruded tumors

Sequential changes of miRNA expression profiles from matched samples, histologically non-neoplastic mu-

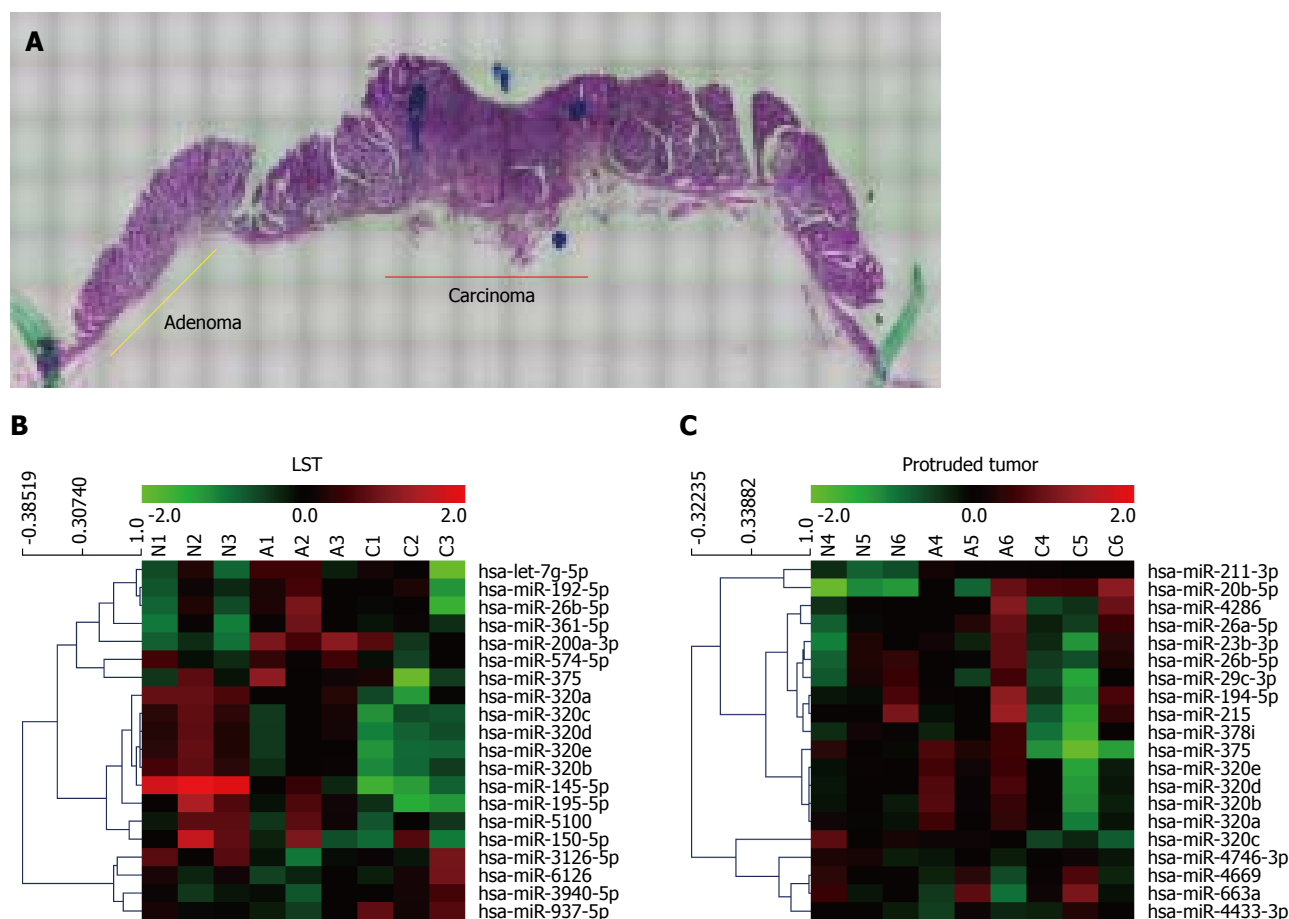


Figure 1 MiRNA expression profiles of matched samples. A: Regions of colorectal carcinoma, pre-existing adenoma, and adjacent non-neoplastic mucosa in a resection specimen were microdissected using laser microdissection (LMD); B and C: The miRNA expression profiles were compared with three matched regions from LSTs ($n = 3$) and protruded tumors ($n = 3$). For each miRNA, red represents higher expression and green represents lower expression than the average expression. LST: Laterally spreading type; C: Carcinoma; A: Adenoma; N: Adjacent non-neoplastic mucosa.

cosa, adenoma, and submucosal invasive carcinoma microdissected by LMD from a tissue sample (Figure 1A), were assessed. To differentiate tumor forms, these analyses were conducted in each of the LSTs ($n = 3$) and protruded tumors ($n = 3$) (Figure 1B and C). All three LSTs were of the granular type. Seven miRNAs in LSTs and 23 miRNAs in protruded tumors showed significantly higher or lower expression in early carcinomas than that in adenomas, with expression in non-neoplastic mucosa as the baseline. The top 10 miRNAs in each form are summarized in Table 1. Comparing these results, six of the 10 miRNAs, including five belonging to the miR-320 family (320a, b, c, d, and e) were identical. In addition, we confirmed the downregulation of miR-195 (LST), miR-375, miR-378 (protruded tumor), and miR-26b (both forms) in early cancer, which has been previously reported in several studies of CRCs^[9,15,16]. Decreased expression of miR-320a and 320b in CRC has also been reported previously^[6,17,18]. It is interesting that these changes in miR-320 family expressions are common in early carcinomas both in LSTs and protruded tumors; therefore, we focused on

the miR-320 family in the subsequent analysis.

miR-320 family is significantly downregulated in colorectal adenoma and submucosal invasive carcinoma

To confirm the downregulation of the miR-320 family in early carcinoma, we conducted qRT-PCR of the miR-320 family using 18 matched colorectal submucosal invasive carcinoma specimens in LSTs (Figure 2A). Expressions of all members of the miR-320 family except miR-320e in adenoma were significantly decreased not only in submucosal invasive carcinomas but also in adenomas compared with those in the non-neoplastic mucosa ($P < 0.05$ and $P < 0.01$, respectively). On comparing adenomas and carcinomas, expression levels of the miR-320 family, except for 320d, in carcinomas were lower than that in adenomas; however, differences were not statistically significant. These findings were confirmed in human colon cancers cell lines by qRT-PCR, and expression of the miR-320 family was found to be decreased in HT29 and SW480 compared with that in InEpc (Figure 2B).

These results indicated that the expression of the miR-320 family decreased from the early stages of the

Table 1 The top 10 miRNA changes between colorectal adenoma and carcinoma

	Systematic name	Expression levels <i>vs</i> normal (mean \pm SD)		<i>P</i> value
		Adenoma	Carcinoma	
Laterally spreading types (<i>n</i> = 3)				
1	hsa-miR-320b	0.649 \pm 0.170	0.402 \pm 0.146	0.00488
2	hsa-miR-320e	0.666 \pm 0.159	0.376 \pm 0.085	0.0213
3	hsa-miR-937-5p	0.842 \pm 0.169	1.446 \pm 0.253	0.0215
4	hsa-miR-574-5p	1.287 \pm 0.477	0.881 \pm 0.339	0.0373
5	hsa-miR-320d	0.705 \pm 0.204	0.425 \pm 0.108	0.0407
6	hsa-miR-320a	0.645 \pm 0.140	0.412 \pm 0.198	0.0417
7	hsa-miR-26b-5p	1.751 \pm 0.307	1.003 \pm 0.600	0.0477
8	hsa-miR-320c	0.676 \pm 0.181	0.401 \pm 0.072	0.0516
9	hsa-miR-361-5p	1.976 \pm 0.124	1.469 \pm 0.378	0.0762
10	hsa-miR-195-5p	0.729 \pm 0.172	0.391 \pm 0.338	0.0772
Protruded tumors (<i>n</i> = 3)				
1	hsa-miR-320d	1.450 \pm 0.388	0.805 \pm 0.369	0.000284
2	hsa-miR-320b	1.456 \pm 0.386	0.834 \pm 0.362	0.00203
3	hsa-miR-375	1.312 \pm 0.163	0.285 \pm 0.174	0.00492
4	hsa-miR-320e	1.350 \pm 0.278	0.772 \pm 0.362	0.00724
5	hsa-miR-320c	0.879 \pm 0.236	0.584 \pm 0.219	0.0105
6	hsa-miR-320a	1.262 \pm 0.208	0.810 \pm 0.263	0.0110
7	hsa-miR-663a	0.985 \pm 0.786	1.317 \pm 0.798	0.0114
8	hsa-miR-211-3p	1.634 \pm 0.193	1.533 \pm 0.189	0.0129
9	hsa-miR-378i	1.110 \pm 0.223	0.640 \pm 0.333	0.0250
10	hsa-miR-26b-5p	1.261 \pm 0.462	0.896 \pm 0.360	0.0253

adenoma-carcinoma sequence.

miR-320 family, except miR-320e, inhibits cell proliferation

To reveal the role of the miR-320 family in CRC, cell proliferations in SW480 transfected with the miR-320 family were analyzed by MTS assay for 5 d. Overexpression of miR-320a, 320b, 320c, or 320d significantly inhibited the cell growth of SW480 (Figure 3), and inhibition of these miRNA significantly promoted proliferation of SW480 (data not shown). However, these findings were not observed with miR-320e.

miR-320a targets CDK6

To find a target gene of the miR-320 family, we used miR-320a as a representative of the miR-320 family in microarray gene expression analysis and miRNA target prediction. Before the gene expression analysis, we confirmed that the expression of miR-320a in SW480 cells transfected with miR-320a mimics was increased by approximately 1600-fold compared with that in controls. Next, the gene expression analysis of SW480 with a mimic control or miR-320a mimics was conducted using microarray, and the expressions of 497 genes were found to be decreased in SW480 with miR-320a mimics (fold-change < 0.67). In addition to these tests, we conducted TargetScan and Pic tar analyses to detect potential targets of miR-320a; seven genes (BLCAP, HOXA10, KCNS3, RASA1, NPAS2, ARPC5, and CDK6) were identified to be common in the results of these three different analyses (Figure 4A). In seven candidate genes, CDK6 expression was shown to be associated with prognosis in patients with CRC^[19]; moreover, the results of miRNA binding-site prediction analyses using bioinformatics tools (Target scan

and microRNA.org) indicate that CDK6 has a putative miR-320 family's binding site that is mapped to the 3' UTR (Figure 4B). From these results, we selected CDK6 as a novel candidate target of the miR-320 family.

mRNA and protein levels of CDK6 suppressed by miR-320 family in colon cancer cell lines

To examine whether the miR-320 family influences both mRNA and protein levels of CDK6, these were analyzed in colon cancer cell lines (HT29 and SW480) with a mimic control or miR-320 family mimics. Both mRNA and protein levels of CDK6 were significantly suppressed in cells with miR-320 family mimics (Figure 4C and D).

Increased CDK6 expression from non-neoplastic mucosa through adenoma to submucosal invasive carcinoma with inverse correlation to the miR-320 family expression

To confirm the changes of CDK6 expression in colorectal tissues, we investigated CDK6 expression in FFPE tissues of 18 matched samples. Two samples were excluded because of difficulty in detecting CDK6 expression. CDK6 expression in submucosal invasive carcinoma compared with non-neoplastic mucosa was significantly increased (*P* < 0.05, Figure 4E) and that in adenoma was also increased but without statistical significance (*P* = 0.10). Finally, we found that there is an inverse correlation between the expression levels of the miR-320 family, except miR-320c, and CDK6 in the 16 matched samples studied (*P* < 0.05, Figure 4F).

DISCUSSION

The main aim of this study was to investigate changes

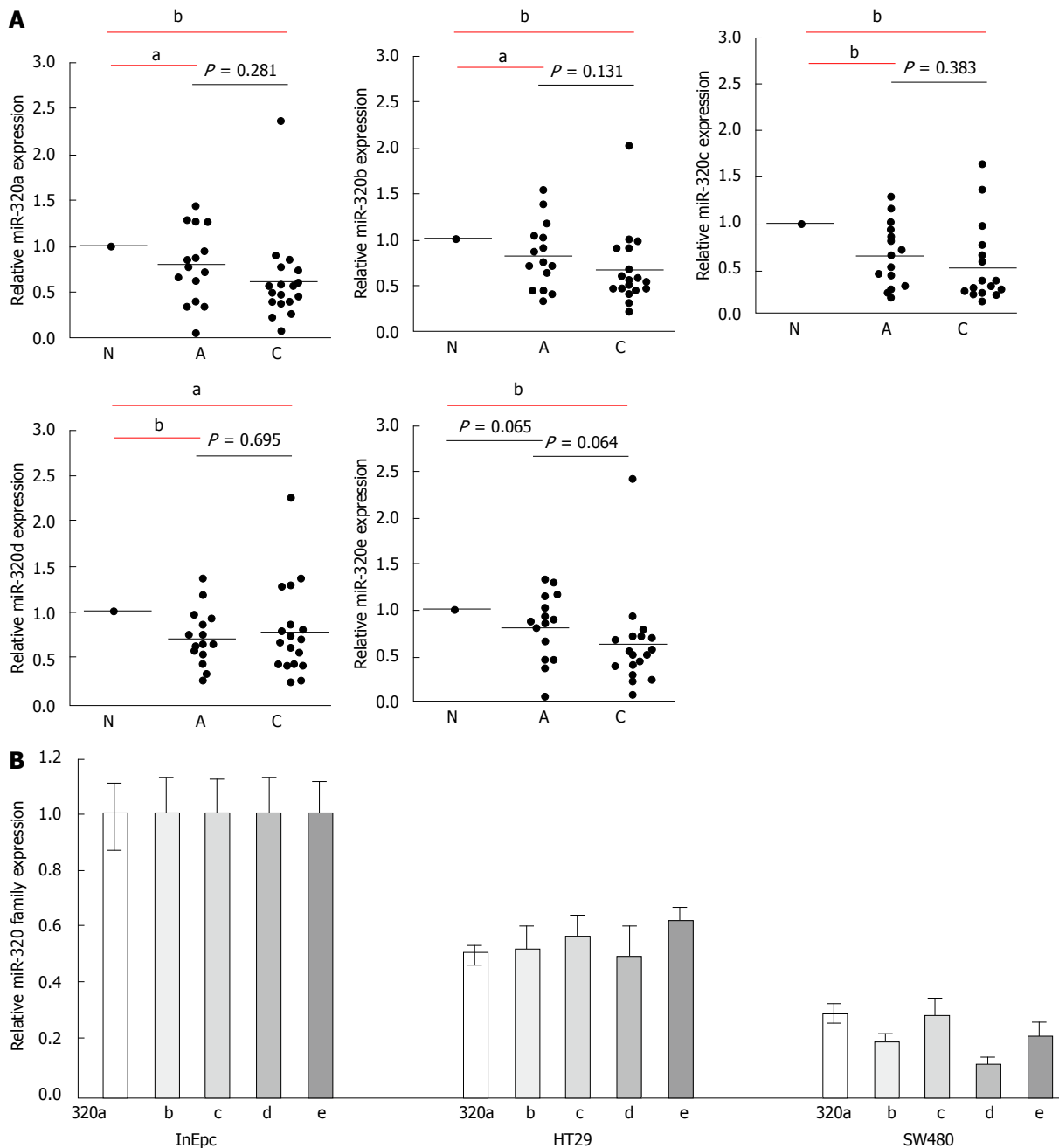


Figure 2 The relative expression levels of the miR-320 family in formalin-fixed, paraffin-embedded samples and cell lines. A: The expression of the miR-320 family in 18 samples (15 colorectal carcinoma with adenoma and 3 colorectal carcinoma without adenoma); B: The expression of the miR-320 family in human colon cancers cell lines (HT29 and SW480) compared with that in human intestinal epithelial cells (InEpC). (^a $P < 0.05$, ^b $P < 0.01$). C: Carcinoma, A: Adenoma, N: Adjacent non-neoplastic mucosa.

in miRNAs corresponding to the adenoma-carcinoma sequence and to clarify the functions of these miRNAs in carcinogenesis. We showed that the miR-320 family (a, b, c, d and e) were important factors in the progression of the early stages of colorectal tumors and found a novel target of the miR-320 family, CDK6. From the results of our comprehensive microarray miRNA analysis, miR-320a, b, c, d and e were downregulated from adenoma to submucosal invasive carcinoma in both LSTs and protruded tumors. It has been previously reported that miR-320a regulates tumor occurrence^[20], progression^[21], and metastasis^[22] in CRC; therefore, we inferred that the miR-320 family may play an important role in colo-

rectal carcinogenesis^[23]. Moreover, 6 of the 10 miRNAs, including the miR-320 family, were identical between the LST-G type and protruded tumors; therefore, there is a possibility that the carcinogenic mechanisms of these two different forms share similar pathways. In contrast, the LST-NG type has been reported to show a significantly higher frequency of submucosal invasion than the LST-G type^[24]; thus, their carcinogenic mechanisms might be different. Further verification of the carcinogenic mechanisms of the LST-NG type is warranted.

To investigate the expression of the miR-320 family in the colorectal tissue, we conducted expression analysis of the miR-320 family in 18 FFPE samples by RT-PCR.

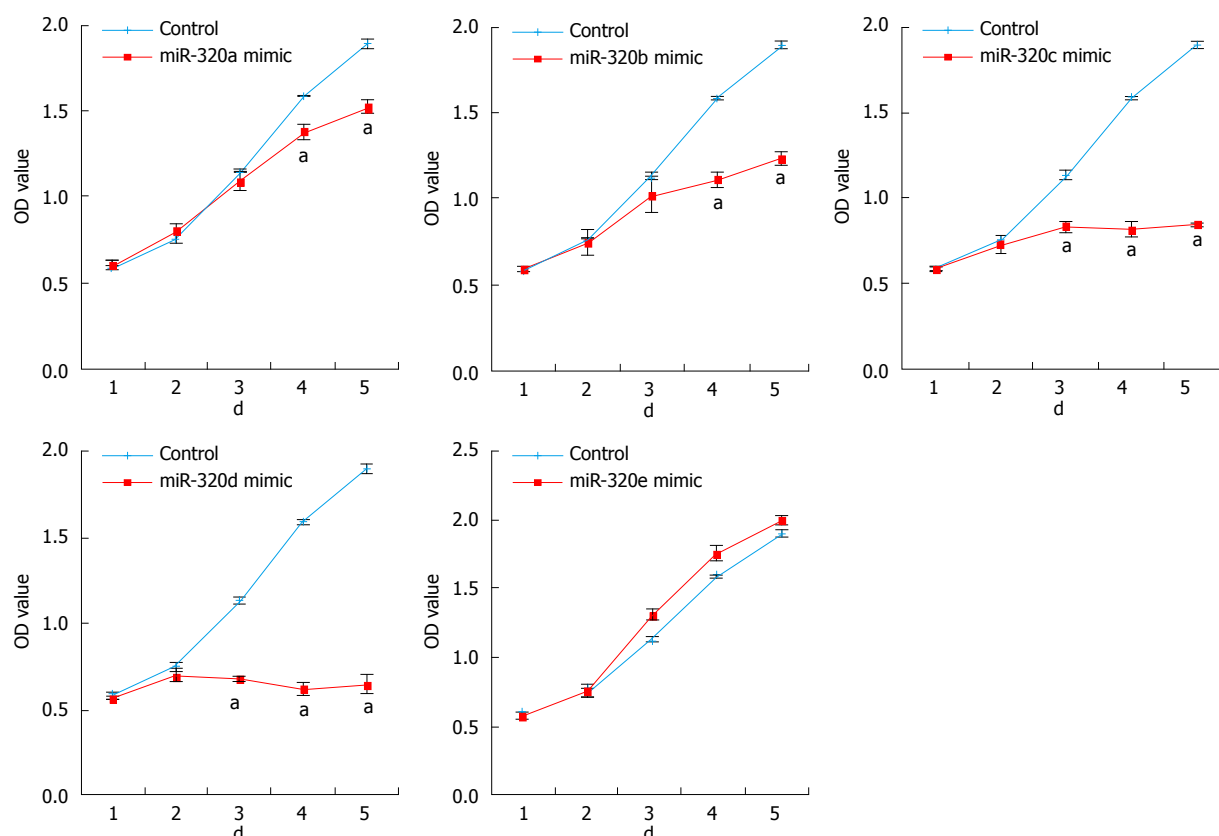


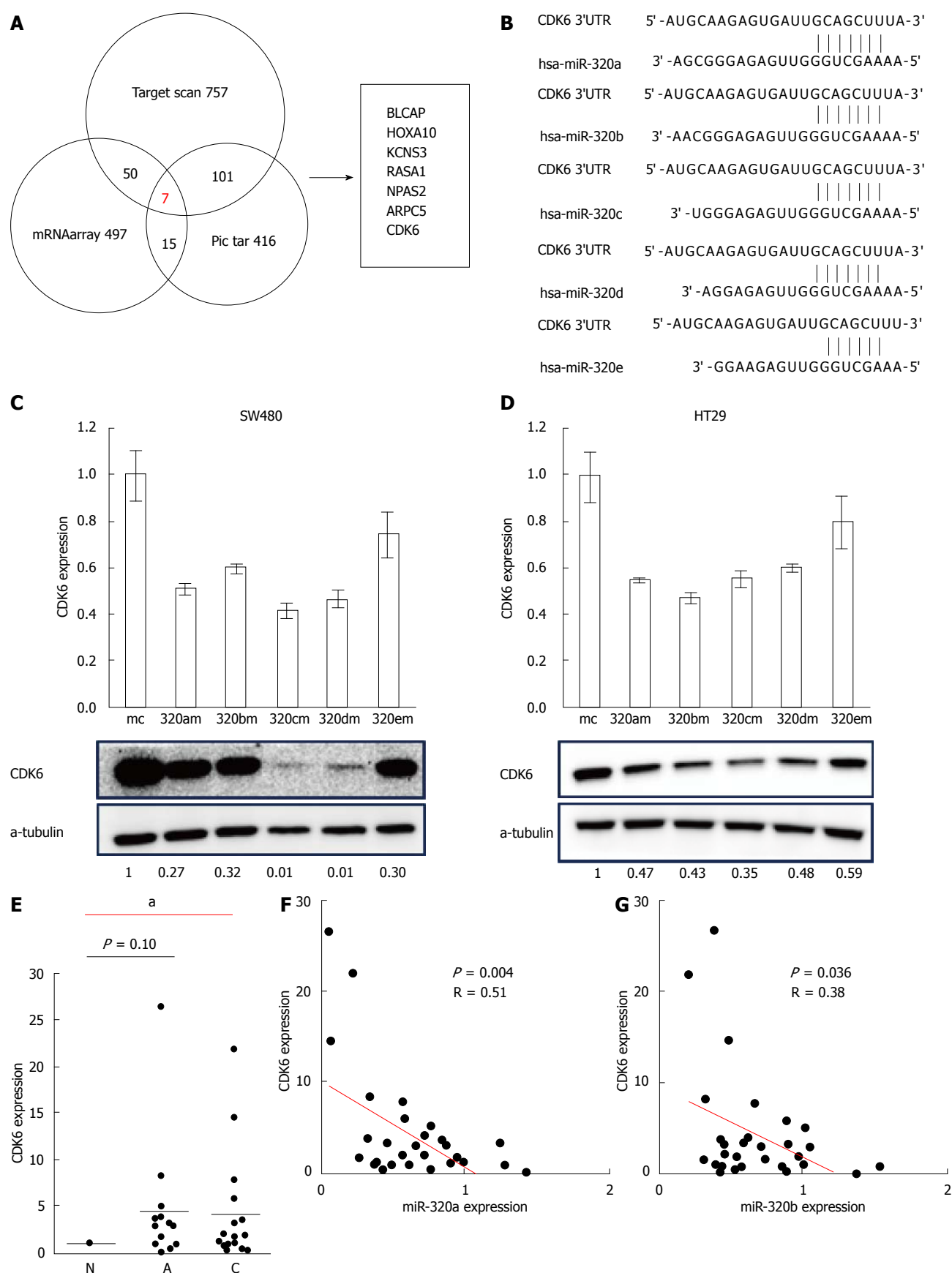
Figure 3 The miR-320 family inhibits colon cancer cell proliferation. Cell proliferation in SW480 transfected with the miR-320 family was assessed by the MTS assay, each day, for up to 5 d ($^aP < 0.05$).

In addition to the results from our miRNA microarray analysis, which showed differences of the miR-320 expression between colorectal adenoma and carcinoma, qRT-PCR analyses showed progressively decreasing expression of the miR-320 family, except miR-320d, from non-neoplastic mucosa through adenoma to submucosal invasive carcinoma. There are some reports of a relationship between miRNA and colorectal adenoma^[6,25], and miR-320a was downregulated from colorectal mucosa to low-grade dysplasia to high-grade dysplasia to adenocarcinomas in the same sample^[6]. Our results support these previous findings, and this article presents the first report of the downregulation of miR-320b in CRA, miR-320c and d in CRA and CRC, and miR-320e in CRC. miR-320a has been shown to effectively regulate proliferation, cell cycle, invasion, migration, and epithelial-mesenchymal transition of CRC, particularly in advanced stages, and is a novel tumor and metastasis suppressor acting by directly targeting mRNAs of neuropilin 1, β -catenin, and other genes^[17]. We confirmed that miR-320a inhibited cell proliferation in cancer cell lines but not how it affects tumor progression in earlier stages of the tumor. Further, the carcinogenic mechanisms of miR-320b, c, d, and e are poorly understood, particularly in CRC. Therefore, we first tried to identify novel targets of the miR-320 family to identify the carcinogenic mechanism in the early stages of the adenoma-carcinoma sequence.

The most commonly used approach to find the target genes of miRNA is through bioinformatics algorithm. Recent reports have provided evidence that miRNAs may downregulate a greater number of transcripts than previously appreciated^[26]; we adopted the results of the mRNA expression array analysis to narrow down the candidates. Finally, we selected seven target genes as common to two bioinformatics algorithms and our results of the mRNA array.

Carcinogenesis is believed to be caused by the dysregulation of the cell-cycle machinery. CDK6, a cyclin-D1-dependent kinase, plays an important role in G₁/S phase transition of the cell cycle and sends signals modulating the control of cell development^[27]. The function of CDK6 in CRC has been shown previously^[19], and there are several reports on the relationships between CDK6 and miRNA in some types of cancers^[28]. Moreover, it was reported that miR-320c inhibited tumor-like behaviors of bladder cancer by targeting CDK6^[29]. However, no studies have examined the relationship between the expression of the miR-320 family and CDK6 expression in CRC.

We confirmed the decreased expression of mRNA and CDK6 in CRC cell lines transfected with the miR-320 family. In addition, we confirmed that CDK6 expression was downregulated in colorectal tumor tissues and that the expressions levels of the miR-320 family, except miR-320c, were negatively correlated with the mRNA expression levels of CDK6. These results suggested that



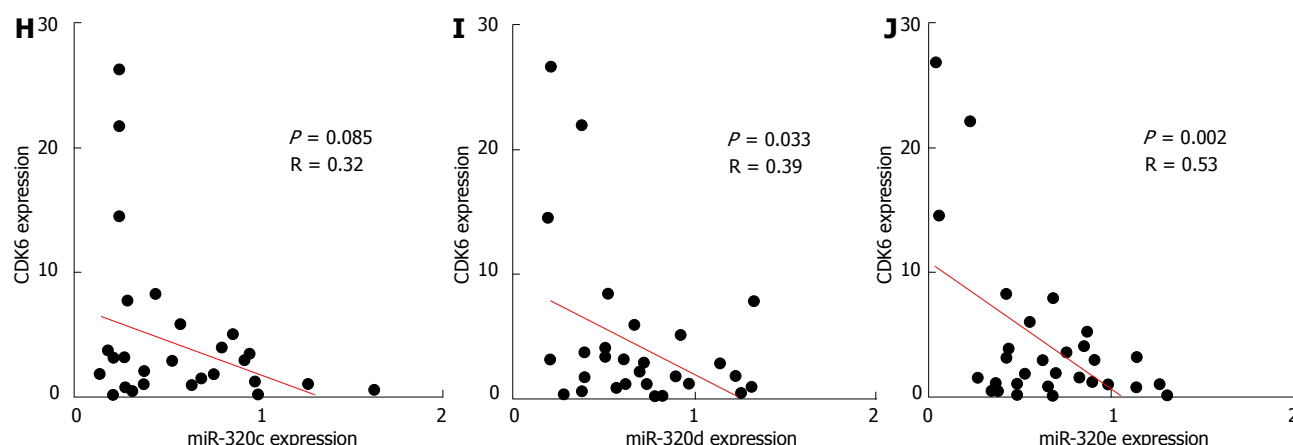


Figure 4 The miR-320 family targets CDK6. A: Seven candidate target genes of miR-320a were identified as being common from the results of expression profiling and two bioinformatics algorithms. The decreased expression of 497 pieces was observed in gene expression profiling of SW480 transfected with an miR-320a mimic and a mimic control (fold-change: < 0.67), and target genes of miR-320a were predicted by TargetScan and Pic tar; B: CDK6 has a putative miR-320 family-binding site mapped to the 3' UTR; C and D: mRNA and protein levels of CDK6 in colon cancer cell lines (HT29 and SW480) transfected with miR-320 family mimics and a mimic control; E: The relative CDK6 expression level compared with that in non-neoplastic mucosa in 16 matched samples; F-J: Pearson's correlation analysis between the expression levels of the miR-320 family and CDK6 in 16 matched samples ($^*P < 0.05$). UTR: Untranslated regions; 320am: miR-320a mimics; 320bm: miR-320b mimics; 320cm: miR-320c mimics; 320dm: miR-320d mimics; 320em: miR-320e mimics; mc: Mimic control; C: Carcinoma; A: Adenoma; N: Adjacent non-neoplastic mucosa.

the miR-320 family is targeted to CDK6 in CRC and has an influence on cell cycle. We report that the miR-320 family suppresses colorectal tumor progression by targeting CDK6.

In our study, the effects on tumor proliferation by overexpression of miR-320e were not observed, whereas overexpression of miR-320c and miR-320d resulted in a particularly strong reduction of tumor proliferation. CDK6 expression after introduction of miR-320e mimics in colon cancer cell lines indicated a similar tendency to be decreased at the mRNA and protein levels, but these were not as significant as the marked changes observed in other members of the miR-320 family. Targets of miRNAs can be predicted by requiring conserved Watson-Crick pairing to the 5' region of the miRNA, known as the miRNA seed^[30]. The seeds of miR-320e for CDK6 were maximum 6 mer. As the seeds of other members of the miR-320 family were maximum 8 mer, this difference in the structure of the miR-320 family might lead to differences in the effects on CDK6 expression. We believe that this results give support the relationship between miR-320 family and CDK6. Further validations of these are required.

In conclusion, we have shown that the miR-320 family, which suppresses tumor proliferation by targeting CDK6, is downregulated in colorectal adenoma and early colorectal carcinoma tissue. The miR-320 family may play an important role in the growth of colorectal tumors and can be considered a biomarker for the early detection of colorectal tumors.

COMMENTS

Background

According to the adenoma-carcinoma sequence, colorectal adenoma (CRA) is considered to be a precursor lesion of colorectal cancer (CRC); the removal of

CRAs by polypectomy has been shown to reduce the incidence and mortality due to CRCs. Thus, detection of colorectal tumors in the early stages has become increasingly important in treatment and prognosis. The adenoma-carcinoma sequence is accompanied by several genetic and epigenetic alterations, such as mutations of cancer-associated genes and epigenetic modifications, including changes in microRNAs (miRNAs). However, the involvement of miRNAs in the mechanism of this sequence remains undetermined.

Research frontiers

miRNAs are frequently dysregulated in human cancers, and alterations of miRNA expression in colorectal tumors have been well documented. For example, miRNA profiles of CRC compared with those of normal mucosa and adenoma. However, limited reports exist on the sequential changes in miRNA expression during histological progression from normal colonic mucosa through colorectal adenoma to early carcinoma in a lesion from a patient. miR-320a has been shown to effectively regulate proliferation, cell cycle, invasion, migration, and epithelial-mesenchymal transition of CRC. However, the carcinogenic mechanisms of miR-320b, c, d, and e are poorly understood, particularly in CRC, and needed further exploration.

Innovations and breakthroughs

The authors investigated for the first time the sequential changes of miRNA expression profiles in colonic lesions from matched samples; histologically, non-neoplastic mucosa, adenoma, and submucosal invasive carcinoma were microdissected from a tissue sample. They have shown for the first time that the miR-320b, miR-320c, miR-320d are downregulated from colorectal adenoma and miR-320e is downregulated from colorectal submucosal carcinoma tissue and the miR-320 family suppresses tumor proliferation by targeting CDK6.

Applications

This study suggests that the miR-320 family affects colorectal tumor proliferation by targeting CDK6, plays an important role in the growth of colorectal tumors. From these results, miR-320 family is considered as a biomarker for early detection of colorectal tumor.

Terminology

The adenoma-carcinoma sequence is accompanied by several genetic and epigenetic alterations, such as mutations of cancer-associated genes and epigenetic modifications, including changes in DNA methylation, histone modifications, and miRNAs. miRNAs are small (19-23 nucleotide) endogenous non-coding RNAs that regulate gene expression by targeting the 3' untranslated

region of mRNA. miRNAs play fundamental roles in various biological processes.

Peer-review

The manuscript by Tadano *et al* investigated the expression of miRNA in colorectal adenoma and submucosal invasive carcinoma and revealed miR-320 family affects colorectal tumor proliferation by targeting CDK6. This article is overall interesting and gives new insight in the field of dysregulation of miRNAs and colorectal cancer.

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Retrospective Cohort Study

Opioid-sparing effect of selective cyclooxygenase-2 inhibitors on surgical outcomes after open colorectal surgery within an enhanced recovery after surgery protocol

Varut Lohsiriwat

Varut Lohsiriwat, Division of Colon and Rectal Surgery, Department of Surgery, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand

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Informed consent statement: As the study is a retrospective review, a waiver of informed consent was approved by the Siriraj Institutional Review Board.

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Data sharing statement: The original anonymous dataset is available on request from the corresponding author at bolloon@hotmail.com.

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Correspondence to: Varut Lohsiriwat, MD, PhD, Associate Professor of Surgery, Division of Colon and Rectal Surgery, Department of Surgery, Faculty of Medicine Siriraj Hospital, Mahidol University, 2 Wang-Lung Road, Bangkok Noi, Bangkok 10700, Thailand. bolloon@hotmail.com

Telephone: +66-2-4198005

Fax: +66-2-4121370

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Abstract

AIM: To evaluate the opioid-sparing effect of selective cyclooxygenase-2 (COX-2) inhibitors on short-term surgical outcomes after open colorectal surgery.

METHODS: Patients undergoing open colorectal resection within an enhanced recovery after surgery protocol from 2011 to 2015 were reviewed. Patients with combined general anesthesia and epidural anesthesia, and those with acute colonic obstruction or perforation were excluded. Patients receiving selective COX-2 inhibitor were compared with well-matched individuals without such a drug. Outcome measures included numeric pain score and morphine milligram equivalent (MME) consumption on postoperative day (POD) 1-3, gastrointestinal recovery (time to tolerate solid diet and time to defecate), complications and length of postoperative stay.

RESULTS: There were 75 patients in each group. Pain score on POD 1-3 was not significantly different between two groups. However, MME consumption and MME consumption per kilogram body weight on POD 1-3 was significantly less in patients receiving a selective COX-2 inhibitor ($P < 0.001$). Median MME consumption per kilogram body weight on POD 1-3 was 0.09, 0.06 and nil, respectively in patients receiving a selective COX-2 inhibitor and 0.22, 0.25 and 0.07, respectively in the comparative group ($P < 0.001$), representing at least 59% opioid

reduction. Patients prescribing a selective COX-2 inhibitor had a shorter median time to resumption of solid diet [1 (IQR 1-2) d vs 2 (IQR 2-3) d; $P < 0.001$] and time to first defecation [2 (IQR 2-3) d vs 3 (IQR 3-4) d; $P < 0.001$]. There was no significant difference in overall postoperative complications between two groups. However, median postoperative stay was significantly 1-d shorter in patients prescribing a selective COX-2 inhibitor [4 (IQR 3-5) d vs 5 (IQR 4-6) d; $P < 0.001$].

CONCLUSION: Perioperative administration of oral selective COX-2 inhibitors significantly decreased intravenous opioid consumption, shortened time to gastrointestinal recovery and reduced hospital stay after open colorectal surgery.

Key words: Selective cyclooxygenase-2 inhibitor; Outcome; Colon surgery; Rectal surgery; Enhanced recovery after surgery; Opioid; Ileus; Non-steroidal anti-inflammatory drug; Pain

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Core tip: This comparative study validates the effectiveness of perioperative administration of oral selective cyclooxygenase-2 (COX-2) inhibitors as a part of multimodal analgesia in an enhanced recovery after surgery protocol to significantly reduce opioid requirement (but not pain score) after open colorectal surgery. Our findings also indicate that opioid-sparing effect of selective COX-2 inhibitor has some important clinical benefits including quicker gastrointestinal recovery and shorter hospitalization.

Lohsiriwat V. Opioid-sparing effect of selective cyclooxygenase-2 inhibitors on surgical outcomes after open colorectal surgery within an enhanced recovery after surgery protocol. *World J Gastrointest Oncol* 2016; 8(7): 543-549 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v8/i7/543.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v8.i7.543>

INTRODUCTION

Effective pain control for open colorectal surgery plays a crucial role in improving patient's recovery. Various analgesic modalities have been utilized to reduce postoperative pain including epidural analgesia and administration of selective cyclooxygenase-2 (COX-2) inhibitors. However, a recent nationwide analysis of the outcomes of epidural analgesia in open colorectal surgery in the United States has shown that epidural analgesia does not add major clinical benefits over conventional analgesia, but it is associated with longer hospital stay and a higher incidence of ileus^[1]. In a large international registry of the enhanced recovery after surgery (ERAS) Compliance Group, prolonged hospitalization was also observed in patients with epidural analgesia^[2]. Moreover,

epidural analgesia needs to be performed by a qualified anesthesiologist and it could lead to some serious complications such as epidural hematoma and epidural abscess^[3]. As a result, the application of epidural analgesia in clinical practice has been limited^[1,4,5].

On the other hand, a selective COX-2 inhibitor, a nonsteroidal anti-inflammatory drug (NSAID) directly targeting COX-2 which is an enzyme primarily responsible for inflammation and pain, are widely available in both oral preparation and injectable form^[6]. Having little or no effect on platelet aggregation, a selective COX-2 inhibitor has currently been used as a part of multimodal analgesia for several surgical procedures including colorectal surgery - which prefers a non-opioid analgesic regimen^[7-9]. Perioperative administration of selective COX-2 inhibitors can reduce opioid requirement^[10], facilitate gastrointestinal recovery and shorten hospital stay after colorectal surgery^[11]. However, there are a limited number of studies examining these outcome benefits in the setting of an ERAS protocol^[10].

In Thailand, an ERAS protocol for colorectal surgery has been introduced into a daily practice since 2011^[9,12]. Regarding perioperative analgesia in our ERAS protocol, selective COX-2 inhibitors will be provided based on patient's comorbidities and their healthcare coverage scheme. Meanwhile, thoracic epidural analgesia is seldom applied due to its technical demand and a limited number of physician anesthesiologists^[4]. Like many developing and underdeveloped countries, a majority of colorectal procedures in Thailand remains an open surgery because of limited resources and the expense of laparoscopic surgery^[13]. The objective of this study was therefore to examine the clinical outcomes of perioperative administration of an oral selective COX-2 inhibitor for open colorectal surgery within an ERAS protocol (without the need of epidural analgesia).

MATERIALS AND METHODS

This non-randomized, comparative, prospective study included adult patients undergoing elective laparotomy for colorectal resection from January 2011 to September 2015 at the Faculty of Medicine Siriraj Hospital. The study was approved by the Siriraj Institutional Review Board (SIRB COA No. Si014/2013). Patients with combined general anesthesia and epidural anesthesia, and those with acute colonic obstruction or perforation were excluded. Clinical outcomes of patients receiving a selective COX-2 inhibitor were compared with those without such a drug, with a ratio of 1 to 1. They were matched for age, gender, body mass index (BMI), the ColoRectal Physiological and Operative Severity Score for the enUmeration of Mortality and Morbidity (CR-POSSUM)^[14], and type of surgical procedure. Of note, all operations were performed by the author under an ERAS protocol. The ERAS protocol has been previously described^[9,12,15]. In brief, only patients with left-sided colon or rectal resection received preoperative mechanical bowel preparation. Right-sided colon resection was preferentially

Table 1 Perioperative pain control regimen

Preoperative period	1 tablet of acetaminophen 500 mg ± 1 tablet of a selective cyclooxygenase-2 inhibitor (either celecoxib 400 mg, etoricoxib 90 mg or etoricoxib 120 mg)
Intraoperative period	Balanced general anesthesia Application of atraumatic O-ring wound retractor (if available)
Postoperative period	Infiltration of 0.5% bupivacaine into fascial layer and muscle around the wound edge 1 tablet of acetaminophen 500 mg every 6 h in the first 3 d ± 1 tablet of a selective cyclooxygenase-2 inhibitor daily for 5-7 d Intravenous patient-controlled morphine (or tramadol) or intermittent intravenous morphine if pain score > 3

done through a transverse incision. Otherwise, a midline laparotomy was performed. No intraabdominal drain or nasogastric tube was used. A diverting stoma was selectively fashioned in cases of coloanal anastomosis and neoadjuvant chemoradiation. Medication for prophylaxis of postoperative nausea and vomiting was administered based on patient's risk factor^[16]. Standard postoperative care was provided including early feeding and scheduled ambulation.

Perioperative analgesia

Approximately 3 h prior to surgery, one tablet of acetaminophen 500 mg with or without one tablet of an oral selective COX-2 inhibitor (either celecoxib 400 mg, etoricoxib 90 mg or etoricoxib 120 mg) were given. Of note, the administration of a selective COX-2 inhibitor was based on patient's co-morbidities, contraindication (i.e., coronary artery disease, ischemic stroke, peripheral arterial disease, uncontrolled hypertension) and their healthcare coverage scheme. An operation was performed under a balanced general anesthesia. An atraumatic O-ring wound retractor was applied during the operation if available^[17]. After a closure of abdominal wall muscle, 0.5% bupivacaine (3-4 mg/kg) was infiltrated into fascial layer and muscle around the wound edge. The wound was then closed primarily. Standard protocol for postoperative pain control was followed in all cases. Basically, intravenous morphine (0.03-0.05 mg/kg per dose every 1-2 h) was administered if pain score was > 3 (using a numeric rating scale of 0-10 with 0 = no pain 10 = worst possible pain). Intravenous patient-controlled morphine or intravenous tramadol (an equivalent of 10 mg tramadol to 1 mg morphine) may be used in some cases^[18]. During the postoperative period, one tablet of acetaminophen 500 mg was given every 6 h in the first 3 d, with or without daily oral selective COX-2 inhibitor for 5-7 d. Perioperative analgesia protocol was summarized in Table 1.

Outcome measures

Primary outcome measures included average pain score on postoperative day (POD) 1-3, intravenous opioid requirement on POD 1-3, gastrointestinal recovery (time to tolerate solid diet and time to defecate), complication according to the Clavien-Dindo classification system^[19], prolonged postoperative ileus^[20], and length of postoperative stay. Pain scores were recorded every 4 h by nursing staff. All pain assessments were noted after

patients were asked to take a deep breath. Should the patients slept at night during the scheduled time for pain assessment, they were not awakened and the actual time the patients were assessed was noted. Unless intravenous patient-controlled opioid was applied, intermittent intravenous morphine was given if pain score was > 3 as in the aforementioned regimen. Total daily intravenous opioid requirement was reported as morphine milligram equivalent (MME).

All patients were offered a clear liquid diet immediately after surgery providing that they were clinically stable. Once the oral intake exceeded 20 mL/kg body weight without nausea and vomiting, the diet was advanced to a low-residual solid diet. Prolonged postoperative ileus was defined as at least two times of nausea/vomiting, inability to tolerate oral diet and absence of flatus over 24 h, and abdominal distension with radiologic confirmation occurring on or after POD 4^[20]. Patients were discharged from the hospital when they had no fever, adequate pain control with oral analgesics, good ambulation, and satisfactory recovery of gastrointestinal function. All patients were scheduled for follow-up at 30 d postoperatively.

Statistical analysis

All data were prepared and compiled using Statistical Package for the Social Sciences (SPSS®) program version 18.0 for Windows (SPSS Inc., Chicago, IL). Values are expressed as median (interquartile range: IQR), mean (SD) or number (%). Continuous variables were compared using the *t*-test or Mann-Whitney *U* test. Categorical variables were compared using the χ^2 test. A *P*-value of less 0.05 was considered statistically significant.

RESULTS

This study included 150 patients (57% male) with the average age of 65 years (range 30-87). There were 75 patients in each group. There was no significant difference in patient's characteristics, intraoperative detail, type of operation and percentage of adherence to the ERAS protocol between the two groups, except patients receiving a selective COX-2 inhibitor had a higher level of preoperative hematocrit and serum albumin (Table 2).

Pain score on POD 1-3 was not significantly different between the two groups. However, MME requirement on POD 1-3 was significantly less in patients receiving

Table 2 Patient characteristics and operative details

	Patients with selective COX-2 inhibitor (<i>n</i> = 75)	Patients without selective COX-2 inhibitor (<i>n</i> = 75)	<i>P</i> -value
Age (yr)	64 (55-73)	65 (59-75)	0.15
Male	43 (57)	42 (56)	0.87
Weight (kg)	68 (51-58)	59 (50-66)	0.93
Body mass index	23.1 (20.9-25.4)	22.5 (20.6-24.6)	0.49
CR-POSSUM predicted mortality	1.8 (1.0-2.5)	1.9 (1.3-3.4)	0.07
Hematocrit (%)	38 (34-41)	35 (31-39)	0.014 ¹
Serum albumin (g/L)	4.0 (3.6-4.3)	3.8 (3.4-4.1)	0.013 ¹
Operative time (min)	180 (120-220)	160 (120-180)	0.21
Blood loss (mL)	150 (50-250)	150 (50-260)	0.75
Operation for malignancy	67 (89)	68 (91)	0.37
Rectal resection	41 (55)	37 (49)	0.51
Operation without bowel restoration	11 (15)	12 (16)	0.82
Use of atraumatic O-ring retractor	66 (88)	59 (79)	0.13
Adherence to ERAS protocol (%)	88 (82-88)	82 (82-88)	0.28

¹*P* < 0.05. COX-2: Cyclooxygenase-2; CR-POSSUM: The ColoRectal Physiological and Operative Severity Score for the enUmeration of Mortality and Morbidity; ERAS: Enhanced recovery after surgery.

Table 3 Postoperative pain score and intravenous opioid requirement

	Patients with selective COX-2 inhibitor (<i>n</i> = 75)	Patients without selective COX-2 inhibitor (<i>n</i> = 75)	<i>P</i> -value
Pain POD1	1.5 (0.7-2.1)	1.5 (0.5-2.7)	0.78
Pain POD2	0.7 (0-2.0)	0.6 (0-1.5)	0.74
Pain POD3	0.5 (0-1.5)	0.5 (0-1.7)	0.38
MME POD1	6 (2-12)	13 (6-20)	< 0.001 ¹
MME POD2	3 (0-17)	20 (4-20)	< 0.001 ¹
MME POD3	0 (0-0)	5 (0-15)	< 0.001 ¹
MME/KG POD1	0.09 (0.03-0.23)	0.22 (0.11-0.42)	< 0.001 ¹
MME/KG POD2	0.06 (0-0.28)	0.25 (0.07-0.37)	< 0.001 ¹
MME/KG POD3	0 (0-0)	0.07 (0-0.25)	< 0.001 ¹

¹*P* < 0.05. COX-2: Cyclooxygenase-2; MME: Morphine milligram equivalent; MME/KG: Morphine milligram equivalent per kilogram body weight; POD: Postoperative day.

a selective COX-2 inhibitor (Table 3). Median MME consumption per kilogram body weight on POD 1-3 was 0.09, 0.06 and nil, respectively in patients receiving a selective COX-2 inhibitor and 0.22, 0.25 and 0.07, respectively in the comparative group (*P* < 0.001), representing at least 59% opioid reduction.

Patients receiving a selective COX-2 inhibitor had a shorter median time to resumption of solid diet [1 (IQR 1-2) d vs 2 (IQR 2-3) d; *P* < 0.001] and time to first defecation [2 (IQR 2-3) d vs 3 (IQR 3-4) d; *P* < 0.001]. There was no significant difference in the rate of overall postoperative complication and prolonged postoperative ileus between the two groups (Table 4). Of note, there were 1 non-fatal acute myocardial infarction and 1 colorectal anastomotic leakage requiring an operation in patients without selective COX-2 inhibitor. Median and average postoperative stay was significantly 1-d shorter in patients prescribing a selective COX-2 inhibitor; [4 (IQR 3-5) d vs 5 (IQR 4-6) d; *P* < 0.001] and [4.3 (SD 3.0) d vs 5.3 (SD 2.5) d; *P* = 0.023], respectively. Three patients (4%) in the selective COX-2 inhibitor group and 1 patient (1%) in the comparative group required readmission within 30 d after the operation (*P* = 0.62). No 30-d death was observed in this study.

DISCUSSION

The main findings of this comparative study are that perioperative administration of an oral selective COX-2 inhibitor - as a part of multimodal analgesic regimen - reduces intravenous opioid requirement, shortens time to gastrointestinal recovery and decreases the length of hospital stay after open colorectal surgery within an ERAS protocol. These results were consistent with a report from a prospective randomized, double-blind, placebo-controlled study examining the influence of pre- and post-administration of a selective COX-2 inhibitor (valdecoxib 40 mg) in major colorectal surgery within a non or partial ERAS protocol^[11]. The randomized clinical trial indicated that patients treated with valdecoxib had a one-third opioid reduction, a 12-h quicker time to first bowel movement and a 2-d shorter hospital stay. However, valdecoxib has been off the market since 2005 due to its potentially life-threatening skin reaction and lack of adequate data on its long-term cardiovascular safety^[21].

Many studies have shown that preemptive analgesia is more effective than postoperative analgesia^[22-24]. A combination of preoperative and postoperative admini-

Table 4 Gastrointestinal recovery, complication and hospital stay

	Patients with selective COX-2 inhibitor (n = 75)	Patients without selective COX-2 inhibitor (n = 75)	P-value
Time to tolerate solid diet (d)	1 (1-2)	2 (2-3)	< 0.001 ¹
Time to defecate (d)	2 (2-3)	3 (3-4)	< 0.001 ¹
Overall complication	9 (11)	17 (23)	0.08
Grade I	4	7	
Grade II	5	7	
Grade III	0	2	
Grade IV	0	1	
Prolonged postoperative ileus	4 (5)	6 (8)	0.75
Postoperative stay (d)			
Median (IQR)	4 (3-5)	5 (4-6)	< 0.001 ¹
Mean (SD)	4.3 (3.0)	5.3 (2.5)	0.023 ¹
Readmission within 30 d	3 (4)	1 (1)	0.62

¹P < 0.05. If a patient had more than one complication, the highest Clavien-Dindo grade was reported. COX-2: Cyclooxygenase-2.

stration of analgesics would have a better pain control. A beneficial outcome effect of perioperative administration of currently available selective COX-2 inhibitors including celecoxib and etoricoxib may be attributed to adequate perioperative nociceptive afferent blockage and to minimize central sensitization (as a preoperative use), and to maintain anti-inflammatory effect after an operation (as a postoperative use). Unlike conventional NSAIDs, a selective COX-2 inhibitor has little or no effect on platelet aggregation and gastrointestinal irritation^[25,26]. These characteristics of selective COX-2 inhibitors are therefore favorable to perioperative administration.

Although there was no difference in postoperative pain score between the two groups, this study showed that a regimen of perioperative pain control in both groups was very effective - which achieved a reasonable level of comfort in the postoperative period (with a median pain score of < 2). However, patients receiving a selective COX-2 inhibitor required less parenteral opioid. Since opioid is well known to cause postoperative nausea/vomiting and gastrointestinal dysfunction^[27], as a result, in part, shorter gastrointestinal convalescence was observed in patients prescribing a selective COX-2 inhibitor. While a reduction in opioid consumption may be responsible for shorter time to gastrointestinal recovery, a selective COX-2 inhibitor alone was shown to diminish a local inflammatory response of the small bowel to surgical manipulation, thus leading to quicker recovery of postoperative intestinal dysfunction^[28]. In animal studies, selective COX-2 inhibitors induced duodenal motility and improved small bowel propulsion in rats subjected to abdominal surgery^[29,30].

In this study, there was a non-significant trend in decreased rates of overall complication and prolonged postoperative ileus in patients receiving a selective COX-2 inhibitor. The clinical relevance of NSAID-induced opioid sparing on favorable postoperative outcomes, including less incidence of postoperative gastrointestinal dysfunction and other complications, has been shown in several studies of non-colorectal surgery^[31-35] and colorectal surgery^[11]. Apart from its opioid-sparing effects,

selective COX-2 inhibitors may be associated with a reduction in postoperative complication by minimizing both inflammatory response and endocrine-metabolic response to surgery^[36].

While it seems clear that a selective COX-2 inhibitor has a positive impact on opioid consumption and gastrointestinal recovery in this study, patients prescribing a selective COX-2 inhibitor are generally at a higher risk for cardiovascular and thromboembolic events compared with a control or placebo drug^[37]. Therefore, selective COX-2 inhibitors should not be used in individuals at increased risk for vascular thrombosis, *e.g.*, coronary artery disease, cerebrovascular disease and peripheral arterial disease. The physicians are also encouraged to use the lowest effective dose for the shortest duration of a selective COX-2 inhibitor. In surgical point of view, the use of any NSAIDs including selective COX-2 inhibitors in the setting of gastrointestinal anastomosis has been concerned because some studies have suggested that NSAIDs may impair anastomotic healing^[38-40]. Recently, a meta-analysis of clinical and experimental studies in 2014 has indicated a strong link between anastomotic leakage and the use of non-selective NSAIDs, but not the use of selective COX-2 inhibitors^[41]. So far, the ERAS society guidelines include NSAIDs and selective COX-2 inhibitors as a component of multimodal analgesia in elective colorectal surgery^[7,8].

Limitations of this study include the fact that it is a non-randomized study. Selective bias and performance bias could occur in the study. However, all patients were operated on by the same surgeon with a relatively high adherence to the ERAS protocol (> 80% compliance in both groups). Moreover, the patients were systematically assessed with a pre-defined objective measurement. It should be noted that not all patients received intravenous patient-controlled analgesia due to a limited number of equipment. To overcome this problem, a standardized protocol for postoperative pain control has been adopted in our institute since 2004. Another limitation is that only patients undergoing open colorectal surgery were included in this study. Whether patients undergoing minimally

invasive surgery, who will have a less inflammatory and metabolic response to surgery compared with open surgery^[42], will be beneficial to the administration of selective COX-2 inhibitors are not investigated.

In conclusion, this study validates the effectiveness of perioperative administration of currently available oral selective COX-2 inhibitors as a part of multimodal analgesia in an ERAS protocol to significantly reduce opioid requirement (but not pain score) after open colorectal surgery. Our findings also indicate that opioid-sparing effect of selective COX-2 inhibitor has some important clinical benefits including quicker gastrointestinal recovery and shorter hospitalization.

COMMENTS

Background

Effective perioperative pain control plays a crucial role in improving patient's recovery especially for an open abdominal surgery. Opioid is very effective analgesia but it has several undesired side effects such as sedation, itching, nausea, vomiting and constipation. Non-opioid analgesia has been recommended as a part of multimodal analgesia in an enhanced recovery after surgery (ERAS) protocol. Selective cyclooxygenase-2 (COX-2) inhibitors have some advantages over conventional non-steroidal anti-inflammatory drugs because they have little or no effect on platelet aggregation and gastrointestinal irritation.

Research frontiers

Several studies have shown perioperative administration of selective COX-2 inhibitors can reduce opioid requirement, facilitate gastrointestinal recovery and shorten hospital stay after colorectal surgery. However, there are a limited number of studies examining these outcome benefits in the setting of an ERAS protocol.

Innovations and breakthroughs

The present study validates the effectiveness of perioperative administration of currently available oral selective COX-2 inhibitors as a part of multimodal analgesia in an ERAS protocol to significantly reduce opioid requirement (but not pain score) after open colorectal surgery. This study also indicates that opioid-sparing effect of selective COX-2 inhibitor has some important clinical benefits including quicker gastrointestinal recovery and shorter hospitalization.

Applications

The study results suggest that perioperative administration of selective COX-2 inhibitors is an effective perioperative pain control regimen - which could be used as a part of multimodal analgesia for open colorectal surgery if no contraindication.

Peer-review

This is a good article.

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Retrospective Study

Management of colorectal neoplasia during pregnancy and in the postpartum period

Erman Aytac, Gokhan Ozuner, Ozgen Isik, Emre Gorgun, Luca Stocchi

Erman Aytac, Gokhan Ozuner, Ozgen Isik, Emre Gorgun, Luca Stocchi, Department of Colorectal Surgery, Digestive Disease Institute, Cleveland Clinic, Cleveland, OH 44195, United States

Author contributions: Aytac E and Isik O collected and analyzed the data; Aytac E and Ozuner G drafted the manuscript; Stocchi L provided analytical oversight; Ozuner G, Aytac E and Gorgun E designed and supervised the study; Aytac E, Ozuner G, Isik O, Gorgun E and Stocchi L revised the manuscript for important intellectual content; Aytac E and Ozuner G offered the technical or material support; Ozuner G and Gorgun E provided administrative support; all authors have read and approved the final version to be published.

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Correspondence to: Gokhan Ozuner, MD, FACS, FASCRS, Department of Colorectal Surgery, Digestive Disease Institute, Cleveland Clinic, 9500 Euclid Ave. A30, Cleveland, OH 44195, United States. ozuner.g@ccf.org
Telephone: +1-216-4446672
Fax: +1-216-4458627

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Abstract

AIM: To report our experience on management of colorectal neoplasia during pregnancy and in the postpartum period.

METHODS: Patients who were diagnosed with colorectal cancer during pregnancy or in the postpartum period (< 6 mo), between 8/1997 and 4/2013, in our department were reviewed. Patient characteristics, operations, fetal health and follow-up during pregnancy, type of delivery and oncologic outcomes were analyzed.

RESULTS: Eight patients met our study criteria. Median age at the time of diagnosis of colorectal cancer was 31 years. Median follow-up after surgery was 36 mo. Median duration of symptoms before diagnosis was 16 wk. Three patients were diagnosed with colorectal cancer during pregnancy and underwent surgery prior to delivery. None of the patients received adjuvant treatment during pregnancy. Five patients were diagnosed with colorectal cancer within a median of 2.1 mo after delivery and underwent surgery. No adverse neonatal outcomes were noted. All deliveries were at term (2 cesarean sections) except for one preterm delivery following low anterior resection on the 34th week of pregnancy.

CONCLUSION: There has been a significant delay in the diagnosis of colorectal cancer which is probably due to overlap of symptoms and signs between these tumors and a normal pregnancy. Surgery for colorectal cancer during pregnancy can be performed safely without compromising

maternal and fetal outcomes.

Key words: Colorectal cancer; Pregnancy; Postpartum; Neoplasia

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Core tip: This paper summarizes the experience of a tertiary referral colorectal center in the United States on the management of colorectal neoplasia during the pregnancy and postpartum period. Eight patients who were diagnosed with colorectal cancer during pregnancy or in the postpartum period between 8/1997 and 4/2013 were reviewed. No maternal and neonatal mortality occurred related to surgical treatment. While surgery for colorectal cancer during pregnancy can be performed safely and may not affect maternal and fetal outcomes adversely, there has been a significant delay in the diagnosis of colorectal cancer which is probably due to overlap of symptoms and signs between these tumors and a normal pregnancy.

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INTRODUCTION

While the incidence of colorectal cancer is steady or falling, some studies report an increased incidence of colorectal cancer in younger patients (< 40 years)^[1], which may occur during the reproductive age and therefore interfere with pregnancy. Around 0.1% of pregnant women develop a malignancy and there is limited experience on the management of colorectal cancer diagnosed during pregnancy or in the postpartum period^[2]. When colorectal cancer is detected in this period, treatment options may be limited. As two patients with possibly conflicting interests need to be managed, many ethical, psycho emotional and medical issues need to be simultaneously addressed. In this study, we analyzed management, complications, maternal and fetal outcomes in patients who were diagnosed with colorectal cancer during pregnancy or in the immediate postpartum period.

MATERIALS AND METHODS

After obtaining the institutional review board approval (IRB), patients who were diagnosed with colorectal cancer during pregnancy or in the postpartum period and treated at the Department of Colorectal Surgery Cleveland Clinic Ohio, from August 1997 to April 2013, were analyzed in the study. The postpartum period is defined as first 6 mo after delivery^[3]. Patient characteristics, cancer

follow-up, history of previous pregnancies, medications used during pregnancy, indication for surgery, operations performed, outcomes after surgery, complications, maternal and fetal morbidity and mortality during perinatal period and type of delivery were analyzed. Data were retrieved from the IRB approved prospectively maintained databases with supplemental information from patient charts. A multidisciplinary team including gastroenterologists, oncologists, obstetricians and colorectal surgeons followed up all patients.

Quantitative data were reported as median (range) and categorical data as numbers.

RESULTS

Eight patients met our study criteria. Median age at the time of diagnosis of colorectal cancer was 31 (24-38) and median body mass index was 24 (19-27) kg/m². Median follow-up after surgery was 36 mo (0.2-192). Two patients had a family history of hereditary non-polyposis colorectal cancer and one had juvenile polyposis syndrome. Four patients were nulliparous, the remaining 4 patients had a history of previous successful pregnancies. The presenting symptoms, duration of symptoms, tumor location and treatment strategy are listed in the Table 1. Median duration of symptoms before diagnosis was 16 (4-43) wk. Three patients were diagnosed with colorectal cancer during pregnancy and underwent surgery prior to delivery. These cases included 1 anterior resection with an end colostomy in the 18th week, 1 low anterior resection in the 24th week and 1 subtotal colectomy during the 8th week of pregnancy. Five patients were diagnosed with colorectal cancer within a median of 2.1 (1-4.2) mo after delivery. One synchronous low anterior resection and liver resection, 1 extensive left colectomy, 1 transanal resection, 1 ileocecal resection, and 1 right colectomy were performed on those patients. No adverse neonatal outcomes were noted. All deliveries were at term, except for one patient who underwent low anterior resection during pregnancy (34th week) and delivered preterm. Two patients underwent a cesarean section. Median APGAR score was 9 (8-9). Median birth weight was 3100 (3000-3800) g.

Adjuvant or neoadjuvant treatments were administered exclusively after delivery. In particular, one patient received neoadjuvant chemoradiotherapy whereas adjuvant chemotherapy was given to 5 patients. The specific chemotherapeutic regimens were 5-fluorouracil with leucovorin (*n* = 2), FOLFOX (*n* = 2) and FOLFIRI (*n* = 1). In long-term follow-up, two patients had further successful pregnancies. One of these patients had a ventral hernia repair. The patient initially treated with transanal excision of a T1 rectal lesion opted in favor of radical surgery after delivery and underwent low anterior resection.

DISCUSSION

Our study shows that surgical intervention can be safe and feasible in patients who are diagnosed with

Table 1 Treatment strategy and patient status at last follow-up

	Stage	Start of symptom (pregnancy week)	Duration of symptoms until diagnosis (wk)	Symptom	Tumor location	NCRT	Postoperative chemotherapy	Postoperative radiotherapy	Status at last follow-up
1 ^{1,2}	I	4	10	Rectal bleeding	Sigmoid colon	-	-	-	Alive (NED)
2 ^{1,2}	III	13	4	Rectal bleeding	Rectum	-	+	-	Alive (NED)
3 ^{1,2}	III	16	7	Rectal bleeding	Rectum	-	+	-	Alive (NED)
4 ^{1,3}	I	36	17	Rectal bleeding	Rectum	-	-	-	Alive (NED)
5 ^{1,3}	III	34	14	Abdominal pain	Right colon	-	-	-	Alive (NED)
6 ^{1,3}	III	20	24	Rectal bleeding	Rectum	-	+	-	Alive (NED)
7 ^{1,3}	IV	30	17	Rectal bleeding	Rectum	+	+	-	Deceased ⁴
8 ³	IV	12	43	Anemia, abdominal pain	Right colon	-	+	-	Deceased

¹Curative surgery; ²Diagnosed during pregnancy; ³Diagnosed after pregnancy; ⁴Recurrent disease. NCRT: Neoadjuvant chemoradio therapy; NED: No evidence of disease.

colorectal cancer during pregnancy or in the postpartum period. All of our patients who were diagnosed with colorectal cancer after delivery, had symptoms during pregnancy. Normal pregnancy can mask the symptoms and signs associated with colorectal malignancy^[2,4,5]. For example, abdominal pain, intermittent rectal bleeding and anemia can occur during the course of pregnancy^[6]. Occasional abdominal pain can be related to an enlarging uterus and uterine cramps. Hemorrhoids or anal fissure can be common causes of rectal bleeding in pregnant women^[7]. Pregnancy can limit the utilization of standard diagnostic and therapeutic tools due to a gravid uterus and a potentially vulnerable fetus^[8], which in particular can hamper the liberal use of colonoscopy and computed tomography. However, all patients in our study group underwent complete colonoscopic evaluation before surgery. The age of diagnosis and tumor characteristics in our patients are similar to other series^[5].

Ultrasonography (USG), magnetic resonance imaging (MRI) or computed tomography (CT) were used for disease staging in our series. USG, MRI and CT can be used during pregnancy after consenting the patients about the associated risks and benefits. Heat effects of the magnetic field can be risky for the fetus, especially in the first trimester^[9,10]. CT scan has a limited role in pregnancy due to radiation and contrast^[11]. Proctosigmoidoscopy may be very helpful for differential diagnosis since more than 85% of colorectal tumors diagnosed during pregnancy appear to develop below the peritoneal reflection^[12]. In addition, common anorectal problems can be excluded with a careful anorectal exam. In our series, 5 patients had rectal and 1 patient had a sigmoid colon cancer. Diagnosis of colorectal cancer at an early stage would result in better outcomes. Gastrointestinal symptoms should not be overlooked in a pregnant woman and should be evaluated with proper diagnostic modalities.

According the American Society of Gastrointestinal Endoscopy guidelines, an endoscopic intervention is safer than radiologically guided or surgical operations^[13]. It is preferable to postpone endoscopy to the second trimester^[12]. However, patients should be informed about the potential side effects including over sedation leading to hypoventilation or hypotension, teratogenic effects of medications used for sedation and premature birth^[13]. We did not experience any complications patient or fetus related in our diagnostic work-up. Familial adenomatous polyposis is a known risk factor for colorectal cancer during pregnancy^[14]. In our cohort, 2 patients had a family history of HNPCC and both of these patients were later confirmed with positive genetic testing. One of our patients had juvenile polyposis syndrome and diagnosed with a right colon cancer but later expired due to metastatic disease. In this particular patient diagnosis was delayed 43 mo and was diagnosed in the postpartum period.

The treatment strategy for colorectal cancer should be no different for pregnant and non-pregnant patients in terms of the aim which is potential curative treatment of the disease. However, the well-being of the fetus should be considered. Termination of ongoing pregnancy or delay of required treatment should be discussed with the patient according to time of pregnancy and patient's preference^[2,6,15]. The first trimester of pregnancy is not appropriate for chemotherapy because of high risk of fetal malformations^[16]. While it is generally recommended that chemotherapy should be given only after delivery, there are some reports suggesting that chemotherapy can be given in the second trimester without causing significant long-term complications^[6,17,18]. However, it has been reported that the administration of 5-fluorouracil during pregnancy may cause spontaneous abortion^[12]. If maternal and/or fetal healths are threatened, pre-

term delivery can be considered^[6]. Walsh *et al.*^[19] have proposed an algorithm to manage colorectal cancer diagnosed during pregnancy and recommend individualized treatment based on the disease stage and time of diagnosis during pregnancy. Acting as a team during follow-up and including the patient in decision making are advised.

While low patient number and retrospective design are the major drawbacks of the study, our study is one of the largest single center experiences on this topic. In our limited experience with three patients who have undergone surgery during pregnancy, no adverse maternal and fetal outcomes were observed. There has been a significant delay in the diagnosis of these tumors which is probably due to overlap of symptoms and signs between colorectal malignancy and a normal pregnancy.

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Poster presentation at the meeting of the American Society of Colon and Rectal Surgeons, 2014. Erman Aytac is an assistant professor of surgery at the Acibadem University in Istanbul, Turkey.

COMMENTS

Background

Colorectal cancer in pregnancy is a rare condition and the literature on this subject is scant with fewer than 300 cases reported. The diagnosis of colorectal cancer in pregnancy is usually delayed because the individuals are young and the diagnosis is not entertained. As two patients with possibly conflicting interests need to be managed, many ethical, psycho emotional and medical issues need to be simultaneously addressed. In this study, they analyzed management, complications, maternal and fetal outcomes in patients who were diagnosed with colorectal cancer during pregnancy or in the immediate postpartum period.

Research frontiers

Around 0.1% of pregnant women develop a malignancy and there is limited experience on the management of colorectal cancer diagnosed during pregnancy or perinatal period. When colorectal cancer is detected in this period, treatment options may be limited.

Innovations and breakthroughs

In the authors' experience with three patients who have undergone surgery during pregnancy, no adverse maternal and fetal outcomes were observed. There has been a significant delay in the diagnosis of these tumors which is probably due to overlap of symptoms and signs between colorectal malignancy and a normal pregnancy.

Applications

The treatment strategy for colorectal cancer should be no different for pregnant and non-pregnant patients in terms of the aim which is potential curative treatment of the disease. Considering the significant delay in the diagnosis of these tumors during pregnancy, new diagnostic modalities with reduced fetal side effects would facilitate diagnosis of colorectal cancer.

Terminology

Patients who were diagnosed with colorectal cancer during pregnancy or in the postpartum period were analyzed in the study. The postpartum period is defined as first 6 mo after delivery.

Peer-review

In this retrospective study by Aytac *et al* the authors are presenting their experiences in the management of colorectal neoplasia during pregnancy and in the postpartum period. This is a well written paper with insightful, thoughtful and helpful observations which are a result of serious and hard work from an experienced team of experts in the field of colorectal surgery.

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Retrospective Study

Prognostic value of inflammation-based markers in patients with pancreatic cancer administered gemcitabine and erlotinib

Jae Min Lee, Hong Sik Lee, Jong Jin Hyun, Hyuk Soon Choi, Eun Sun Kim, Bora Keum, Yeon Seok Seo, Yoon Tae Jeen, Hoon Jai Chun, Soon Ho Um, Chang Duck Kim

Jae Min Lee, Hong Sik Lee, Jong Jin Hyun, Hyuk Soon Choi, Eun Sun Kim, Bora Keum, Yeon Seok Seo, Yoon Tae Jeen, Hoon Jai Chun, Soon Ho Um, Chang Duck Kim, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Korea University College of Medicine, Seoul 02841, South Korea

Author contributions: Lee HS designed the study; Lee JM and Hyun JJ modified the study methods and performed the study; Choi HS and Kim ES collected the data; Seo YS and Jeon YT analyzed the data; Lee JM wrote the draft; Keum B, Chun HJ, Um SH and Kim CD revised the draft; Lee HS corrected the final draft and approved the manuscript.

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Correspondence to: Hong Sik Lee, MD, PhD, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Korea University College of Medicine, Incheon-ro 73, Seongbuk-gu, Seoul 02841, South Korea. hslee60@korea.ac.kr
 Telephone: +82-2-9205312
 Fax: +82-2-9531943

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Abstract

AIM: To evaluate the value of systemic inflammation-based markers as prognostic factors for advanced pancreatic cancer (PC).

METHODS: Data from 82 patients who underwent combination chemotherapy with gemcitabine and erlotinib for PC from 2011 to 2014 were collected retrospectively. Data that included the neutrophil-to-lymphocyte ratio (NLR), the platelet-to-lymphocyte ratio, and the C-reactive protein (CRP)-to-albumin (CRP/Alb) ratio were analyzed. Kaplan-Meier curves, and univariate and multivariate Cox proportional hazards regression analyses were used to identify the prognostic factors associated with progression-free survival (PFS) and overall survival (OS).

RESULTS: The univariate analysis demonstrated the prognostic value of the NLR ($P = 0.049$) and the CRP/Alb ratio ($P = 0.047$) in relation to PFS, and a positive

relationship between an increase in inflammation-based markers and a poor prognosis in relation to OS. The multivariate analysis determined that an increased NLR (hazard ratio = 2.76, 95%CI: 1.33-5.75, $P = 0.007$) is an independent prognostic factor for poor OS. There was no association between the PLR and the patients' prognoses in those who had received chemotherapy that comprised gemcitabine and erlotinib in combination. The Kaplan-Meier method and the log-rank test determined significantly worse outcomes in relation to PFS and OS in patients with an NLR > 5 or a CRP/Alb ratio > 5.

CONCLUSION: Systemic inflammation-based markers, including increases in the NLR and the CRP/Alb ratio, may be useful for predicting PC prognoses.

Key words: Pancreatic cancer; Neutrophil-to-lymphocyte ratio; C-reactive protein; Albumin; Prognostic factor

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Core tip: This retrospective study validates the value of systemic inflammation-based markers as prognostic factors for pancreatic cancer (PC). The neutrophil-to-lymphocyte ratio and the C-reactive protein-to-albumin ratio, which can be determined from routine blood tests before chemotherapy, can be used as useful biomarkers in PC to predict a patient's response to chemotherapy.

Lee JM, Lee HS, Hyun JJ, Choi HS, Kim ES, Keum B, Seo YS, Jeon YT, Chun HJ, Um SH, Kim CD. Prognostic value of inflammation-based markers in patients with pancreatic cancer administered gemcitabine and erlotinib. *World J Gastrointest Oncol* 2016; 8(7): 555-562 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v8/i7/555.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v8.i7.555>

INTRODUCTION

Pancreatic cancer (PC) is a devastating malignant tumor that has a poor prognosis^[1]. Although surgical resection offers a good prognosis and prolongs survival, only 10%-20% of patients are eligible for a curative resection at the time of diagnosis^[2,3]. Systemic chemotherapy is a major treatment modality for unresectable PC, and the National Comprehensive Cancer Network recommends gemcitabine-based chemotherapy as standard therapy for advanced or metastatic PC. The findings from recent studies have demonstrated that gemcitabine and erlotinib (Tarceva®) administered in combination improve therapeutic response rates and overall survival (OS)^[4-6]. However, the PC prognosis remains extremely poor, and it is difficult to predict in advanced PC before chemotherapy. Hence, to administer effective treatment, better prognostic predictors are required than those that are currently available.

Evidence is accumulating that supports the relationship between the inflammatory response and cancer development^[7,8]. Bhatti *et al.*^[9] proposed that hematologic inflammation-based markers could be used as prognostic markers in resectable PC. C-reactive protein (CRP) levels, and leukocyte, neutrophil, lymphocyte, and platelet counts, as well as the neutrophil-to-lymphocyte ratio (NLR) and the platelet-to-lymphocyte ratio (PLR), could be prognostic markers for patients with PC^[10,11]. Recently, an increase in the CRP/albumin (CRP/Alb) ratio has been reported to correlate with poor prognoses in patients with malignant tumors^[12-14]. However, the relationship between the CRP/Alb ratio and the PC prognosis has not been studied. Since cancer progression depends on the systemic inflammatory response^[15], we hypothesized that the status of the peripheral blood at the time of diagnosis reflects the inflammatory response and the disease activity associated with PC.

This study aimed to evaluate the prognostic value of systemic inflammation-based markers within the peripheral blood of patients with advanced or metastatic PC, and to determine their usefulness in predicting patients' responses to chemotherapy.

MATERIALS AND METHODS

Patients and study design

We retrospectively collected data from patients with PC who were admitted to two tertiary hospitals, namely, Anam Hospital and Ansan Hospital in South Korea, between January 2011 and December 2014. Only consecutive patients with primary PC were included in the study. All of the patients met the following criteria: (1) the presence of a pathologically confirmed pancreatic adenocarcinoma; (2) receipt of first-line chemotherapy comprising gemcitabine and erlotinib administered in combination; and (3) the presence of locally advanced or metastatic PC. Gemcitabine was administered at 1000 mg/m² three times per week, which was followed by rest for 1 wk. Erlotinib was administered as a single oral 150 mg dose during chemotherapy. Patients who had undergone previous curative resections of their primary pancreatic tumors, or who had undergone first-line chemotherapy that involved other chemotherapeutic agents, including 5-fluorouracil, were excluded from this study.

The patients' demographic, clinical, and laboratory data, including the WBC and differential counts, the platelet count, and information about tumor markers, were collected and analyzed. All of the laboratory data were obtained on the day of or on the day that followed hospital admission. The NLR was calculated by dividing the neutrophil count by the lymphocyte count, and the PLR was calculated by dividing the platelet count by the lymphocyte count. The CRP/Alb ratio was determined as the CRP level divided by the serum albumin level. The follow-up duration was defined as the period from the first day of treatment to the day of death or August 2015.

Table 1 Patient demographics and laboratory findings

Characteristic	
No. of patients, <i>n</i>	82
Age, mean \pm SD, yr	63.5 \pm 10.7
Male, <i>n</i> (%)	49 (60)
Laboratory findings	
WBC count, mean \pm SD	6259 \pm 2667
Platelet count, mean \pm SD, \times 1000	225 \pm 94
Neutrophil count, mean \pm SD	4175 \pm 2139
Lymphocyte count, mean \pm SD	1462 \pm 729
CRP, mean \pm SD, mg/dL	12.5 \pm 23.8
Albumin, mean \pm SD, g/dL	3.5 \pm 0.6
CA19-9, median, IU/mL	503.8
CEA, median, ng/mL	2.8
ECOG performance status score, <i>n</i> (%)	
0	22 (27)
1	48 (58)
2	12 (15)
Inflammatory markers	
NLR, median, range	3.1 (1-48)
PLR, median, range	141 (44-921)
CRP/albumin ratio, median, range	0.5 (0-37.7)
Staging	
Locally advanced, <i>n</i> (%)	14 (17)
Metastatic, <i>n</i> (%)	68 (83)

WBC: White blood cell; CRP: C-reactive protein; NLR: Neutrophil-to-lymphocyte ratio; PLR: Platelet to lymphocyte ratio; CA19-9: Carbohydrate antigen 19-9; CEA: Carcinoembryonic antigen; ECOG: Eastern Cooperative Oncology Group.

Statistical analysis

An increased NLR was defined as > 5 , and an increased PLR was defined as > 150 ^[14]. The CRP/Alb ratio cutoff value was 0.5, which was based on a previous study^[13]. Progression was defined as a 25% or more increase in total tumor size and/or the appearance of new lesions at any site. Progression-free survival (PFS) was defined as the time from treatment to the first observation of progression. OS was defined as the date of the first treatment to the date of death. The Kaplan-Meier method and the log-rank test were used to compare the PFS and OS rates, and the 95%CI were calculated. The univariate and multivariate analyses were carried out using the Cox proportional hazards model, and student's *t*-test was used to analyze the response and survival time results. A *P* value < 0.05 was considered statistically significant. The statistical analyses were conducted using IBM® SPSS® software version 20.0 (IBM Corporation, Armonk, NY, United States).

RESULTS

Patient characteristics

Table 1 presents the characteristics of the 82 patients who met all of the study's eligibility criteria. The mean age of the patients when they were diagnosed with PC was 63.5 ± 10.7 years, and 60% of the patients were men. Most of the patients (85%) had favorable performance statuses with Eastern Cooperative Oncology Group (ECOG) scores of 0 or 1. The median values for the inflammatory

Table 2 Univariate analysis of the clinical parameters for the prediction of progression-free survival

Parameter	<i>n</i>	Univariate analysis	
		HR (95%CI)	<i>P</i> value
Sex			
Woman	33	1	
Man	49	1.37 (0.82-2.3)	0.232
Age (yr)			
< 65	41	1	
≥ 65	41	0.85 (0.52-1.41)	0.536
Staging			
Locally advanced	14	1	
Metastatic	68	1.39 (0.68-2.82)	0.367
ECOG performance status score			
0-1	70	1	
2	12	1.49 (0.77-2.87)	0.234
CA19-9 (IU/mL)			
< 1000	48	1	
≥ 1000	34	1.33 (0.81-2.21)	0.264
CEA (ng/mL)			
< 5	46	1	
≥ 5	29	1.24 (0.72-2.13)	0.441
NLR			
≤ 5	62	1	
> 5	20	1.80 (1.04-3.19)	0.049
PLR			
≤ 150	46	1	
> 150	36	1.22 (0.73-2.02)	0.448
CRP/albumin ratio			
≤ 0.5	42	1	
> 0.5	40	1.72 (1.07-2.80)	0.047

ECOG: Eastern Cooperative Oncology Group; CA19-9: Carbohydrate antigen 19-9; CEA: Carcinoembryonic antigen; NLR: Neutrophil-to-lymphocyte ratio; PLR: Platelet-to-lymphocyte ratio; CRP: C-reactive protein; HR: Hazard ratio.

markers were as follows: NLR: 3.1 (range, 1-48); PLR: 141 (range, 44-921); and CRP/Alb ratio: 0.5 (range, 0-38). All of the patients were finally diagnosed with pancreatic adenocarcinoma based on pathologic examinations. Fourteen patients (17%) had locally advanced PC and 68 patients (83%) had metastatic lesions when they were diagnosed with PC.

Prognostic value of the factors associated with PC

Univariate analyses were performed using sex, age, the tumor stage, the ECOG performance status score, the tumor markers, and the inflammatory markers as possible variables for PFS (Table 2), and it determined that an NLR > 5 ($P = 0.049$) and a CRP/Alb ratio > 0.5 ($P = 0.047$) were significant predictors of a poor prognosis. Univariate and multivariate analyses were also performed in relation to OS (Table 3). The univariate analysis revealed that the presence of distant metastasis ($P = 0.017$), an ECOG performance status score of 2 ($P = 0.002$), an NLR > 5 ($P = 0.008$), and a CRP/Alb ratio > 0.5 ($P = 0.011$) were significantly associated with poor OS. The multivariate analysis showed that an ECOG performance status score of 2 [hazard ratio (HR) = 2.94, 95%CI: 1.42-6.08, $P = 0.004$] and an NLR > 5 (HR = 2.76, 95%CI: 1.33-5.75,

Table 3 Univariate and multivariate analysis of the clinical parameters for the prediction of overall survival

Parameter	n	Univariate analysis		Multivariate analysis	
		HR (95%CI)	P value	HR (95%CI)	P value
Sex					
Woman	33	1			
Man	49	1.26 (0.73-2.17)	0.418		
Age (yr)					
≥ 65	41	1			
< 65	41	1.27 (0.75-2.17)	0.379		
Staging					
Locally advanced	14	1		1	
Metastatic	68	2.87 (1.20-6.83)	0.017	2.10 (0.85-5.18)	0.108
ECOG performance status score					
0-1	70	1		1	
2	12	2.96 (1.49-5.89)	0.002	2.94 (1.42-6.08)	0.004
CA19-9 (IU/mL)					
< 1000	48	1			
≥ 1000	34	1.45 (0.79-2.66)	0.224		
CEA (ng/mL)					
< 5	46	1			
≥ 5	29	1.67 (0.90-3.10)	0.107		
NLR					
≤ 5	62	1		1	
> 5	20	2.61 (1.29-5.27)	0.008	2.76 (1.33-5.75)	0.007
PLR					
≤ 150	46	1			
> 150	36	1.43 (0.79-2.60)	0.24		
CRP/albumin					
≤ 0.5	42	1		1	
> 0.5	40	2.13 (1.19-3.81)	0.011	1.60 (0.84-3.04)	0.151

ECOG: Eastern Cooperative Oncology Group; CA19-9: Carbohydrate antigen 19-9; CEA: Carcinoembryonic antigen; NLR: Neutrophil-to-lymphocyte ratio; PLR: Platelet-to-lymphocyte ratio; CRP: C-reactive protein; HR: Hazard ratio.

$P = 0.007$) were independent factors associated with the prognosis of PC.

Inflammation-based factors and PC outcomes

By the time this study was completed, 57 patients had died because of disease progression. The patients were categorized according to the NLR and the CRP/Alb ratio and subgroup analyses were performed. The groups were compared with respect to the duration of chemotherapy and the time until death (Table 4). Patients with initial NLRs ≤ 5 continued chemotherapy with gemcitabine and erlotinib for longer. The time to disease progression was significantly longer when the patients' NLRs did not increase. The mean time until death was longer in patients who had NLRs ≤ 5 compared with patients who had NLRs > 5 . The mean time until death was shorter in patients with CRP/Alb ratios > 0.5 compared with patients with CRP/Alb ratios ≤ 0.5 .

Prognostic comparisons based on the NLR and the CRP-to-albumin ratio

The NLR has been identified as a prognostic indicator in patients with PC who are undergoing gemcitabine-based chemotherapy; therefore, we compared the cancer prognosis in a group of patients with NLR ≤ 5 with that in a group of patients with NLR > 5 . Kaplan-Meier analyses determined that PFS was significantly better in patients

with NLRs ≤ 5 (4.9 ± 0.5 mo) compared with those with NLRs > 5 (3.1 ± 0.7 mo) ($P = 0.043$) (Figure 1A), and that OS was significantly better in patients with NLRs ≤ 5 (11.1 ± 1.2 mo) compared with those with NLRs > 5 (5.8 ± 0.9 mo) ($P = 0.005$) (Figure 1B). PFS for patients with CRP/Alb ratios > 0.5 (3.2 ± 0.4 mo) was significantly worse compared with those with CRP/Alb ratios ≤ 5 (5.3 ± 0.7 mo) ($P = 0.034$) (Figure 2A), and OS for patients with CRP/Alb ratios > 0.5 (7.9 ± 1.2 mo) was significantly worse compared with those with CRP/Alb ratios ≤ 5 (12.7 ± 1.2 mo) ($P = 0.007$) (Figure 2B).

DISCUSSION

Systemic chemotherapy is recommended as palliative therapy for PC^[16], but only a limited number of patients benefit from chemotherapy. Gemcitabine-based combination therapy is considered an effective first-line treatment for advanced PC^[4,17-19]. While the tumor stage and the carbohydrate antigen 19-9 levels have been used to predict patients' prognoses^[20-22], predicting the therapeutic effect of or a patient's response to chemotherapy is difficult.

Findings from recent studies of different malignant tumors have suggested that increases in the levels of systemic inflammation are indicative of poor survival^[23,24]. Inflammatory cells within the tumor microenvironment play important roles in tumor development and in the

Table 4 Mean times to disease progression and death according to the neutrophil-to-lymphocyte ratio and the C-reactive protein/albumin ratio

Variable	NLR ≤ 5	NLR > 5	<i>P</i> value	CRP/albumin ratio ≤ 0.5	CRP/albumin ratio > 0.5	<i>P</i> value
Time until disease progression, mean \pm SD, mo	3.0 \pm 1.7	1.9 \pm 1.2	0.016	3.0 \pm 1.8	2.3 \pm 1.4	0.08
Time until death, mean \pm SD, mo	9.3 \pm 5.9	4.7 \pm 3.5	0.014	10.0 \pm 5.4	6.0 \pm 5.5	0.01

NLR: Neutrophil-to-lymphocyte ratio; CRP: C-reactive protein.

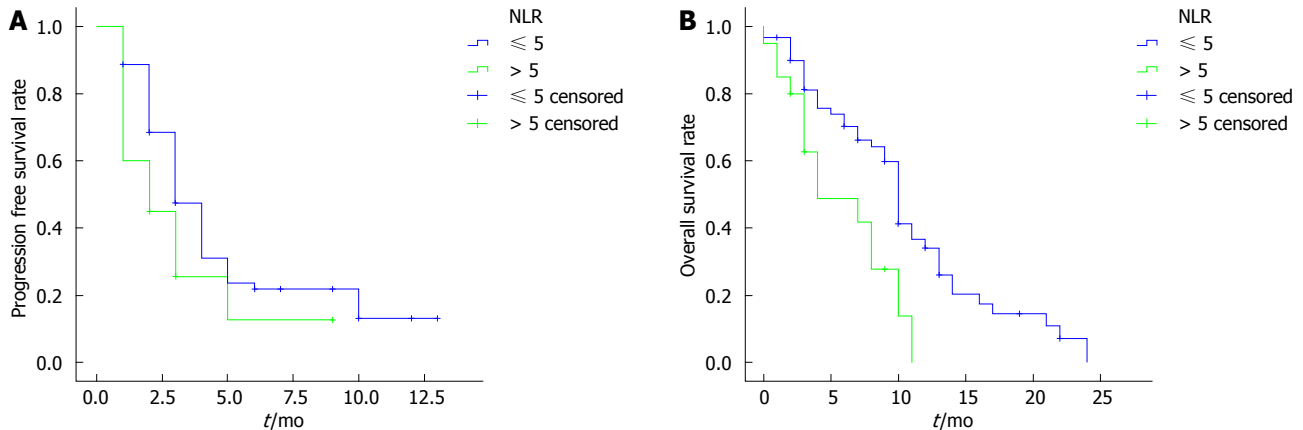


Figure 1 Kaplan-Meier curves for progression-free survival and overall survival according to the neutrophil-lymphocyte ratio. A: PFS stratified according to the NLR; B: Overall survival stratified according to the NLR. NLR: Neutrophil-to-lymphocyte ratio; PFS: Progression-free survival.

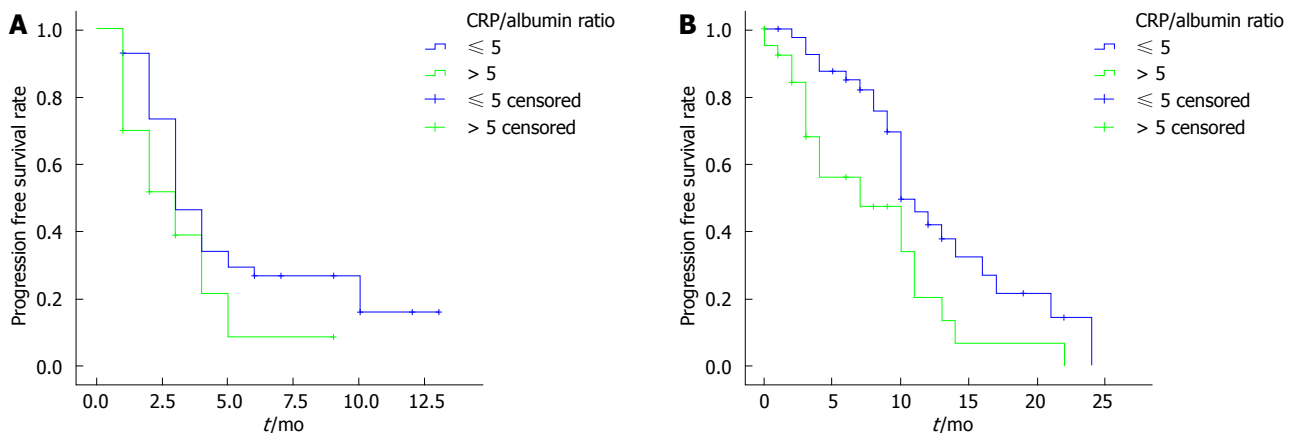


Figure 2 Kaplan-Meier curves for progression-free survival and overall survival according to the C-reactive protein /albumin ratio. A: Progression-free survival stratified according to the CRP/albumin ratio; B: Overall survival stratified according to the CRP/albumin ratio. CRP: C-reactive protein.

survival of malignant cells^[25,26]. Therefore, systemic inflammation-based markers may be indicators of cancer prognosis and of patients' responses to therapy. Since these markers can be readily measured in peripheral blood samples, its usefulness would be greatly expectable in practice. Indeed, some investigators have described the prognostic value of these systemic inflammatory response markers in advanced PC^[11,27-29].

The current investigation scrutinized the value of a number of clinical parameters, including the NLR, PLR, and the CRP/Alb ratio, as prognostic predictors in patients with PC who received combination chemotherapy that comprised gemcitabine and erlotinib. The univariate and multivariate analyses determined that a higher

ECOG performance status score, metastatic disease, a higher NLR, and a higher CRP/Alb ratio were associated with poor outcomes. A multivariate analysis of the significant inflammation-related factors determined that the NLR was independently associated with OS. In the patients with PC, a higher NLR was associated with significantly worse OS (2.6 mo) compared with a lower NLR (8.5 mo), but the PLR was not determined to be an independent prognostic factor. A higher CRP/Alb ratio was associated with a poor prognosis according to the univariate analysis, but the multivariate analysis did not show that it was an independent prognostic factor.

The mechanism that underlies the association between inflammation-based markers and poor PC outcomes

has not been clarified. Systemic inflammatory changes would be reflected in increases in the neutrophil levels, and these could be induced by tumor invasion and disease progression, despite the administration of chemotherapy. Inflammatory responses can inhibit the immune system by suppressing the cytolytic activity of the immune cells, including that associated with the lymphocytes, activated T cells, and natural killer cells^[30]. Furthermore, inflammatory responses can promote tumor angiogenesis, invasion, and metastasis by recruiting regulatory T lymphocytes and activating cytokine production^[31,32]. Since an increase in the neutrophil count or a decrease in the lymphocyte count within the WBC count will present as a higher NLR, the NLR will be strongly associated with the prognosis for a patient with a malignant tumor. Moreover, cancer progression against chemotherapy activates inflammatory processes within the tumor microenvironment^[33], and the WBC ratios may change under these conditions.

The prognostic value of the preoperative NLR has been described in patients with resectable PC^[34-36], but the response of the NLR to chemotherapy and its value as a prognostic marker have not been established. We observed that the mean times until disease progression or death were significantly shorter in patients with NLRs > 5 compared with those whose NLRs were not elevated. Elevated neutrophil counts may aid cancer progression by providing a favorable environment for tumor growth. Furthermore, lymphocytopenia, which can be induced by many of the inhibitory immunologic mediators that are released by tumor cells, results in a weakened immune system that would contribute to poor patient outcomes during systemic chemotherapy.

To the best of our knowledge, this is the first study to evaluate the value of the CRP/Alb ratio in advanced PC. Another strength of our study is that the data were only collected from patients who were receiving a unified chemotherapy regimen that comprised gemcitabine and erlotinib. While other studies of PC have included both resectable and unresectable tumors^[9,37], we excluded patients with PC who had undergone surgery from the analysis. However, there are several limitations to our study. First, this was a retrospective study that involved a relatively small number of patients. While an elevated NLR was related to the PC prognosis, we could not validate the prognostic value of the PLR in this study. More data obtained from larger numbers of patients will be required to determine the true value of the PLR for predicting PC prognoses. Second, while we excluded those patients who had been diagnosed with acute pancreatitis or other infections, patients with early infections may have been included during the selection process. Since pancreatic duct obstruction and biliary tract invasion are relatively frequent in PC, patients with potentially aggressive disease may have been allocated to the group containing patients with higher NLRs. Finally, this study only evaluated patients who received combination chemotherapy with gemcitabine and erlotinib; hence, it is difficult to extrapolate the data to

all patients with PC. The therapeutic strategy for PC may differ considerably in relation to a patient's socioeconomic status, comorbidities, and other factors. However, since gemcitabine-based chemotherapy is widely recommended as first-line palliative chemotherapy for PC throughout the world, the NLR, which is easily calculated, would assist clinicians to predict patients' therapeutic responses and PC prognoses.

In summary, our results strongly support the idea that systemic inflammation-based parameters may be useful prognostic markers for patients with advanced PC. The NLR when determined at the time of a diagnosis of PC could be a valuable marker for predicting a patient's response to chemotherapy with gemcitabine and erlotinib. Furthermore, the CRP/Alb ratio may be valuable as a prognostic factor in PC. More prospective studies are needed to verify the usefulness of these inflammation-based markers in patients with PC.

COMMENTS

Background

Inflammation based markers have been known to have a prognostic value predicting the outcome of various cancers. Since the status of the peripheral blood reflects the inflammatory response at the time of diagnosis, it could be used the systemic inflammation based markers [neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio, C-reactive protein (CRP)-to-albumin ratio, etc.] as a prognostic biomarker in advanced pancreatic cancer (PC).

Research frontiers

It is important to be able to predict the outcome and response to chemotherapy in advanced PC. This study assessed the prognostic value of systemic inflammation based markers in patients with palliative chemotherapy due to inoperable PC.

Innovations and breakthroughs

Although prior investigators had studied about the prognostic value of NLR in malignancy, there was no study about the CRP-to-albumin ratio in PC. The present study showed that both NLR and CRP-to-albumin ratio can be useful and easy biomarkers to predict the response and outcome of PC.

Applications

It can be easily calculated NLR or CRP-to-albumin ratio from routine blood tests. The systemic inflammation-based markers can be useful tool to predict the outcome in patients with PC.

Terminology

The NLR was calculated by dividing the neutrophil count by the lymphocyte count, and the PLR was calculated by dividing the platelet count by the lymphocyte count. The CRP/Alb ratio was determined as the CRP level divided by the serum albumin level.

Peer-review

The manuscript by Lee *et al* aims to identify inflammation-based markers in patients with pancreatic cancer treated with gemcitabine and erlotinib. Eighty-two pancreatic cancer patients were enrolled in this retrospective study. Patients received combination chemotherapy with gemcitabine and erlotinib. Multivariate analysis demonstrated that an increased neutrophil-to-lymphocyte ratio (hazard ratio = 2.76, 95%CI: 1.33-5.75, $P = 0.007$) was an independent prognostic factor for poor overall survival. CRP/albumin ratio was related to progression free survival. The manuscript is in general well written and the topic is of interest.

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Observational Study

Undernutrition, risk of malnutrition and obesity in gastroenterological patients: A multicenter study

Massimiliano Rizzi, Silvia Mazzuoli, Nunzia Regano, Rosa Inguaggiato, Margherita Bianco, Gioacchino Leandro, Elisabetta Bugianesi, Donatella Noè, Nicoletta Orzes, Paolo Pallini, Maria Letizia Petroni, Gianni Testino, Francesco William Guglielmi

Massimiliano Rizzi, Silvia Mazzuoli, Nunzia Regano, Francesco William Guglielmi, Gastroenterology Unit, San Nicola Pellegrino Hospital, 76125 Trani, Italy

Donatella Noè, Nicoletta Orzes, Paolo Pallini, Maria Letizia Petroni, Gianni Testino, Francesco William Guglielmi, AIGO-Committee on Nutrition and Alcoholism, 20153 Milano, Italy

Rosa Inguaggiato, Margherita Bianco, Gioacchino Leandro, Gastroenterology Unit I, Saverio De Bellis IRCCS, 70013 Castellana Grotte, Italy

Gioacchino Leandro, UCL, Department of Liver and Digestive Health, London NW3 2PF, England

Elisabetta Bugianesi, Division of Gastro-Hepatology, Department of Medical Sciences, University of Torino, 10126 Torino, Italy

Donatella Noè, Dietetics and Clinical Nutrition Unit, S. Carlo Borromeo Hospital, 20153 Milano, Italy

Nicoletta Orzes, Gastroenterology Unit, S. Giovanni di Dio Hospital, 34170 Gorizia, Italy

Paolo Pallini, Gastroenterology Unit, Umberto I Hospital, 30174 Mestre, Italy

Maria Letizia Petroni, Nutritional and Gastroenterological Unit, IRCCS, 28824 Piacavallo, Italy

Gianni Testino, Alcoholism and Related Conditions Unit, IRCCS-AOU S. Martino-IST, 16132 Genova, Italy

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Correspondence to: Francesco William Guglielmi, Professor, Gastroenterology Unit, Regional Centre of Home Artificial Nutrition, "San Nicola Pellegrino" Hospital, 76125 Trani, Italy. guglielmifw@libero.it
Telephone: +39-883-483209
Fax: +39-883-483342

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Abstract

AIM: To investigate the prevalence of undernutrition, risk of malnutrition and obesity in the Italian gastroenterological population.

METHODS: The Italian Hospital Gastroenterology Association conducted an observational, cross-sectional multicenter study. Weight, weight loss, and body mass index were evaluated. Undernutrition was defined as unintentional weight loss > 10% in the last three-six months. Values of Malnutrition Universal Screening Tool (MUST) > 2, NRS-2002 > 3, and Mini Nutritional Assessment (MNA) from 17 to 25 identified risk of malnutrition in outpatients, inpatients and elderly patients, respectively. A body mass index ≥ 30 indicated obesity. Gastrointestinal pathologies were categorized into acute, chronic and neoplastic diseases.

RESULTS: A total of 513 patients participated in the study. The prevalence of undernutrition was 4.6% in outpatients and 19.6% in inpatients. Moreover, undernutrition was present in 4.3% of the gastrointestinal patients with chronic disease, 11.0% of those with acute disease, and 17.6% of those with cancer. The risk of malnutrition increased progressively and significantly in chronic, acute and neoplastic gastrointestinal diseases in inpatients and the elderly population. Logistical regression analysis confirmed that cancer was a risk factor for undernutrition (OR = 2.7; 95%CI: 1.2-6.44, $P = 0.02$). Obesity and overweight were more frequent in outpatients.

CONCLUSION: More than 63% of outpatients and 80% of inpatients in gastroenterological centers suffered from significant changes in body composition and required specific nutritional competence and treatment.

Key words: Obesity; Malnutrition; Risk of Malnutrition; NRS2002; Gastrointestinal disease

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Core tip: The relevance of this study concerns the finding that in patients with gastroenterological disease, both prevalence of undernutrition and risk of malnutrition were higher in patients admitted to the hospital and in patients with cancer disease, while obesity and overweight were more frequently detected in outpatients. In conclusion, we can attest that two-thirds of gastroenterological patients suffered from abnormalities in body composition and required targeted nutritional treatments.

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INTRODUCTION

Malnutrition is defined as a structural and functional alteration of the body composition. Although the term malnutrition is commonly used in the sense of undernutrition, it encompasses both weight loss (undernutrition) and weight gain (overweight and obesity). The physiological basis of undernutrition and obesity is the deficit (undernutrition) or the excess (obesity) of calories that results in measurable adverse effects on clinical outcomes^[1-5]. The equilibrium between the total energy requirements, nutrient intake and utilization is mediated by hormonal and cytokine stimuli that induce the activation of intracellular metabolic pathways.

Previous papers have found that the prevalence of hospital undernutrition varies between 27% to more than 50% depending on the identification criteria, the medical or surgical setting and the age of the patients^[6-10].

It is important to identify and treat undernutrition because it has been associated with a higher likelihood of hospitalization, a prolonged (by 29%-65%) hospital stay^[11-15] and an increase in costs of up to 68%^[16,17]. Moreover, it must be emphasized that hospital undernutrition worsens if untreated^[18]. Today, many health care workers do not recognize malnutrition and do not consider nutrition as one of the more relevant aspects of the clinical management of patients^[19].

The risk of undernutrition can be identified using different validated nutritional screening tools, such as the NRS-2002, MUST, and the MNA^[20-22].

Of note, the prevalence of overweight and obesity has been steadily growing in the general population and among inpatients and represent important risk factors of morbidity and mortality^[23-27].

Currently few data are available in the literature about the prevalence of undernutrition and the risk of malnutrition among Italian inpatients and outpatients, particularly in the gastroenterological setting. The Italian multicenter HOMIS study identified undernutrition in 19.1% and obesity in 24.8% of its patients^[28]. Another Italian multicenter study, PIMAI, reported a prevalence rate of undernutrition of 30.7%, a risk of malnutrition, evaluated by the NRS-2002, of 28.6% and a rate of obesity of 21%^[29,30].

The nutritional state of Italian gastroenterological patients has been described in an Italian multicenter study^[31] that involved twenty-seven gastroenterology units. The study highlighted a mean of 27% of undernourished patients with a wide range (4% to 55%), probably due to the lack of appropriate nutritional assessment.

Consistent data on the prevalence of malnutrition in gastroenterology units have been limited to a few subsets of patients such as those with cirrhosis^[32-36] and Crohn's disease^[37-39].

Similarly, the prevalence of obesity in Italian gastroenterology centers has not been adequately studied, although it is an important risk factor for many gastroenterological diseases^[40-42], and it seems to affect approximately 12% of discharged patients^[43].

The aim of this study is to measure the prevalence rate of undernutrition, risk of malnutrition and obesity in the Italian gastroenterological population suffering from acute, chronic and neoplastic disease.

MATERIALS AND METHODS

Study design

This was an observational, cross-sectional and prospective multicenter study that was urged and supported by the Italian Hospital Gastroenterology Association (AIGO).

Nineteen Italian Gastroenterological Units and Services participated in the study, as disclosed by the AIGO website.

Inclusion criteria

All inpatients and outpatients (day hospital and ambulatory) admitted between January 14 and 20, 2013 were consecutively enrolled in the study and submitted to nutritional assessment. As this was an observational study, the sample size was determined by the number of inpatients and outpatients who visited the various centers during the study week.

Patient population

In this study, we enrolled three different categories of patients with gastrointestinal disease: (1) subjects with a diagnosis of acute gastrointestinal disease: Dysphagia, dyspepsia, heartburn, other abdominal pains, acute diarrhea, exacerbation of chronic diarrhea, acute constipation, digestive hemorrhage, acute hepatitis, cholangitis, rectum hemorrhage, inflammatory bowel disease (IBD) exacerbation, or acute pancreatitis; (2) patients with chronic gastroenterological disease: Reflux disease, achalasia, chronic gastritis, chronic viral hepatitis, chronic alcoholic hepatitis, non-alcoholic fatty liver disease, other chronic hepatitis, cirrhosis, chronic pancreatitis, Crohn's disease and ulcerative proctocolitis, other chronic enteropathy, or chronic constipation; and (3) patients with gastrointestinal cancer: Esophagus, gastric, small bowel, colon, pancreatic, or biliary cancer and hepatocarcinoma. Patients were categorized as having (1) chronic disease, if the symptoms, signs and diagnosis had persisted for ≥ 6 mo; (2) acute disease, if the symptoms, signs and diagnosis had recently appeared, regardless of the presence of chronic disease; or (3) cancer, if affected by gastroenterological neoplastic disease, regardless of the presence of acute and/or chronic disease, as it was assumed that the presence of neoplastic disease itself was sufficient to determine further deterioration of a patient's nutritional status.

Nutritional parameters

Weight (kg) and height (centimeters) were measured in the morning in fasting patients dressed in their underwear and without shoes.

The body mass index (BMI) was calculated by dividing the weight in kg by the square of height in meters. Weight loss was calculated as the difference between the

current weight and the weight in the last three-six months as reported by the patient. Patients were considered undernourished when they presented an unintentional (*i.e.*, without voluntary dietary restriction) weight loss $> 10\%$ in the last 3-6 mo. Obesity was defined as a BMI ≥ 30 , and overweight a BMI between 25 and 29.9. Obese patients were considered undernourished when they lost more than 10% of their weight while receiving their usual diet. The risk of malnutrition was calculated using three different specific tools, as shown in Table 1.

Specifically, the calculation of the three methods are described in detail in the following text: (1) the Malnutrition Universal Screening Tool (MUST) was used for outpatients. This method was based on the BMI, the unintentional weight loss and the presence of acute disease that was able to significantly reduce nutrient intake in the following five days. The total score ranged from 0-6; a score of 0 indicated null or low risk of malnutrition, a score of 1 suggested a moderate risk of malnutrition, and a score ≥ 2 was indicative of a severe risk of malnutrition^[44]. We considered a score ≥ 2 to identify patients at nutritional risk.

The Nutritional Risk Screening Score 2002 (NRS-2002) has been recommended by ESPEN to screen inpatients. This test evaluates the nutritional risk (score: 0-3) and the severity of disease (score: 0-3) with an additional point for patients ≥ 70 years old. The final score ranges from 1 to 7. The patient is considered at nutritional risk for scores higher than 3^[45].

The Mini Nutritional Assessment (MNA) is a tool for elderly patients (≥ 65 years). This tool has two parts. The first part consists of six items and results in a score between 0 and 14; a score lower than 12 is considered indicative of risk of undernutrition and leads to the patient answering the second part of the tool, which is composed of 12 items with a possible maximal score of 16. A total score < 17 is indicative of malnutrition, a score between 17 and 25 indicates risk of undernutrition, while scores > 25 are indicative of well-nourished patients^[46-48].

We used the MUST and NRS-2002 to evaluate the risk of malnutrition in < 65 -year-old patients and the MNA for those ≥ 65 years old.

Ethical considerations

This study was designed with the aim of obtaining epidemiological data and anthropometric measurements that did not compromise patient's safety. All data were anonymous. Patients were referred using the first two letters of their name and surname and with a consecutive number.

A written informed consent statement was obtained from each patient prior to study inclusion. The study was conducted with the consensus of the local ethical committees of each center and of all the patients.

Statistical analysis

The results were presented as the mean \pm SD. Categorical data were described as frequencies. The nonparametric Mann-Whitney *U* test was used to compare continuous

Table 1 Subscores of the three nutritional risk screening tests used in the study

Test	Subscore			Nutritional risk score
MUST	BMI (score: 0-2)	Unintentional weight loss (score: 0-2)	Acute disease able to significantly reduce nutrient intake in the following 5 d (score: 2)	≥ 2
NRS-2002	Pre-test: BMI < 20.5; weight loss, reduced caloric intake, acute disease	Nutritional risk: Weight loss, BMI, caloric intake (score: 0-3)	Severity of disease (score: 0-3)	Age ≥ 70 -yr-old (score: 1)
MNA	First part (six items: Caloric food intake, weight loss, motility, psychological stress, neuropsychological disease, BMI (score: 0-14)	If subscore ≤ 11 : Complete second part	Second part (12 items: Home and nutritional autonomy, drugs, bedsores, daily meals, brachial and calf circumference; (maximal score of 16)	17-25

MUST: Malnutrition Universal Screening Tool; NRS: Nutritional Risk Screening; MNA: Mini Nutritional Assessment.

variables, and χ^2 test was used to compare categorical data. Two-tailed *P* values less than 0.05 were considered statistically significant.

Univariate and multivariate analyses were used to identify potential predictors of malnutrition. A linear trend test was used to assess associations with severity of disease. A stepwise backward logistical regression analysis, adjusted for age and gender and considering acute, chronic and neoplastic disease independent of each other, was performed to identify significant independent predictors of malnutrition. Statistical analyses were performed using SPSS software version 12.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

Five hundred and ninety-seven patients were enrolled. We excluded from the statistical analysis five patients for incomplete data and 79 cirrhotic patients because of the negative effects of ascites and peripheral edema on nutritional parameters.

In Table 2 we report the anthropometric and nutritional data of the 513 patients enrolled in the 19 gastroenterology units participating in the study: 51.4% were males and 48.6% females, and the average age was 59.8 ± 17.8 years without a significant difference between the centers.

In total, 39.8% of the patients required hospitalization, 12.1% were DH patients and 48.1% were ambulatory patients. Approximately 30.8% of the patients were urgently hospitalized, 21.2% of the cases had a planned admission, 22.2% needed a follow-up evaluation and 25.8% were at their first medical visit.

In Table 3 we report the age, gender, medical evaluation setting and disease category of patients according to their age category.

Gastrointestinal diseases were acute in 72.1% of the cases, chronic in 16.7% and neoplastic in 11.1%. In particular, chronic diseases were more frequent in younger participants (22.6% vs 9.0% in older patients, $P < 0.001$), while neoplastic diseases were more represented in the elderly patients (5.6% in younger patients vs 18.8%, $P < 0.001$).

Table 4 shows the prevalence of each pathology included in the three categories: Acute disease, chronic diseases and cancers according to age.

Undernutrition

The mean prevalence of undernutrition in our population was 12.1%:4.6% in the ambulatory and DH settings and 19.6% in patients requiring hospitalization.

The undernourished patients were affected by chronic, acute and neoplastic disease at rates of 8%, 74% and 18%, respectively.

In Table 5 we report the distribution of undernutrition, risk of malnutrition, obesity and overweight among inpatients and outpatients in both categories of age. In both categories of age, the rate of undernourished inpatients was significantly higher compared to that of outpatients.

As shown in Figure 1, the prevalence of undernutrition in chronic, acute and neoplastic gastrointestinal diseases was 4.3%, 11.0% and 17.6%, respectively, with a prevalence significantly higher in neoplastic patients than in those with chronic disease ($P = 0.03$). The linear trend test showed that the prevalence of undernutrition was significantly higher in hospital admitted patients ($P = 0.01$).

The logistical regression analysis corrected for age, gender and acute, chronic and neoplastic disease showed that gastrointestinal cancer was a risk factor for undernutrition with an odds ratio of 2.7 (95%CI: 1.2-6.4, $P = 0.02$).

Risk of malnutrition

The mean prevalence of risk of malnutrition in our population was 23.8%. In Table 5 we show the prevalence of risk of malnutrition among inpatients and outpatients, and in Table 6 we report the prevalence of risk of malnutrition according to the three screening tests and age group. Of the < 65-year-old patients, the 24.7% of inpatients were at risk of malnutrition; in the elderly participants, the MNA screening tool revealed a risk of malnutrition in inpatients that was higher than that of elderly outpatients (69.2% vs 40.6%, respectively, $P < 0.001$).

Table 2 Characteristics of the patients in the 19 gastroenterological units participating in the study

Centers	<i>n</i>	M	F	Age (average ± DS)	Ambulatory	DH	Inpatients	MUST	NRS	MNA	Weight (kg)	Weight loss (%)	BMI
Bolzano	21	61.90%	38.10%	67.5 ± 17.2	0.00%	0.00%	100.00%	-	2.2 ± 1.5	16.8 ± 4.3	71.6 ± 16.4	-4.7 ± 6.2	24.8 ± 4.4
Fiemme del Cavalese	17	57.1%	42.9%	56.2 ± 25.1	57.1%	0.0%	42.9%	0.8 ± 1.3	1.7 ± 1.4	19.0 ± 9.9	66.9 ± 5.1	-2.5 ± 6.9	23.1 ± 2.4
Pordenone	14	25.0%	75.0%	48.7 ± 24.0	100.0%	0.0%	0.0%	1.0 ± 1.2	-	22.0 ± 0.0	58.3 ± 12.7	0.0 ± 0.0	21.3 ± 3.6
Udine	10	50.0%	50.0%	51.6 ± 8.7	70.0%	0.0%	30.0%	0.4 ± 0.7	-	13.0 ± 0.0	75.5 ± 16.0	-2.0 ± 5.7	25.9 ± 5.2
Como	12	72.2%	27.8%	65.3 ± 17.9	0.0	22.2%	77.8%	1.7 ± 2.1	2.3 ± 1.5	18.6 ± 5.4	73.5 ± 22.7	-7.6 ± 9.4	25.8 ± 6.7
Milano	17	64.0%	36.0%	64.9 ± 19.2	0.0	0.0%	100.0%	-	1.7 ± 1.4	9.5 ± 3.1	69.4 ± 15.7	-2.8 ± 7.4	25.2 ± 4.7
Torino	38	50.0%	50.0%	58.9 ± 16.1	0.0	55.3%	44.7%	1.6 ± 1.7	2.2 ± 2.2	15.8 ± 4.9	65.3 ± 16.6	-0.9 ± 9.1	23.9 ± 4.5
Alto Vicentino di Santorso	32	40.6%	59.4%	68.2 ± 12.7	97.3	0.0%	2.7%	0.2 ± 0.6	0.5 ± 0.7	15.4 ± 4.6	71.3 ± 12.8	-2.3 ± 4.9	25.0 ± 3.9
Bassano del Grappa	25	48.0%	52.0%	48.3 ± 17.9	60.0	0.0%	40.0%	0.5 ± 0.9	0.8 ± 1.4	17.6 ± 4.8	68.0 ± 14.5	0.1 ± 9.0	23.9 ± 4.3
Legnano	16	87.5%	12.5%	53.0 ± 16.1	0.0	0.0%	100.0%	-	1.1 ± 1.6	15.2 ± 4.6	66.1 ± 13.5	-4.2 ± 12.3	23.0 ± 2.7
Ravenna	66	41.5%	58.5%	57.4 ± 19.4	52.5	21.9	25.6%	0.8 ± 1.3	1.3 ± 1.4	19.6 ± 2.8	67.7 ± 17.4	-1.5 ± 7.2	24.3 ± 5.4
Ancona	33	39.4	60.6	58.9 ± 19	100.0	0.0	0.0	0.1 ± 0.3	-	13.5 ± 4.8	70.8 ± 13.5	0.5 ± 2.5	25.7 ± 4.8
Napoli	26	53.8	46.1	59.1 ± 15.8	7.7	30.8	61.5	0.5 ± 0.9	1.3 ± 1.2	12.2 ± 2.7	67.9 ± 14.9	-1.2 ± 3.8	24.7 ± 5.6
Foggia	33	39.4	60.6	57.7 ± 18.2	66.6	6.1	27.3	0.3 ± 0.9	0.4 ± 0.8	15.0 ± 3.3	76.5 ± 21.9	-1.5 ± 3.0	28.6 ± 7.6
Trani	53	58.5	41.5	55.5 ± 18.1	62.1	10.3	27.6	0.5 ± 0.9	0.7 ± 1.1	13.4 ± 3.2	71.6 ± 14.5	1.1 ± 5.5	27.1 ± 5.5
Cosenza	58	53.9	46.1	58.8 ± 18.4	38.1	15.9	46.0	1.3 ± 1.7	2.1 ± 1.1	15.3 ± 3.7	67.0 ± 15.9	-3.8 ± 7.9	24.5 ± 4.8
Palermo	15	64.7	35.3	64.2 ± 10.1	0.0	0.0	100.0	-	1.3 ± 0.6	13.0 ± 4.5	77.5 ± 21.3	-1.3 ± 1.6	27.6 ± 6.7
Marsala	17	41.2	58.8	70.4 ± 9.5	88.2	0.0	11.8	0.6 ± 0.8	2.6 ± 0.9	18.0 ± 5.4	72.2 ± 12.4	-2.2 ± 3.5	27.7 ± 3.7
Siracusa	10	60.0	40.0	67.9 ± 1.3	20.0	0.0	80.0	0.8 ± 1.2	2.6 ± 1.5	15.4 ± 4.6	61.8 ± 7.8	-1.6 ± 4.9	23.9 ± 2.6
Total	513	51.4	48.6	59.8 ± 17.8	48.1	12.1	39.8	0.7 ± 0.5	1.5 ± 0.8	15.8 ± 2.9	69.7 ± 16.0	-1.7 ± 6.9	25.3 ± 5.3

M: Male; F: Females; DH: Day Hospital; MUST: Malnutrition Universal Screening Tool; NRS: Nutritional Risk Screening; MNA: Mini Nutritional Assessment; BMI: Body Mass Index.

Table 3 Clinical features of evaluated patients according to age group *n* (%)

	Total cohort (<i>n</i> = 513)	< 65 yr (<i>n</i> = 289)	≥ 65 yr (<i>n</i> = 218)	<i>P</i> value
Age (yr) (Mean ± SD)	59.8 ± 17.8	47.4 ± 12.8	76.1 ± 6.9	< 0.001 ¹
Male gender,	260 (51.4)	138 (47.8)	122 (56.2)	0.06 ²
Setting				< 0.001 ²
Inpatient	204 (39.8)	93 (32.2)	110 (50.7)	
Outpatient	308 (60.2)	196 (67.8)	107 (49.3)	
Disease				< 0.001 ²
Acute	414 (72.1)	232 (71.8)	177 (72.2)	
Chronic	96 (16.7)	73 (22.6)	22 (9.0)	
Neoplastic	64 (11.1)	18 (5.6)	46 (18.8)	

P value: ¹*P* < 0.05. ²Wilcoxon rank-sum (Mann-Whitney) test; ³χ² test. Significant standardized adjusted residual.

Of the outpatients at risk of malnutrition, acute and chronic gastrointestinal diseases had the same prevalence (50%); ten outpatients were affected by cancer, but none were at risk of malnutrition. In this population, only the 17.7% of patients with chronic gastrointestinal disease and 8.8% of those with acute gastrointestinal disease were at risk of malnutrition.

The inpatients at risk of malnutrition were affected by acute disease in 61.5% and by gastrointestinal cancer in 38.5% of the cases.

The elderly patients at risk of malnutrition presented chronic, acute and neoplastic disease in 5.2%, 67.0%, and 27.8% of the cases, respectively.

Figure 2 shows that the frequency of risk of malnutrition increased progressively with hospitalization, reaching statistical significance (*P* < 0.007) in the presence

of neoplasm.

Overweight and obesity

The prevalence of overweight and obesity in our population was, respectively, 28.6% and 15.6%. In Table 5, we report the rate of overweight and obesity in outpatients (31.7% and 18.3%, respectively) and in inpatients (25.5% and 13.0%, respectively) according to age group. Moreover, the rate of overweight and obesity did not show a difference in age. The prevalence of overweight and obesity was, respectively, 28.7% and 19.1% in chronic, 29.1% and 15.7% in acute, and 26.4% and 11.3% in neoplastic disease.

In our population, we found that no obese outpatients presented a weight loss > 10% in the last 3-6 mo, while the prevalence of risk of malnutrition was 15% higher

Table 4 Prevalence and number of patients with chronic disease, acute disease and cancer according to age group

Chronic disease	%	< 65 yr	≥ 65 yr	P value	Acute disease	%	< 65 yr	≥ 65 yr	P value	Cancer	%	< 65 yr	≥ 65 yr	P value
GERD	8.90	35	18	0.123	Dysphagia	2.7	7	9	0.199	Esophagus	0.7	0	4	0.033
Achalasia	0.20	1	0	0.577	Dyspepsia	9.3	25	30	0.046	Stomach	1.3	3	5	0.219
Chronic gastropathy	5.90	17	18	0.174	Epigastralgia	12.6	44	31	0.44	Small-large intestine	3	5	13	0.01
Chronic viral hepatitis	4.20	17	8	0.194	Other abdominalgia	17.7	53	52	0.78	Pancreas	0.5	1	2	0.394
Chronic alcoholic hepatitis	3.20	14	5	0.113	Acute diarrhea	2.5	8	6	0.602	Liver	3.4	7	13	0.036
NAFLD	4.50	13	14	0.206	Acute constipation	1.5	7	2	0.18	Biliary tree	2	3	9	0.024
Other chronic hepatitis	1.30	3	5	0.211	Digestive bleeding	5.6	13	20	0.027					
Compensated cirrhosis	14.70	49	38	0.441	Acute hepatitis	3.4	13	7	0.315					
Chronic pancreatitis	1.20	4	3	0.632	Cirrhosis complicated	11.5	39	29	0.478					
Non-IBD chronic diarrhea	3.50	11	10	0.391	Hematochezia	3.5	7	14	0.022					
Crohn's disease	6.60	33	6	0.0002	IBD exacerbation	6.1	33	3	0.000033					
Ulcerative colitis	4.5	24	3	0.00036	Acute pancreatitis	4.9	17	12	0.515					
Chronic constipation	5.2	13	18	0.053										
Celiac disease	1.7	10	0	0.004										

GERD: Gastric esophagitis reflux diseases; NAFLD: Non-alcoholic fatty liver disease; IBD: Inflammatory bowel disease.

Table 5 Patients with nutritional abnormalities in the 19 gastroenterological centers participating in the study *n* (%)

	Outpatients		Inpatients		P value
	< 65 yr	> 65 yr	< 65 yr	> 65 yr	
Undernutrition	8 (4.2)	6 (5.7)	24 (23.9) ¹	15 (15.6) ²	¹ P < 0.001 ² P
Risk of Malnutrition	24 (12.2)	43 (40.6)	21 (24.7)	64 (69.2) ³	³ P
Obesity	34 (18.3)	19 (17.5)	15 (17.2)	10 (10.5)	NS
Overweight	52 (26.5)	46 (42.5)	18 (20.7)	27 (28.4)	NS

¹P < 0.001 out- vs inpatient < 65; ²P = 0.02 out- vs inpatient ≥ 65; ³P < 0.001 out- vs inpatient > 65.

Table 6 Prevalence of risk of malnutrition according to age group

Nutritional risk test	Total cohort (n = 513)	< 65 yr (n = 289)	≥ 65 yr (n = 218)	P value
MUST				0.45
< 2 (%)	274 (89.0)	172 (87.8)	97 (90.7)	
≥ 2 (%)	34 (11.0)	24 (12.2)	10 (9.3)	
NRS 2002				0.002
< 3 (%)	112 (63.3)	61 (75.3)	51 (53.1)	
≥ 3 (%)	65 (36.7)	20 (24.7)	45 (46.9)	
MNA 17%-25%	115 (54.2)	-	115 (54.5)	

MUST: Malnutrition Universal Screening Tool; NRS: Nutritional Risk Screening; MNA: Mini Nutritional Assessment.

in elderly subjects (36.8%). The rate of undernutrition was 12% in the hospitalized obese patients, and the prevalence of risk of malnutrition was 13.3% in young obese inpatients and increased up to 80% in those > 65

years old.

Of the overweight outpatients, the rate of undernutrition was 2%, while it was 8.9% in overweight inpatients. The rate of overweight outpatients at risk of malnutrition was

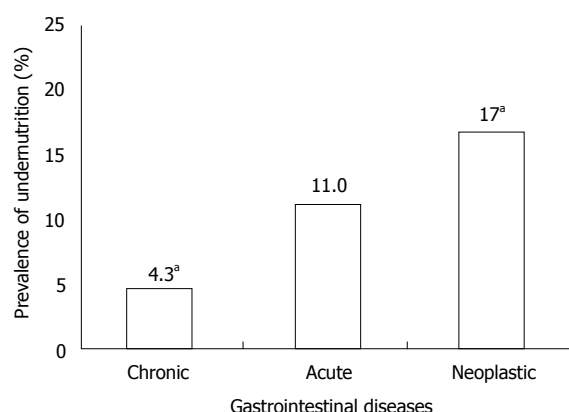


Figure 1 Prevalence of undernutrition in presentations of acute, chronic and neoplastic gastrointestinal diseases. ^a $P = 0.03$, neoplastic vs chronic. Linear trend test: $P = 0.01$.

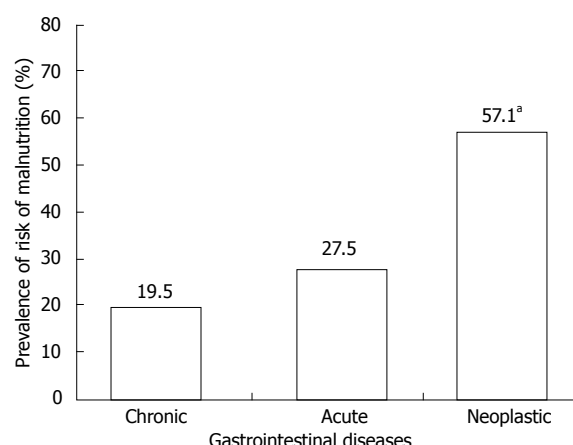


Figure 2 Prevalence of risk of malnutrition in presentations of acute, chronic and neoplastic gastrointestinal diseases. ^a $P < 0.007$, neoplastic vs chronic and acute.

28.3%, but this rate reached 33.3% in those hospitalized.

DISCUSSION

This study was designed to define the nutritional state of patients with gastroenterological disease. Regarding the diseases included in this study, we want to specify that cirrhotic patients were excluded because of the obscuring effect of compartmentalized ascites on body weight^[31,36,49]. However, diseases causing reduced albumin levels, which decreases oncotic pressure and allows for diffuse fluid leakage into the interstitial space, were not excluded, as fluid retention has to be considered a direct consequence of malnutrition.

Our data found that undernutrition affected approximately 12% of all patients; in particular, we documented that the rate of prevalence of undernutrition was significantly higher in inpatients (20%) than in outpatients. This is a consequence of the fact that acute diseases requiring hospitalization reduce caloric intake because of the presence of abdominal pain, diarrhea and vomiting. The use of tools to evaluate the risk of malnutrition should allow us to focus our attention on patients who have not yet begun to lose weight.

It was also interesting to note that younger and neoplastic inpatients were more frequently malnourished than the other groups, and this was due to the high rate of chronic inflammatory gastrointestinal diseases in our younger population (IBD: 11.4% vs 1.4% and celiac disease: 3.5% vs 0% in < 65-year-old and > 65-year-old patients, respectively) and the metabolic abnormalities characterizing cancer cachexia. Indeed, logistic regression confirmed that (1) the rate of undernutrition increased with the severity of disease; and (2) gastrointestinal cancer was a risk factor for undernutrition.

Very little data can be found in the literature concerning the prevalence rates of undernutrition in gastroenterology units. In our population, the prevalence of undernutrition was similar to those reported in other European^[8] and Italian studies^[28] but differed from other studies^[9,29,30,50] because of both (1) the different

tools used to evaluate malnutrition; and (2) the diverse stages of disease. In this study, we reported for the first time (1) the rate of undernutrition in gastroenterology outpatients; and (2) the correlation of undernutrition with hospital admission.

Regarding the risk of malnutrition, we want to emphasize that all of the screening tools we used were well-validated in identifying the patients who would develop undernutrition in the absence of an adequate nutritional care plan and that the NRS-2002 has shown a good predictive value for mortality, length of stay and complications. Few data have been published about the risk of malnutrition in the gastroenterology population^[9,10,50]. In this study, we demonstrated that, in the gastroenterology department, inpatient and elderly patients had a greater frequency of risk of malnutrition than outpatients and younger patients, especially if affected by cancer; additionally, this risk was lower for those with chronic gastrointestinal disease. We must also highlight the fact that the NRS-2002 in inpatients was more frequently influenced by a reduction in caloric intake than by weight loss.

In our population, the NRS-2002 disclosed a risk of malnutrition in 36.7% of the inpatients. This value was lower than that reported in a Danish study evaluating gastro-surgery patients^[9] but higher than data from Romanian gastroenterology departments^[50].

Moreover, while young inpatients were undernourished or at risk of malnutrition at a similar prevalence, elderly inpatients were at a greater risk of malnutrition. These data indicate that the management of malnutrition in gastrointestinal departments should have different targets according to patients' age: We should probably treat undernutrition with artificial nutrition in younger patients, while oral supplementation should be recommended to prevent malnutrition in elderly patients. Very limited data are available on the evaluation of the risk of malnutrition in outpatients. In this setting, our data indicated that patients with nutritional risk were more often affected by chronic disease, in which the catabolic

effects of inflammatory cytokines influence nutritional status^[4,51,52].

Overweight and obesity represent another aspect of malnutrition that entails excessive fat mass in body composition. In recent years, a number of works have reported an increasing incidence of obesity in the general population, but data on the prevalence of obesity and overweight in the gastroenterological population have been limited. The average prevalence of overweight-obesity in our study was 22.7%; this rate of obesity was lower than those reported in a hospital setting in previous Italian^[28-30] reports but was higher than the Italian general population rate, especially in outpatients.

We also found that approximately 10% of the obese and overweight hospitalized patients were undernourished and that the risk of malnutrition was present in more than one-third of the obese and overweight gastroenterological patients, with rates that reached 80% in > 65-year-old obese inpatients.

The prevalence of obesity and overweight in outpatients was higher than in inpatients but was not statistically significant.

It is not surprising that in obese patients, undernutrition and risk of malnutrition could be present at the same time. An involuntary weight loss > 10% and/or reduction in caloric intake allowed us to identify patients who, despite having excess weight, met the criteria for malnutrition or risk of malnutrition. These data indicate that we must keep in mind that even obese patients can be malnourished and that we must investigate the risk of malnutrition especially among elderly gastroenterological patients, regardless of their weight at admission.

A possible bias of this study was the voluntary participation of the gastrointestinal units including centers that paid major attention to nutritional aspects. Another limitation of this work was the difference in the population size and in the frequency of disease, which was often determined by each center's particular experience; it was thus difficult to study undernutrition and the risk of malnutrition for each single disease.

Overall, our data noted that 55% of inpatients and 22% of outpatients were undernourished and at risk of malnutrition and that half of the outpatients and nearly one-third of inpatients were obese or overweight. In our population, only 19.7% and 36.6% of inpatients and outpatients, respectively, did not present nutritional abnormalities (weight loss, risk of malnutrition, overweight or obesity). These data indicate that 80.3% of inpatients and 63.4% of outpatients would require nutritional competence in gastrointestinal units to assess the degree of malnutrition, to correctly design appropriate therapeutic programs to improve protein-caloric alterations and to prevent complications.

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COMMENTS

Background

Malnutrition has adverse effects on clinical outcomes, but many health care workers do not adequately consider it as a relevant aspect of the clinical management of patients. In the literature, few data are available regarding the prevalence of malnutrition (undernutrition, obesity,) and risk of malnutrition in European and Italian gastroenterology departments, with rates that depend on the criteria adopted for their identification, the medical or surgical setting and the age of the patients.

Research frontiers

A better understanding of the prevalence of malnutrition and nutritional risk according to the severity of disease (chronic, acute and cancer) in outpatients and inpatients would facilitate the identification of patients with impairment of nutritional status and with adequate nutritional management.

Innovations and breakthroughs

For the first time, they studied undernutrition, risk of malnutrition (using three different nutritional screening tools) and obesity according to the severity of gastroenterological disease (chronic, acute and cancer) in both admitted patients and outpatients.

Applications

The authors' data showed that there was a different distribution of undernutrition, risk of malnutrition and obesity according to the severity of disease and age group among inpatients and outpatients, which indicates that an appropriate nutritional care plan in gastrointestinal departments to achieve different nutritional targets may be needed.

Terminology

Undernutrition: When patients presented an unintentional (*i.e.*, without voluntary dietary restriction) weight loss > 10% in the last 3-6 mo; The MUST (Malnutrition Universal Screening Tool), NRS-2002 (Nutritional Risk Screening Score 2002) and MNA (Mini Nutritional Assessment) are three screening nutritional tests that identify patients at risk of malnutrition; BMI: Body mass index (calculated by dividing the weight in kg by the square of height in meters). Obesity: BMI \geq 30; overweight: BMI between 25 and 29.9.

Peer-review

The authors have excluded cirrhotics because of the obscuring effect of ascitis and or edema. Inflammatory diseases may cause a drop in serum albumin

levels that decrease oncotic pressure and favors fluid leakage to the interstitial space, that may reach up to 5 l before edema is clinically evident.

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Systematic review of laparoscopic vs open surgery for colorectal cancer in elderly patients

Shoichi Fujii, Mitsuo Tsukamoto, Yoshihisa Fukushima, Ryu Shimada, Koichi Okamoto, Takeshi Tsuchiya, Keiji Nozawa, Keiji Matsuda, Yojiro Hashiguchi

Shoichi Fujii, Mitsuo Tsukamoto, Yoshihisa Fukushima, Ryu Shimada, Koichi Okamoto, Takeshi Tsuchiya, Keiji Nozawa, Keiji Matsuda, Yojiro Hashiguchi, Department of Surgery, Teikyo University School of Medicine, Tokyo 173-8605, Japan

Author contributions: Fujii S wrote the paper; Tsukamoto M, Fukushima Y, Shimada R, Okamoto K, Tsuchiya T, Nozawa K and Matsuda K performed the collected the data; Hashiguchi Y generalized and guided the paper production.

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Correspondence to: Shoichi Fujii, MD, PhD, Department of Surgery, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 173-8605, Japan. sfujii631011@med.teikyo-u.ac.jp
Telephone: +81-3-39641231
Fax: +81-3-53756097

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Abstract

AIM: To verify the safety and validity of laparoscopic surgery for the treatment of colorectal cancer in elderly patients.

METHODS: A meta-analysis was performed of a systematic search of studies on an electronic database. Studies that compared laparoscopic colectomy (LAC) in elderly colorectal cancer patients with open colectomy (OC) were retrieved, and their short and long-term outcomes compared. Elderly people were defined as 65 years old or more. Inclusion criteria were set at: Resection of colorectal cancer, comparison between laparoscopic and OC and no significant difference in backgrounds between groups.

RESULTS: Fifteen studies were identified for analysis. LAC was performed on 1436 patients, and OC performed on 1810 patients. In analyses of short-term outcomes, operation time for LAC was longer than for OC (mean difference = 34.4162, 95%CI: 17.8473-50.9851, $P < 0.0001$). The following clinical parameters were lower in LAC than in OC: Amount of estimated blood loss (mean difference = -93.3738, 95%CI: -132.3437 to -54.4039, $P < 0.0001$), overall morbidity (OR = 0.5427, 95%CI: 0.4425-0.6655, $P < 0.0001$), incisional surgical site infection (OR = 0.6262, 95%CI: 0.4310-0.9097, $P = 0.0140$), bowel obstruction and ileus (OR = 0.6248, 95%CI: 0.4519-0.8638, $P = 0.0044$) and cardiovascular complications (OR = 0.4767, 95%CI: 0.2805-0.8101, $P = 0.0062$). In analyses of long-term outcomes (median follow-up period: 36.4 mo in LAC, 34.3 mo in OC), there was no significant difference in overall survival (mean difference = 0.8321, 95%CI: 0.5331-1.2990, $P = 0.4187$) and disease specific survival (mean difference = 1.0254, 95%CI: 0.6707-1.5675, $P = 0.9209$). There was also no significant difference in the number of dissected lymph nodes (mean difference = -0.1360, 95%CI: -4.0553-3.7833, $P = 0.9458$).

CONCLUSION: LAC in elderly colorectal cancer patients had benefits in short-term outcomes compared with OC except operation time. The long-term outcomes and oncological clearance of LAC were similar to that of OC. These results support the assertion that LAC is an effective procedure for elderly patients with colorectal cancer.

Key words: Laparoscopic surgery; Systematic review; Meta-analysis; Colorectal cancer; Elderly patient

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Core tip: Safety and effectiveness of laparoscopic surgery (LAC) in elderly has been unknown. A meta-analysis was performed of a systematic search of studies on an electronic database. Studies that compared LAC in elderly colorectal cancer patients with open colectomy (OC) were retrieved, and their short and long-term outcomes compared. Fifteen studies which had 1436 LAC and 1810 OC were identified. In short-term outcomes, blood loss, morbidity, incisional surgical site infection, bowel obstruction and cardiovascular complications were superior in LAC except operation time. There was no significant difference in long-term outcomes. LAC is an effective procedure for elderly with colorectal cancer.

Fujii S, Tsukamoto M, Fukushima Y, Shimada R, Okamoto K, Tsuchiya T, Nozawa K, Matsuda K, Hashiguchi Y. Systematic review of laparoscopic vs open surgery for colorectal cancer in elderly patients. *World J Gastrointest Oncol* 2016; 8(7): 573-582 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v8/i7/573.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v8.i7.573>

INTRODUCTION

People are living longer across the globe. According to the World Health Organization, 6.9% of the world was over the age of 65 in 2000 with an estimated increase to 10.4% in 2025 and a further rise to 16.4% in 2050^[1]. This estimation is valid in all regions of the world. Average life expectancies in 2025 are estimated to be 77 years old in the Americas and Europe and 72 years old in Asia. Colorectal cancer is the third most common malignant neoplasm in the world and aging is assumed to be one of the risk factors for colorectal carcinogenesis^[2]. Elderly patients have a higher American Society of Anesthesiologists score, higher cardiac and pulmonary comorbidity rate and lower preoperative nutritional conditioning than younger patients^[3-5]. Therefore, there is a high risk associated with even minimally invasive surgery in elderly patients. Several studies have reported the benefits of laparoscopic colorectal surgery in elderly patients^[6-10]. Most studies concluded that laparoscopic surgery had a lower postoperative morbidity rate and shorter length of hospital stay when compared to open surgery. Several large-scale systematic reviews that

compare laparoscopic colorectal surgery with open surgery have been published in recent years^[11,12]. They report that laparoscopic surgery has lower mortality, lower overall morbidity, lower cardiac and respiratory complications, lower wound infection and shorter length of hospital stay. However, they analyzed both colorectal cancer and benign diseases together. The surgical procedure for colorectal cancer differs from that for benign disease because optimal lymph node dissection and resection, with a securing safety margin, are vital in malignant neoplasm surgery. Therefore, a study analyzing laparoscopic surgery that targeted only colorectal cancer was required.

Moreover, the results of previous reviews reported only short-term outcomes. The evaluation of long-term outcomes is very important in the analysis of treatment efficacy for malignant neoplasia. The purpose of the present review is to clarify the benefits of laparoscopic surgery in elderly patients with colorectal cancer. We analyzed not only short-term but also long-term outcomes.

MATERIALS AND METHODS

Eligibility criteria

Elderly people were defined as 65 years old or more, as outlined by the World Health Organization^[1]. All studies were limited to randomized controlled or comparative studies. The subject of each study was limited to colorectal cancer and studies that included any benign disease were excluded. Backgrounds were similar between both groups, and had at least 15 patients in one group. The results had to include a comparison between laparoscopic and open surgery.

Outcomes

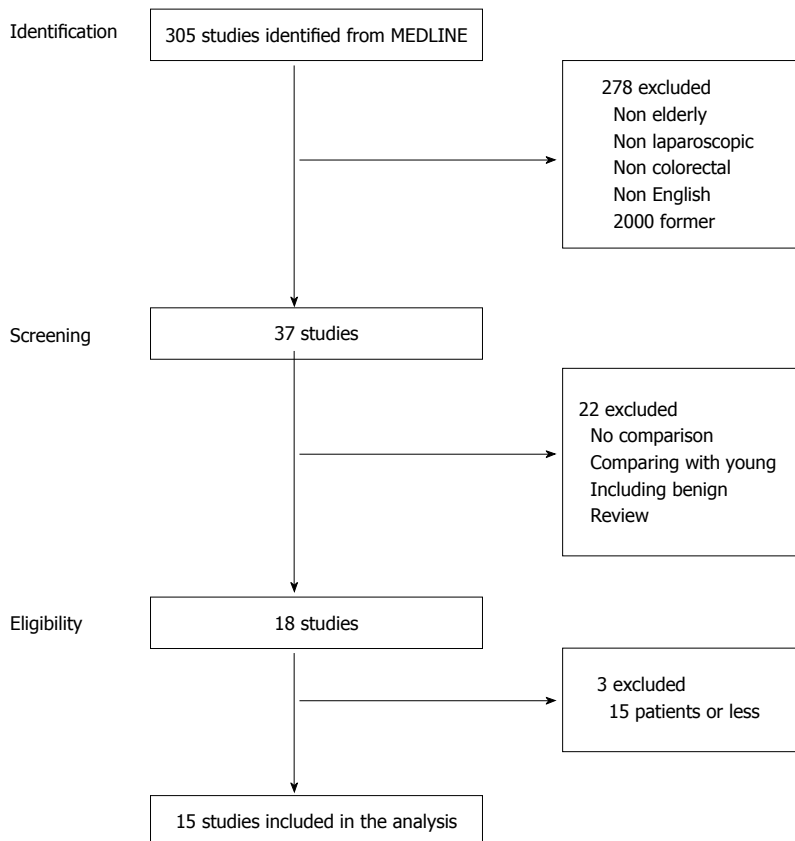
Short-term outcomes analyzed in the present study were as follows: Operative time, amount of estimated blood loss, mortality, overall morbidity, incisional surgical site infection, deep surgical site infection, anastomotic leakage, bowel obstruction and ileus, pneumonia, cardiovascular complication, time of normal bowel function and length of postoperative hospital stay. Duration of short-term was defined after the operation within 30 d.

The overall and disease specific survival rates were measured as long-term outcomes.

The number of dissected lymph nodes was used as an indicator of oncological clearance.

Study selection

The literature search was performed electronically using PubMed (MEDLINE). The search terms were as follows: Elderly or old, colorectal cancer or colon cancer, and laparoscopic surgery or laparoscopic colectomy (LAC) in combination with Boolean operators AND or OR. The language was limited to English. Studies were selected from those published after 2000 because they included the long-term results of several randomized



controlled studies that compared laparoscopic and open surgery^[13-18]. Moreover, developments in laparoscopic surgery instrumentation might influence short-term results in studies conducted in more recent years.

Assessment of study quality

The number of randomized controlled study was only one in this meta-analysis^[19]. The randomized controlled study was assessed for methodological quality using the Cochrane Handbook^[20]. Five of six items were at low risk of bias. Blinding of the study was not possible.

The comparative studies were assessed by the Newcastle-Ottawa Quality Assessment Scale (NOS)^[21]. Twelve of 14 studies had 6 or more star points on the NOS scale.

Statistical analysis

The odds ratios (ORs) for each study and 95% CIs were calculated from event numbers of categorical variables of short-term results. Pooled ORs were calculated using a random effect model. The mean value difference between continuous variables of short-term results and the number of dissected lymph nodes was also calculated using a random effect model. In the analysis of long-term results, 95% CIs of survival comparison and the number of patients in each study were synthesized using a random effect model. Synthesis of data was performed using the DerSimonian-Laird method^[22]. Study heterogeneity was checked by means of Cochran's Q statistic. If the P value of the heterogeneity test was

less than 0.05 in significance level, a null hypothesis of homogeneity was dismissed and study heterogeneity was proved. Publication bias among the studies was checked using the Egger test or Begg test accordingly. If the P value for publication bias was less than 0.10 a null hypothesis of no bias was dismissed and publication bias was confirmed.

RESULTS

Study profile

Thirty seven studies were identified by the first screening of MEDLINE. The reviews and studies that included benign disease cases or no data comparison between laparoscopic and open surgery were excluded. Finally, 15 studies were selected for analysis (Figure 1)^[19,23-36]. The types of studies were as follows: 1 randomized controlled, 2 case-matched, 1 prospective comparative and 11 retrospective comparative studies. In total, 1436 laparoscopic surgeries and 1810 open surgeries were analyzed. Conversion to open surgery was described in 9 studies. The range of conversion rate was between 0% and 13.9%, and the incidence of total patients was 4.5%. A summary of study characteristics is shown in Table 1.

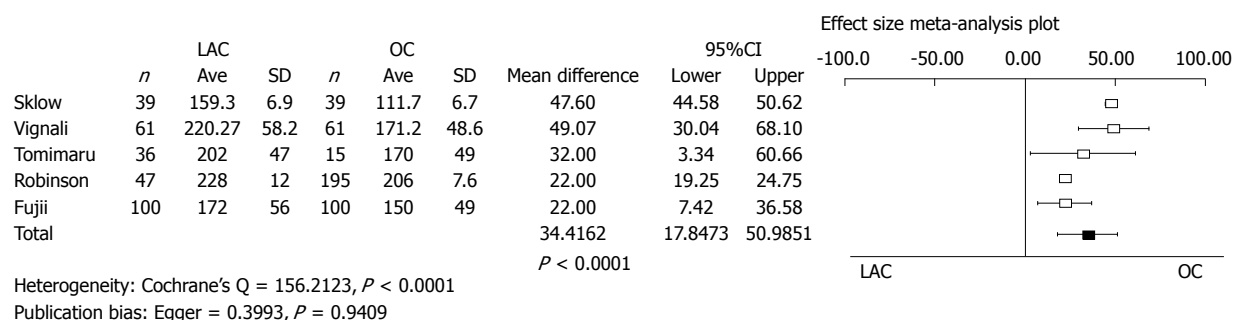
Short-term outcomes

Operation time: Five studies reported operative time as the mean value with standard deviation. The operation time of LAC was significantly longer than OC (mean difference = 34.4162, 95%CI: 17.8473-50.9851, $P <$

Table 1 Characteristics of studies

Ref.	Year	Study type	Age	Age		Patient	No. of LAC	No. of OC	Convert (%)	ASA (1-2/3-4)		Gender (M/F)		NOS
				LAC	OC					LAC	OC	LAC	OC	
Sklow <i>et al</i> ^[23]	2003	Case-matched	76	81.4 ± 0.83	81.8 ± 0.91	All cancer	39	39		19/20	10/29	22/17	21/18	6
Vignali <i>et al</i> ^[24]	2005	Case-matched	80	82.3 ± 2.3	83.1 ± 3.1	All cancer	61	61	4 (6.6)	2.5 ± 0.1	2.6 ± 0.6	29/32	29/32	6
Feng <i>et al</i> ^[25]	2006	Retro, comparative	71	77.8 ± 5.1	76.9 ± 6.1	All cancer	51	102	2 (3.9)					5
Tei <i>et al</i> ^[26]	2009	Retro, comparative	71	75.5 (71-89)	76.0 (71-93)	All cancer	51	78	3 (5.9)	Apr-37	63/15	32/19	43/35	6
Akiyoshi <i>et al</i> ^[27]	2009	Retro, comparative	75	79 (75-90)	79 (75-86)	Rectal cancer	44	43	0 (0)	Jun-38	Jul-36	21/23	23/20	3
Tomimaru <i>et al</i> ^[28]	2011	Retro, comparative	76	82.0 ± 4.6	81.9 ± 5.7	Colon cancer	36	15	5 (13.9)	20/16	8/7	13/23	7/8	8
Robinson <i>et al</i> ^[29]	2011	Retro, comparative	65	74 (65-86)	75 (65-91)	All cancer	47	195		1/46	9/162	47/0	191/4	8
She <i>et al</i> ^[30]	2013	Retro, comparative	75	80 (75-94)	80 (75-95)	All cancer	189	245	9 (4.8)	122/66	134/101	90/99	120/125	6
Scarpa <i>et al</i> ^[31]	2013	Retro, comparative	70	77 (74-80)	75 (72-80)	All cancer	33	24				14/19	8/16	6
Fujii <i>et al</i> ^[19]	2014	RCT	75	79.8 ± 3.6	80.1 ± 4.2	All cancer	100	100	3 (3)	Sep-91	85/15	50/50	60/40	NA
Hinoi <i>et al</i> ^[32]	2014	Retro, comparative	80	83 (81-85)	83 (81-85)	All cancer	459	459		362/107	355/104	215/244	222/237	8
Miyasaka <i>et al</i> ^[33]	2014	Retro, comparative	70	75 (70-86)	78 (70-94)	All cancer	28	79		6/22	48/31	13/15	27/52	6
Vallribera Valls <i>et al</i> ^[34]	2014	Prospective, comparative	75			All cancer	134	133		59/75	71/62	88/46	88/45	8
Zeng <i>et al</i> ^[35]	2015	Retro, comparative	70	74 (70-87)	74 (70-88)	Rectal cancer	112	182	7 (6.3)	66/46	92/90	62/50	98/84	6
Shigeta <i>et al</i> ^[36]	2015	Retro, comparative	80	82 (81-84)	83 (81-87)	All cancer	52	55	0 (0)	Apr-48	Apr-81	28/24	26/29	7

LAC: Laparoscopic surgery; OC: Open surgery; NOS: Newcastle-Ottawa scale stars.

**Figure 2** Forest plot of the mean difference for operative time. LAC: Laparoscopic surgery; OC: Open surgery.

0.0001). The heterogeneity was statistically significant (Cochrane's $Q = 156.2123$, $P < 0.0001$). Publication bias was not evident (Egger = 0.3993, $P = 0.9409$) (Figure 2).

Amount of estimated blood loss: Six studies reported the amount of estimated blood loss as a mean value with standard deviation. The operation time of LAC was significantly less than OC (mean difference = -93.3738, 95%CI: -132.3437 to -54.4039, $P < 0.0001$). Heterogeneity was statistically evident (Cochrane's $Q = 74.1364$, $P < 0.0001$). Publication bias was not evident (Egger = 0.9129, $P = 0.7776$) (Figure 3).

Mortality: Four studies reported mortality. There was no significant difference between LAC and OC in mortality (OR = 0.5052, 95%CI: 0.2438-1.0467, $P = 0.0662$). Heterogeneity and publication bias were not evident (Cochrane's $Q = 2.0911$, $P = 0.5537$, Egger = -0.6646, $P = 0.5883$).

Overall morbidity: Thirteen studies reported incidence of overall morbidity. The overall morbidity of LAC was significantly less than for OC (OR = 0.5427, 95%CI: 0.4425-0.6655, $P < 0.0001$). Heterogeneity was not evident (Cochrane's $Q = 14.7867$, $P = 0.2533$). Publication

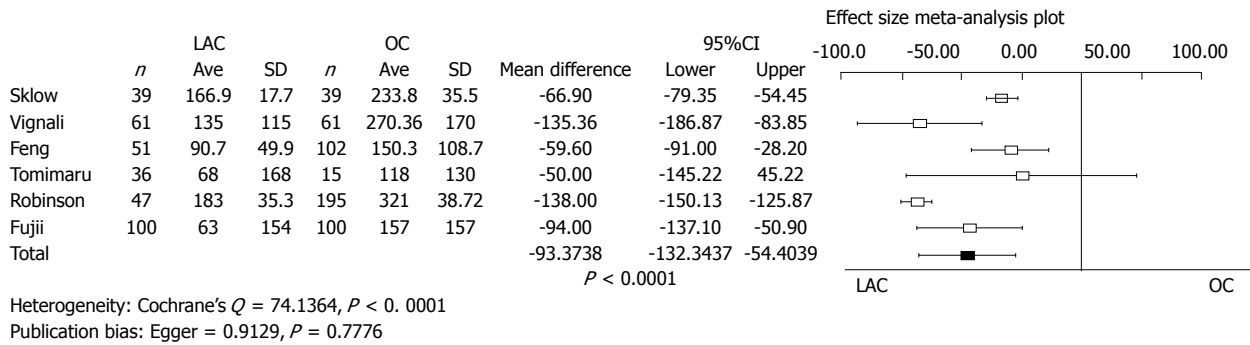


Figure 3 Forest plot of the mean difference for amount of estimated blood loss. LAC: Laparoscopic surgery; OC: Open surgery.

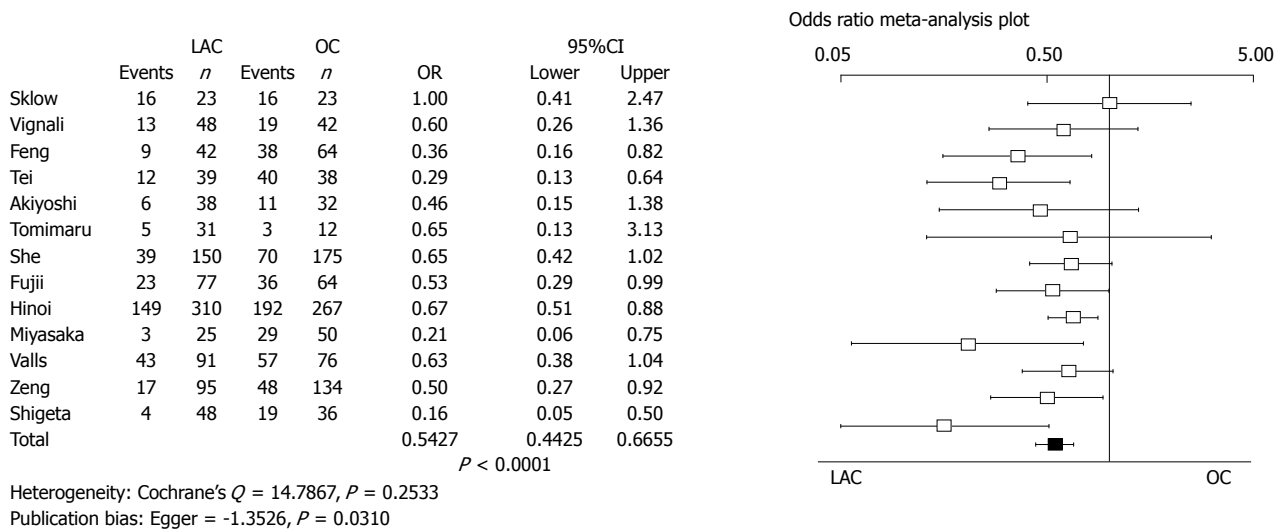


Figure 4 Forest plot of the odds ratio for overall morbidity. LAC: Laparoscopic surgery; OC: Open surgery.

bias was statistically evident (Egger = -1.3526, $P = 0.0310$) (Figure 4).

Incisional surgical site infection: Twenty studies reported the incidence of incisional surgical site infection. The incisional surgical site infection of LAC was significantly less than for OC (OR = 0.6262, 95%CI: 0.4310-0.9097, $P = 0.0140$). Heterogeneity and publication bias were not evident (Cochrane's $Q = 15.2636$, $P = 0.1707$, Egger = -0.3638, $P = 0.6557$) (Figure 5).

Deep surgical site infection: Four studies reported the incidence of deep surgical site infection. There was no significant difference between LAC and OC in deep surgical site infection (OR = 0.8234, 95%CI: 0.3298-2.0556, $P = 0.6771$). Heterogeneity and publication bias were not evident (Cochrane's $Q = 6.3512$, $P = 0.0957$, Egger = -3.0524, $P = 0.1922$).

Anastomotic leakage: Twenty studies reported the incidence of anastomotic leakage. There was no significant difference between LAC and OC in anastomotic leakage (OR = 0.9138, 95%CI: 0.5667-1.4735, $P =$

0.7115). Heterogeneity and publication bias were not evident (Cochrane's $Q = 8.0075$, $P = 0.7126$, Egger = 0.0396, $P = 0.9632$) (Figure 6).

Bowel obstruction and ileus: Ten studies reported the incidence of bowel obstruction and ileus. Bowel obstruction and ileus of LAC was significantly less than for OC (OR = 0.6248, 95%CI: 0.4519-0.8638, $P = 0.0044$). Heterogeneity and publication bias were not evident (Cochrane's $Q = 8.7612$, $P = 0.4596$, Egger = -1.1383, $P = 0.1602$) (Figure 7).

Pneumonia: Three studies reported the incidence of pneumonia. There was no significant difference between LAC and OC in the incidence of pneumonia (OR = 0.4526, 95%CI: 0.1976-1.0365, $P = 0.0608$). Heterogeneity and publication bias were not evident (Cochrane's $Q = 2.3251$, $P = 0.3127$, Egger = 0.1846, $P = 0.9743$).

Cardiovascular complication: Eight studies reported the incidence of cardiovascular complication. Cardiovascular complications of LAC was significantly less than for OC (OR = 0.4767, 95%CI: 0.2805-0.8101, $P = 0.0062$). Heterogeneity was not evident (Cochrane's Q

	LAC		OC			95%CI	
	Events	<i>n</i>	Events	<i>n</i>	OR	Lower	Upper
Sklow	3	36	3	36	1.00	0.19	5.29
Vignali	5	56	9	52	0.52	0.16	1.64
Tei	4	47	25	53	0.18	0.06	0.56
Akiyoshi	3	41	2	41	1.50	0.24	9.45
Tomimaru	3	33	1	14	1.27	0.12	13.32
Robinson	9	38	38	157	0.98	0.44	2.20
She	6	183	4	241	1.98	0.55	7.10
Fujii	5	95	10	90	0.47	0.16	1.44
Hinoi	37	422	43	416	0.85	0.54	1.34
Miyasaka	1	27	10	69	0.26	0.03	2.09
Valls	4	130	10	123	0.38	0.12	1.24
Zeng	8	104	31	151	0.37	0.17	0.85
Total					0.6262	0.4310	0.9097

Heterogeneity: Cochrane's $Q = 15.2636$, $P = 0.1707$

Publication bias: Egger = -0.3638, $P = 0.6557$

Odds ratio meta-analysis plot

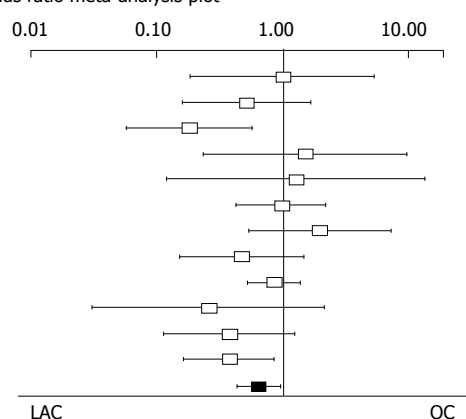


Figure 5 Forest plot of the odds ratio for incisional surgical site infection. LAC: Laparoscopic surgery; OC: Open surgery.

	LAC		OC			95%CI	
	Events	<i>n</i>	Events	<i>n</i>	OR	Lower	Upper
Vignali	4	57	3	58	1.36	0.29	6.33
Tei	2	49	2	76	1.55	0.21	11.38
Akiyoshi	1	43	2	41	0.48	0.04	5.46
Robinson	2	45	6	189	1.40	0.27	7.17
She	1	188	2	243	0.65	0.06	7.18
Scarpa	1	32	3	21	0.22	0.02	2.25
Fujii	5	95	8	92	0.61	0.19	1.92
Hinoi	8	451	2	457	4.05	0.86	19.19
Miyasaka	1	27	1	78	2.89	0.17	47.80
Valls	7	127	9	124	0.76	0.27	2.10
Zeng	2	110	6	176	0.53	0.11	2.69
Shigeta	1	51	2	53	0.52	0.05	5.91
Total					0.9138	0.5667	1.4735

Heterogeneity: Cochrane's $Q = 8.0075$, $P = 0.7126$

Publication bias: Egger = 0.0396, $P = 0.9632$

Odds ratio meta-analysis plot

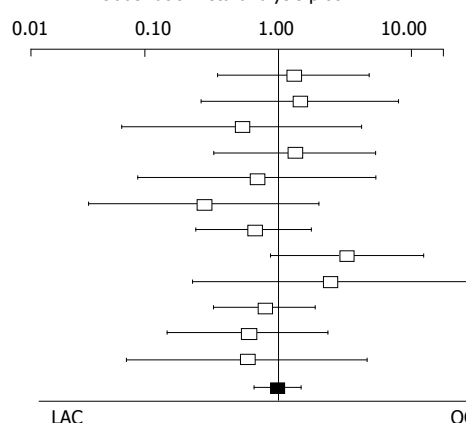


Figure 6 Forest plot of the odds ratio for anastomotic leakage. LAC: Laparoscopic surgery; OC: Open surgery.

= 6.6316, $P = 0.4682$). Publication bias was statistically evident (Egger = 1.5152, $P = 0.0521$) (Figure 8).

Recovery time of normal bowel function: Five studies reported the recovery time of normal bowel function as the mean value with standard deviation. There was no significant difference in the recovery time to normal bowel function between LAC and OC (mean difference = -0.8573, 95%CI: -1.8778 to 0.1632, $P = 0.0997$). Heterogeneity was statistically evident (Cochrane's $Q = 379.9427$, $P < 0.0001$). Publication bias was not evident (Egger = 5.4503, $P = 0.5226$).

Length of postoperative hospital stay: Three studies reported the length of postoperative hospital stay as mean value with standard deviation. There was no significant difference in the length of postoperative hospital stay between LAC and OC (mean difference = -1.3336, 95%CI: -3.3995 to 0.7322, $P = 0.2058$).

Heterogeneity was not evident (Cochrane's $Q = 3.9019$, $P = 0.1421$). Publication bias was statistically evident (Egger = -1.4308, $P = 0.0689$).

Long-term outcomes

Overall survival: Hinoi *et al.*^[32] reported overall survival in colon and rectal cancer separately. Three analyses of two studies reported the overall survival with 95%CI. There was no significant difference in the overall survival between LAC and OC (mean difference = 0.8321, 95%CI: 0.5331 to 1.2990, $P = 0.4187$). Heterogeneity and publication bias were not evident (Cochrane's $Q = 3.3977$, $P = 0.1829$, Egger = -1.9819, $P = 0.3846$) (Figure 9).

Disease specific survival: Hinoi *et al.*^[32] reported disease specific survival in colon and rectal cancer separately. Three analyses of two studies reported the disease specific survival with 95%CI. There was no

	LAC		OC		OR	95%CI	
	Events	<i>n</i>	Events	<i>n</i>		Lower	Upper
Sklow	3	36	4	35	0.73	0.15	3.50
Vignali	3	58	6	55	0.47	0.11	1.99
Tei	1	50	8	70	0.18	0.02	1.44
Robinson	5	42	37	158	0.51	0.19	1.37
She	12	177	12	233	1.32	0.58	3.00
Fujii	4	96	12	88	0.31	0.10	0.98
Hinoi	25	434	33	426	0.74	0.43	1.27
Valls	5	129	13	120	0.36	0.12	1.03
Zeng	2	110	3	179	1.08	0.18	6.60
Shigeta	3	49	8	47	0.36	0.09	1.44
Total					0.6248	0.4519	0.8638

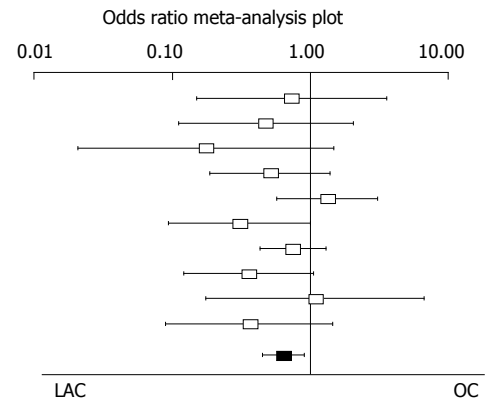
 $P = 0.0044$ Heterogeneity: Cochrane's $Q = 8.7612$, $P = 0.4596$ Publication bias: Egger = -1.1383, $P = 0.1602$ 

Figure 7 Forest plot of the odds ratio for bowel obstruction and ileus. LAC: Laparoscopic surgery; OC: Open surgery.

	LAC		OC		OR	95%CI	
	Events	<i>n</i>	Events	<i>n</i>		Lower	Upper
Sklow	1	38	4	35	0.23	0.02	2.16
Vignali	4	57	4	57	1.00	0.24	4.19
Robinson	4	43	40	155	0.36	0.12	1.06
She	7	182	28	217	0.30	0.13	0.70
Scarpa	2	31	1	23	1.48	0.13	17.37
Fujii	2	98	1	99	2.02	0.18	22.65
Hinoi	1	458	2	457	0.50	0.05	5.52
Miyasaka	1	27	1	78	2.89	0.17	47.80
Total					0.4767	0.2805	0.8101

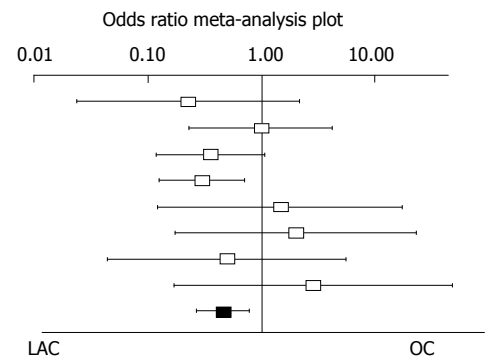
 $P = 0.0062$ Heterogeneity: Cochrane's $Q = 6.6316$, $P = 0.4682$ Publication bias: Egger = 1.5152, $P = 0.0521$ 

Figure 8 Forest plot of the odds ratio for cardiovascular complication. LAC: Laparoscopic surgery; OC: Open surgery.

	<i>n</i>	OR	Lower	Upper
Hinoi (Colon)	804	1.02	0.749	1.38
Hinoi (Rectum)	114	0.90	0.432	1.857
Shigeta	107	0.43	0.18	1.02
Total		0.8321	0.5331	1.2990

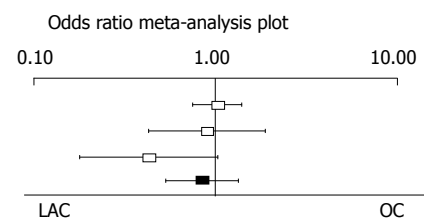
 $P = 0.4187$ Heterogeneity: Cochrane's $Q = 3.3977$, $P = 0.1829$ Publication bias: Egger = -1.9819, $P = 0.3846$ 

Figure 9 Forest plot of the odds ratio for overall survival. LAC: Laparoscopic surgery; OC: Open surgery.

significant difference in the disease specific survival between LAC and OC (mean difference = 1.0254, 95%CI: 0.6707 to 1.5675, $P = 0.9209$). Heterogeneity and publication bias were not evident (Cochrane's $Q = 0.1648$, $P = 0.9209$, Egger = -0.4921, $P = 0.1559$) (Figure 10).

Oncological clearance

The number of dissected lymph nodes: Two studies reported the number of dissected lymph nodes as the mean value with standard deviation. There was no significant difference in the number of dissected lymph nodes between LAC and OC (mean difference = -0.1360, 95%CI: -4.0553 to 3.7833, $P = 0.9458$). Heterogeneity

and publication bias were not evident (Cochrane's $Q = 3.2471$, $P = 0.0716$, Kendall tau rank correlation coefficient by Begg test = 1.0000, $P = 0.3173$).

Study quality

There was only one randomized controlled study^[19] which contained the following; random sequence generation, allocation concealment, blinding of participants, personnel and outcome assessors, incomplete outcome data, selective reporting and other potential threats to validity.

The study which had 5 or less star points on the NOS scale related to 5 short-term outcomes; amount of estimated blood loss, overall morbidity, incisional surgical site infection, anastomotic leakage and time of normal

	<i>n</i>	OR	Lower	Upper
Hinoi (Colon)	804	1.06	0.657	1.726
Hinoi (Rectum)	114	0.97	0.339	2.76
Shigeta	107	0.75	0.14	4.05
Total		1.0254	0.6707	1.5675

$P = 0.9209$

Heterogeneity: Cochrane's $Q = 0.1648$, $P = 0.9209$

Publication bias: Egger = -0.4921 , $P = 0.1559$

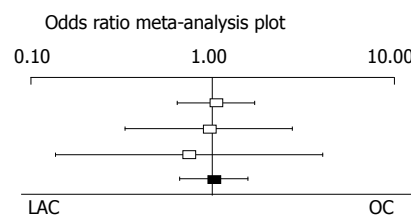


Figure 10 Forest plot of the odds ratio for disease specific survival. LAC: Laparoscopic surgery; OC: Open surgery.

bowel function. These outcomes were synthesized with studies which had 6 or more star points. Results were similar to the primary analyzed results and there was no conversion of interpretation.

DISCUSSION

Two systematic reviews that compare LAC with OC report benefits in short-term outcome. Grailey *et al.*^[11] report that LAC reduces the length of hospital stay, intraoperative blood loss, pneumonia, time to normal bowel function, cardiac complication and wound infection. Antoniou *et al.*^[12] report that LAC had a decreased risk for mortality, overall morbidity, plus cardiac and respiratory complications. Their results are similar to those reported in this review. However, they included analyses for both colorectal cancer and benign disease. Large scale, randomized studies and reviews that compare long-term results between LAC and OC in all generations report no difference in colon cancer patients^[37]. However, long-term results of randomized studies and reviews with elderly patient have not yet been reported. This meta-analysis, which compared LAC and OC in elderly colorectal cancer patients, demonstrates advantages in short-term and equivalency with respect to long-term outcomes and oncological clearance. These results will be useful in informing the selection of operative approach in elderly patients.

In analyses of the amount of estimated blood loss, overall morbidity, incisional surgical site infection and cardiovascular complication were all reduced in LAC. These results are similar to previous reports^[11,12]. It has been suggested that decreases in blood loss and postoperative pain reduce the stress of surgery, and therefore reduce overall morbidity. The reduction in cardiovascular complications might also be due to decrease in blood loss. Bowel obstruction and ileus was also reduced in LAC. Bowel obstruction and ileus were not distinguished in this analysis, because the definition was not clear in some studies and data was assigned to both conditions. This was not shown in previous reviews and it is supposed that the incidence of ileus increase is due to the extent of lymph node dissection in colorectal cancer. The exposure of intestines and major trauma to the abdominal wall might explain the increase in incidence of bowel paralysis and adhesion in OC.

The operative time of LAC was longer than OC. This

result was consistent with past reports, too. However, pneumonia was not increased and overall morbidity was decreased in LAC. Mean difference in operative time was about 34 min. The increase in operative time and pneumoperitoneum may not cause adverse effects on postoperative morbidity.

In this meta-analysis, there were no significant differences in mortality, incidence of pneumonia and recovery time of normal bowel function, which is not consistent with past reports. However, all LAC results tended to be lower than OC and p-values were close to being significantly different (mortality; OR = 0.5052, 95%CI: 0.2438- 1.0467, $P = 0.0662$, pneumonia; OR = 0.4526, 95%CI: 0.1976-1.0365, $P = 0.0608$, recovery time of normal bowel function; mean difference = -0.8573 , 95%CI: -1.8778 to 0.1632 , $P = 0.0997$). These inconsistent results may be due to the fact that patients who underwent elective colorectal surgery could be considered to be at relatively low risk. The reason for there being no significant difference in recovery time of normal bowel function is unknown. There might have been a significant difference if the time period of the data collection was a number of days not hours.

The incidences of deep surgical site infection and anastomotic leakage were similar between LAC and OC (deep surgical site infection; OR = 0.8234, 95%CI: 0.3298-2.0556, $P = 0.6771$, anastomotic leakage; OR = 0.9138, 95%CI: 0.5667-1.4735, $P = 0.7115$). It has been suggested that surgical invasiveness of the retroperitoneal dissection and anastomotic procedure are similar between LAC and OC in colorectal cancer surgery.

There was no significant difference in length of postoperative hospital stay.

This may be due to differences in the standard for hospital discharge in each study and may also be related to differences in the insurance systems in each country. Thus there might be a large bias in social factors between studies.

In analyses of long-term outcomes, both overall and disease specific survival rates were similar. There was also no significant difference in the number of dissected lymph nodes. This reveals the fact that LAC had similar treatment success to OC. The results of randomized studies and Cochrane review were also supported by this meta-analysis in elderly colorectal cancer surgery

patients^[13-18,37].

The limitation of this review is that it consists of only one randomized controlled study. Thus there were publication biases in analyses of overall morbidity, cardiovascular complication and length of postoperative hospital stay. Analysis of high risk elderly patients with impaired cardiac and pulmonary function might be required in the future. A secondary limitation of this study is that long-term outcomes were limited to three data sets from two studies. The analysis of more long-term results, that include details on the specific form of relapse, may thus be required.

LAC in elderly colorectal cancer patients had benefits in short-term outcomes such as amount of estimated blood loss, overall morbidity, incidences of incisional surgical site infection, bowel obstruction and ileus and cardiovascular complications. The only area where LAC did not show a benefit over OC was for operative time. The long-term outcomes and oncological clearance of LAC were similar to that of OC. These results support the view that LAC is an effective and safe procedure for elderly patients with colorectal cancer.

COMMENTS

Background

Laparoscopic surgery for colorectal cancer is increasing rapidly, particularly among elderly patients. However, neither the safety nor the effectiveness of laparoscopic surgery in this demographic has yet been determined.

Research frontiers

Some systematic reviews that compare laparoscopic colectomy (LAC) with open colectomy for elderly had reported benefits in short-term outcome.

Innovations and breakthroughs

However, past reports included benign diseases, and no report about long-term results. The authors analyzed for elderly colorectal cancer only and long-term outcomes.

Applications

Some short-term outcomes were superior in LAC except operation time. There was no significant difference in long-term outcomes. LAC is an effective procedure for elderly with colorectal cancer.

Terminology

LAC: Laparoscopic colectomy.

Peer-review

Good review article, scientific and rigorous analysis.

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REVIEW

- 583 Apoptotic pathways as a therapeutic target for colorectal cancer treatment

Abraha AM, Ketema EB

- 592 Regulation of CTNNB1 signaling in gastric cancer and stem cells

Tanabe S, Aoyagi K, Yokozaki H, Sasaki H

- 599 Road map for pain management in pancreatic cancer: A review

Lahoud MJ, Kourie HR, Antoun J, El Osta L, Ghosn M

ORIGINAL ARTICLE

Basic Study

- 607 Plasma chitinase 3-like 1 is persistently elevated during first month after minimally invasive colorectal cancer resection

Shantha Kumara HMC, Gaita D, Miyagaki H, Yan X, Hearth SAC, Njoh L, Cekic V, Whelan RL

- 615 Immunohistochemical analysis of the Wnt/ β -catenin signaling pathway in pancreatic neuroendocrine neoplasms

Weiss V, Dueber J, Wright JP, Cates J, Revetta F, Parikh AA, Merchant NB, Shi C

Observational Study

- 623 Activated systemic inflammatory response at diagnosis reduces lymph node count in colonic carcinoma

Kennelly RP, Murphy B, Larkin JO, Mehigan BJ, McCormick PH

- 629 Academic hospital staff compliance with a fecal immunochemical test-based colorectal cancer screening program

Vlachonikolou G, Gkolfakis P, Sioulas AD, Papanikolaou IS, Melissaritou A, Moustafa GA, Xanthopoulou E, Tsilimidos G, Tsironi I, Filippidis P, Malli C, Dimitriadis GD, Triantafyllou K

Randomized Clinical Trial

- 635 Utility of different serum fibrosis markers in diagnosing patients with chronic pancreatitis and pancreatic adenocarcinoma

Kozak A, Talar-Wojnarowska R, Kaczka A, Borkowska A, Czupryniak L, Malecka-Panas E, Gqiorowska A

Contents

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World Journal of Gastrointestinal Oncology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-85381891
Fax: +86-10-85381893
E-mail: editorialoffice@wjnet.com
Help Desk: <http://www.wjnet.com/esps/helpdesk.aspx>
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Baishideng Publishing Group Inc
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Fax: +1-925-223-8243
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Apoptotic pathways as a therapeutic target for colorectal cancer treatment

Aman M Abraha, Ezra B Ketema

Aman M Abraha, Ezra B Ketema, Department of Medical Biochemistry, College of Health Sciences, Mekelle University, Mekelle 1871, Ethiopia

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Correspondence to: Ezra B Ketema, MSc, Department of Medical Biochemistry, College of Health Sciences, Mekelle University, Mekelle 1871, Ethiopia. aga.bely@gmail.com
Telephone: +251-912-912586
Fax: +251-344-416681

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Abstract

Colorectal cancer is the second leading cause of death from cancer among adults. The disease begins as a benign adenomatous polyp, which develops into an advanced adenoma with high-grade dysplasia and then progresses

to an invasive cancer. Appropriate apoptotic signaling is fundamentally important to preserve a healthy balance between cell death and cell survival and in maintaining genome integrity. Evasion of apoptotic pathway has been established as a prominent hallmark of several cancers. During colorectal cancer development, the balance between the rates of cell growth and apoptosis that maintains intestinal epithelial cell homeostasis gets progressively disturbed. Evidences are increasingly available to support the hypothesis that failure of apoptosis may be an important factor in the evolution of colorectal cancer and its poor response to chemotherapy and radiation. The other reason for targeting apoptotic pathway in the treatment of cancer is based on the observation that this process is deregulated in cancer cells but not in normal cells. As a result, colorectal cancer therapies designed to stimulate apoptosis in target cells would play a critical role in controlling its development and progression. A better understanding of the apoptotic signaling pathways, and the mechanisms by which cancer cells evade apoptotic death might lead to effective therapeutic strategies to inhibit cancer cell proliferation with minimal toxicity and high responses to chemotherapy. In this review, we analyzed the current understanding and future promises of apoptotic pathways as a therapeutic target in colorectal cancer treatment.

Key words: Colorectal cancer; Apoptotic pathways; Drug resistance; Colorectal cancer therapies; Apoptosis

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Core tip: Evasion of apoptosis has been established as a prominent hallmark of several human cancers, contributing to both tumor progression and chemo-resistance. In colorectal cancer development, the balance between the rates of cell growth and apoptosis that maintains intestinal epithelial cell homeostasis is impaired progressively. Recent studies indicated that failure of apoptosis may be an important factor in the evolution of colorectal cancer and its poor response to chemotherapy

and radiation. We herein discussed the mechanisms of apoptosis, abnormal expression of apoptosis-related genes and future promises of apoptotic pathways as a therapeutic target for colorectal cancer chemoprevention and treatment.

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INTRODUCTION

Colorectal is the second driving reason for death from malignancy among adults^[1]. The diseases starts as a benign adenomatous polyp, which changes into a propelled adenoma with high-rate dysplasia that advances to aggressive tumor^[2]. The development of colorectal cancer (CRC) shows that tumor is a multistep procedure. The continuous process of cell division and differentiation of intestinal epithelium can be subverted by genetic alteration that switch the progenitor cells into tumor cells. This change is not prompt but occurs progressively through various stages as the cells get increasingly mutated, depending on the activation of oncogenes and the inactivation of tumor suppressors^[3]. The aim of this review was to summarize evidences on the role of apoptotic pathways in the initiation and progression of CRC and their potentials as a therapeutic target.

APOPTOSIS AND ITS ROLE IN CANCER

Apoptosis is an implicit cell suicide pathway that helps to remove cells that are no more required or have extreme injury to their DNA and cytoskeleton^[4]. Since defined by Kerr *et al.*^[5] in the 1970's, apoptosis remains amongst the most explored topics in biologic investigation. Being a very selective process, apoptosis is vital both in normal and pathological processes.

Mainly three fundamental sorts of biochemical changes are seen in apoptosis: (1) stimulation of caspases; (2) DNA and protein degradation; and (3) membrane alterations and detection by phagocytic cells. Specific morphological changes of apoptotic cells comprises nuclear compression and disintegration, cell contraction, active membrane blebbing, and loss of linkage to extracellular lattices or to neighboring cells. These alterations involve chromosomal DNA cleavage into internucleosomal rubbles, translocation of phosphatidylserine, and stimulation of proteases distinguished as the caspases^[6,7].

The unique characteristics of apoptosis is the activation various enzymes that are cysteine protease families called caspases^[7]. Activated caspases slice several key cellular proteins and degraded the nuclear

scaffold and the cytoskeleton. They additionally stimulate DNAase, which advances nuclear DNA degradation processes^[8].

MECHANISMS OF APOPTOSIS

Since they are both the initiators and destroyers, caspases play a key role in apoptotic processes. Caspases are initiated by three separate pathways: The intrinsic (or mitochondrial), the extrinsic (or death receptor) and the intrinsic endoplasmic reticulum pathways^[9]. The extrinsic pathway is initiated by triggering cell death receptors on the cell surface which leads to activation of the intracellular apoptotic machinery. The signals are originated from the activation of specific proapoptotic membrane receptors, a subclass of tumour necrosis factor (TNF) receptor family, through ligands, for example, FasL23/CD95L (receptor Fas/CD95) and Apo2 ligand TNF-linked apoptosis-initiating binding groups (Apo2L/TRAIL) (receptors DR4 and DR5)^[10]. These receptors have an internal components that mobilize core proteins including TNF receptor-associated death domain (TRADD) and fas-associated death domain (FADD)^[11]. Upon binding of the ligand to its receptor, the death-inducing signaling complex (DISC) that causes stimulation of caspase 8 formed^[9]. Thusly, caspase 8 activate the remaining downstream caspase enzymes. In some group of cells, the stimulation of caspase 8 could be the only prerequisite conditions to accomplish death, but in other cell types; caspase 8 cooperates with the intrinsic apoptotic processes by splitting Bid (a proapoptotic member of the Bcl-2 protein), causing the successive release of cytochrome-c^[12,13].

As the name suggests, the intrinsic pathway starts inside the cell. Intracellular stimuli such as irreversible genetic impairment, hypoxia, high calcium (Ca^{2+}) concentrations and elevated oxidative stress are amongst the triggers of intrinsic mitochondrial pathway. Irrespective of the inducers, this pathway caused an increase in mitochondrial porousness and the discharge of cytochrome-c and other proapoptotic proteins into the cytosol^[14]. This process is strictly controlled by Bcl-2 family proteins, termed after the *Bcl-2* gene originally detected at the chromosomal breakpoint of the translocation of chromosome 18 to 14 in follicular non-Hodgkin lymphoma^[15]. There are two classes of the Bcl-2 proteins: The pro-apoptotic proteins (such as Bax, Bakand, Bad, Bcl-Xs, Bid, Bik, Bimand Hrk) and the anti-apoptotic proteins (such as Bcl-2, Bcl-XL, Bcl-W, Bfl-1 Mcl-1). While anti-apoptotic proteins control apoptosis by delaying the mitochondrial release of cytochrome-c, the pro-apoptotic proteins accomplish by stimulating such releases. It is not the total amount, but rather the equilibrium between the pro- and anti-apoptotic proteins that regulate whether apoptosis should be started or not^[16]. Additional apoptotic factors that are released from the mitochondria into the cytosol contains apoptosis-inducing factor (AIF), second mitochondria-derived activator of caspase (SMAC), inhibitor of apoptosis

proteins (IAP), binding protein with low pI (DIABLO) and Omi/high-temperature requirement protein A (HtrA2). The cytoplasmic release of cytochrome c stimulates caspase 3 through the formation of a complex called apoptosome made up of cytochrome c, apoptotic protease activating factor 1 (APAF-1) and caspase 9. Active caspase-9 then activates caspase-3, which continually triggers the remaining of the caspase cascade and resulted in apoptosis^[17]. On the other hand, SMAC/DIABLO or Omi/HtrA2 stimulates caspase activation by binding to inhibitor of apoptosis proteins (IAPs) which then leads to disturbance in the interaction of IAPs with caspase 3 or 9^[16]. The intrinsic and extrinsic apoptotic pathways come together at caspase-3, which splits the inhibitor of the caspase-activated deoxyribonuclease, and the caspase-activated deoxyribonuclease causes nuclear apoptosis. The triggered enzymes cleave regulatory and structural molecules, ending in the death of the cell^[9]. Caspases also disturb the cytoskeletal structure, cell cycle and signaling process, eventually leading to the morphological indicators of apoptosis, for instance, DNA condensation and disintegration, and membrane blebbing^[4]. In early stage of apoptosis, there is expression of phosphatidylserine (PS) in cell membrane, which is overturned from the inside. This lets prompt detection of deceased cells by macrophages, causing phagocytosis without the release of pro-inflammatory mediators^[18].

The equilibrium between pro- and anti-apoptotic pathways comprises a vital role in the control of cell death, and disturbances in the balance between proteins have been associated in increased carcinogenesis by reducing apoptosis in tumor cells^[19,20].

ABNORMAL EXPRESSIONS OF GENES CONTROLLING APOPTOSIS IN CRC

CRC development is defined by the consecutive accumulation of mutations in genes controlling epithelial cell growth and differentiation^[21]. Genomic instability (gene mutations) and epigenetic alterations are the major mechanisms for CRC development^[22]. Approximately 60%-80% of CRC display genomic instability^[23]. In CRC, genomic instability takes several forms each with a different cause including mutations in specific genes that control mitosis, sequential inactivation of tumor-suppressor genes, such as adenomatous polyposis coli (APC) (chromosome 5q), P53 (chromosome 17p), and deleted in CRC (DCC), mothers against decapentaplegic homolog 2 (SMAD2), and SMAD4 (chromosome 18q) through numerous changes in chromosomal copy number and structure^[21]. The loss of genomic stability can initiate the evolution of CRC by enabling the acquisition of different cancer-related mutations^[24]. The building up of genomic modifications may bring changes in the level of apoptotic cell death, and many of these genes have been found to control apoptosis. Tumor-suppressor genes and oncogenes genes commonly associated with apoptosis

and CRC development are the tumor suppressor genes: Adenomatous polyposis coli (APC), P53 and the proto-oncogene Bcl-2.

ADENOMATOUS POLYPOSIS COLI

Changes in the adenomatous polyposis coli (APC) gene have been linked to about 60% of colorectal neoplasia signifying that APC mutations may be a central event in the development of colorectal carcinogenesis^[25-27]. APC protein is synthesized in normal epithelial cells as they moved towards the uppermost part of the crypt. Interruption of normal APC function distracts the equilibrium between new cell development at the bottom of the crypts and cell death at the top of the crypts, promoting the expansion of the descendants of APC-mutant cells^[28,29].

The absence of functional APC leads to an inappropriately and constitutively activation of the Wnt signaling pathway, thereby promoting colorectal tumor initiation^[30,31]. Stimulation of the Wnt pathway overwhelms the phosphorylation of the onco-protein β -catenin, causing its stabilization and nuclear translocation. β -catenin then cooperates with T-cell factor/lymphocyte enhancer factor (TCF/LEF) to induce expression of Wnt target pro-proliferative and anti-apoptotic genes. In normal cells, where Wnt signaling is controlled, cytoplasmic β -catenin is phosphorylated by the glycogen synthase kinase 3 β (GSK-3 β) within a complex comprising APC and Axin, leading to the degradation of β -catenin via the ubiquitin proteasome pathway. The complex also activates transcription of TCF target genes such as c-myc, cyclin-D1, and peroxisome proliferator-activated receptor delta which are known to influence cell proliferation and apoptosis^[32-34]. APC, as constituent of this multifaceted complex, not only contributes to the β -catenin degradation but also hinders its nuclear positions, thereby abrogating transcription of Wnt target genes^[35].

P53 GENE

The tumor suppressor gene, P53 functions to assimilate a diversity of cellular strains into an array of reactions that include apoptosis. Its product attaches to particular sequences in DNA and control transcription of various pro-apoptotic genes such as Bax and the BH3 only proteins puma and noxa^[36]. These genes and proteins inactivate anti apoptotic proteins Bcl-2 and Bcl-xL and cause the release of cytochrome c from mitochondria. P53 also promotes the synthesis of apoptosis effector proteins such as APAF-1 and caspase 6^[37]. Moreover, P53 has main components of the extrinsic apoptosis pathway such as the death receptor Fas and DR5 as well as the BH3 only protein Bid that couples the extrinsic pathway to the intrinsic pathway^[38]. Although not adequately investigated, P53 repress the key inhibitor of apoptosis proteins (IAP) gene, which may stop caspase activity. P53 also blocks survival pathways including the PI3 kinase/AKT survival pathway that neutralize

apoptosis by promoting transcription of the PI_3 kinase inhibitor, phosphatase and tensin homolog (PTEN) and in doing so prevents inhibition of P53 by mouse double minute 2 homolog (MDM2)^[39]. Furthermore, P53 can pledge cell cycle detention, DNA healing, and senescence, besides apoptosis. The P53 protein may possibly also react to DNA injury by activating either growth seizure at G1 or G2 phase of the cell cycle, or automated cell decease. By similar fashion, P53 defend the cells from scheduled to duplicate injured DNA^[37]. Thus, deletion of P53 gene through the CIN pathway is undutiful to cause the transition of adenoma to carcinoma in CRC^[24]. It has also been documented that P53 efficiently affects reactions to chemotherapeutic medications utilized as a part of CRC treatment^[40].

Reports showed that P53 of gene deletions and mutations has been noticed in up to 85% of colorectal tumors and typically occurs throughout the transition from adenoma to adenocarcinoma^[41]. Cells with mal-functional P53 are tolerant to chromosomal instability coming from telomere restriction and have an influential selection benefit. The adenoma/carcinoma shift of CRC is amongst occasions where failure of apoptosis is pivotal in the progression of malignant clones. In contrast, few immune-histochemical studies did not back the main role of mutant P53 protein as an inhibitor of apoptosis in CRC progression^[42].

BCL-2 PROTEINS

As mentioned above, the intrinsic pathway is tightly controlled by Bcl-2 family proteins^[43]. Bcl-2 protein is regularly expressed only in the bottom half of the crypts of the colon, consistent to the stem cell compartment, where Bcl-2 is supposed to defend stem cells from apoptosis^[44]. Most colonic adenomas express Bcl-2 protein at abnormal states all through the neoplastic epithelium while non-neoplastic polyps have regular Bcl-2 expression. In this manner, overexpression of Bcl-2 may contribute to the switch between hyperplastic epithelium and adenomas. Bcl-2 protein expression in colorectal carcinomas is greater than in ordinary mucosa but lesser than in adenomas^[45-47]. On the other hand, tumor necrosis factor (TNF)- α could promote the expression of nuclear factor (NF)- κ B and triggers anti-apoptotic Bcl-2 proteins^[4]. High expression of Bcl-2 leads to resistance to chemotherapeutic drugs and radiation therapy, whereas diminished Bcl-2 expression promotes apoptotic reactions to chemotherapeutic drugs. Furthermore, overexpression of Bcl-2 could lead to gathering of cells in the G0 phase of cell cycle and subsidizing to chemoresistance^[48]. Additionally, overproduction of Bcl-2 in the intrinsic pathway may perhaps inhibit extrinsic mediated apoptosis. Subsequently, the *Bcl-2* gene could be one of the genes that regulate the occurrence of apoptotic cell death in colorectal neoplasms. Certainly, alterations in the expression of other members of the Bcl-2 proteins has been revealed during evolution of colorectal tumors, such as the anti-apoptotic proteins Bcl-XL, mcl-1 and the

pro-apoptotic protein Bak, that may be more important than Bcl-2^[49].

TARGETING APOPTOSIS PATHWAYS IN CRC TREATMENT

Proper apoptotic signaling is basically central to conserve a healthy balance between cell death and survival and in keeping genome stability^[50]. As a rule, it is thought that the equilibrium between the rates of cell growth and apoptosis sustains intestinal epithelial cell homeostasis, and this stability gets disturbed during cancer expansion^[51,52]. In accordance with this, there is an expanding proof to support the speculation that failure of apoptosis might be an essential component in the expansion of colorectal tumor and its poor response to chemotherapy and radiation.

Interruption of apoptosis causes an irregularity in typical tissue homeostasis by promoting cell development. It additionally permits the survival of hereditarily modified cells, adding to both tumor growth and progression^[53]. Thus, apoptotic pathways can be modified by tumor cells transcriptionally, translationally, and post-translationally. Altogether, these mechanisms are not limited and tumor cells can utilization various strategies to escape apoptosis^[54]. The other explanation behind focusing on apoptosis in the treatment of malignancy depends on the perception that this procedure is dysregulated in tumor cells yet not in ordinary cells^[4]. Tumor cells have acquired blocks to apoptosis but are continually driven to recruit it by genomic and other aberrations. Accordingly, if proapoptotic pathways could be invigorated, these cells would be more helpless to death than ordinary cells^[55].

Researchers are actively searching on a targets in every single defect in the apoptotic pathways to re-establish the apoptotic signaling pathways and eradicate tumor cells^[56]. Study of apoptosis has hinted the basis for novel targeted therapies that can bring death in malignant cells or prepare them to proven cytotoxic agents and radiation therapy^[55]. Consequently, the activation of apoptosis is emerging as a key approach to treat colorectal tumor^[57] and substantial effort has been devoted in isolating pro-apoptotic and apoptotic targets comprising the extrinsic and/or intrinsic pathways with therapeutic potential^[58]. Directed stimulation of the extrinsic pathway by pro-apoptotic receptors, inactivation of Bcl-2 proteins, caspase modulation, and IAP inhibition are amongst these methods.

EXTRINSIC PATHWAY AS A THERAPEUTIC TARGET

Several members of the extrinsic apoptotic pathway hold potential biomarkers and therapeutic targets in colorectal malignancy. The death receptor Fas (CD95), tumor necrosis family receptor (TNFR), and its ligand are assumed to be participated in colorectal tumor progression. Aberrations in these death-signaling

pathways are described in the circumvention of the extrinsic apoptotic pathway. This could be as a result of FasL overexpression in colon cancer cells, that enables these cells to escape cell death by the immune reactions^[59]. The pro-apoptotic Fas ligand possesses strong cytotoxic activity towards various types of tumors cells; despite, hepatotoxic side effects limit its usage. In contrast, activation of the pro-apoptotic receptors DR4 and DR5 by enhanced soluble recombinant human Apo2L/TRAIL (rhApo2L/TRAIL) signifies a better approach, providing effective antitumor activity but marginal cytotoxicity in healthy cells^[55]. Besides, tumor cells that are not amenable to apoptosis induced through FasL are sensitive to Apo2L/TRAIL^[60]. These directed therapies play a key role in cancer therapy by increasing the extent of apoptotic cell death through stimulation of TRAIL receptor. From the candidates, recombinant TRAIL and monoclonal agonist antibodies directed against the TRAIL receptors are highly promising targets^[61]. There are limited studies investigating the role of TRAIL and its receptors DR4 and DR5 as colon cancer biomarkers. In contrast to colorectal tumors and matched normal mucosa, reports indicated that tumor expression of TRAIL is often lesser in normal mucosa^[62,63]. The results of preliminary studies verified the ability of Apo2L/TRAIL to act synergistically with common chemotherapies in a wide variety of malignancies and are especially convincing^[64]. This interaction may be ascribed to the additional activation of the caspase cascade by the extrinsic and intrinsic pathways^[65]. Besides, Apo2L/TRAIL has been found to overcome tumor cell confrontation to chemotherapy in some experimental models^[66] and to work together with chemotherapy even in cells lacking P53. Based on these facts, numerous pro-apoptotic receptor agonists (PARAs) are in clinical trial^[67]. Since the extrinsic pathway activates apoptosis independently, treatments that initiate this mechanism have also the potential to induce cell death in cancers with a variety of responses to common drugs^[57].

INTRINSIC MITOCHONDRIAL PATHWAY AND BCL-2 FAMILY PROTEINS AS A THERAPEUTIC TARGETS

Common cancer treatments seem to trigger apoptosis mainly by the intrinsic pathway, and failure of this pathway could contribute to the development of drug resistance^[68]. So far, approaches targeting the intrinsic apoptosis pathway were focusing on Bcl-2 family proteins^[69]. Bcl-2 was initially qualified as a compelling anticancer target in studies that revealed its capacity to cooperate with the *myc* oncogene in driving B cell lymphomas in mice^[55]. The disequilibrium between pro- and anti-apoptotic Bcl-2 proteins can promote cancer cell survival^[20]. This can be due to an under-expression or over-expression of the pro-apoptotic proteins and anti-apoptotic proteins, respectively, or a combination of both^[70,71]. About 30%-94% of human CRC has been

found to over-express Bcl-2^[72]. Overexpression of anti-apoptotic Bcl-2 family proteins is often correlated with aggressive cancer, recurrence, poor prognosis, and chemo-resistance to cancer therapeutics^[73]. In addition, Bcl-2 and Bax are inversely correlated in colon cancer and over-expression of Bcl-2 in primary CRC specimens is a negative prognostic factor^[72]. Accordingly, these proteins are labeled as exceedingly promising therapeutic targets to design pharmacological manipulation of cell death by inactivating anti-apoptotic Bcl-2 family proteins^[20]. Recent studies found that inactivation of Bcl-2 proteins through small interfering RNAs (siRNAs) caused induction of cell apoptosis and then a decrease in tumor growth. Consequently, several microRNAs such as miR-195, miR-24-2, and miR-365-2 have been identified. These miRNAs regulate Bcl-2 expression, act as negative regulators of Bcl-2 *via* direct binding to the 3'-UTR of the *Bcl-2* gene, showing their therapeutic potentials^[20,74].

An additional approach to improve the response of apoptotic stimuli could be stimulation of pro-apoptotic protein expression. Drugs that simulate the action of the BH3 domain by binding to Bcl-2 like proteins and triggering apoptosis are under investigation^[75]. Both genetic and epigenetic alterations of several pro-apoptotic members of the Bcl-2 family have been described^[20]. Under the physiological condition, the pro-apoptotic and anti-apoptotic members of the Bcl-2 family can cooperate to maintain a dynamic balance. Small molecule inhibitors of Bcl-2 such as HA14-1 or Bcl-2 antisense Oblimersen have been tested in experimental therapy for CRC. Preliminary data suggest that Oblimersen alone only had limited therapeutic efficacy, but this agent can significantly sensitize cancer cells to other therapeutic agents induced apoptosis^[72].

TARGETING P53

Inhibition of P53 is one of common mutations, arising in more than half of tumors, and gives a key resistance machinery that supports these tumor cells to escape apoptosis initiation in response to various injuries caused by common therapies^[68]. P53 serves as a transcription factor controlling downstream genes that have role in stopping cell cycle, DNA reparation, and apoptosis. The main role that P53 plays is shown by the numerous tumors that have mutation in this gene. Impairment of *P53* gene in several tumors resulted in genomic instability, impaired cell cycle control, and inhibition of apoptosis. When DNA damages, P53 keeps the cell at a checkpoint until the damage is reversed. If the damage is permanent, apoptosis is activated^[76]. One of the most crucial roles of P53 is its capacity to activate apoptosis by both transcription-dependent and transcription-independent means. The disturbance of this process can cause tumor progression and chemo-resistance^[20,77].

There are various methods that target P53 to activate apoptosis, such as targeting P53 family proteins, inactivation of P53-MDM2 contact, returning mutated

P53 back to their normal function, removing mutant P53, producing P53-based vaccines, and gene therapy to repair P53 mutation^[20,78]. The role of P53 is adversely controlled by onco-proteins such as MDM2 (known as HDM2 in humans) and MDMX by an interaction with the P53 transactivation domain (P53TAD)^[20,79]. Hence, one of the key targets for cancer therapy is inactivation of the P53TAD-MDM2/MDMX contact by the small molecule MDM2 antagonist that maintain P53 by avoiding its interaction with MDM2 and selectively brings senescence in tumor cells. Some of these small-molecule MDM2 inhibitors, for example R7112 (Nutlin-3, analogs of cis-imidazoline) and JNJ-26854165 (a tryptamine derivative) are now under clinical investigations^[20,80,81].

TARGETING INTRACELLULAR CASPASE INHIBITORS

Since caspases are important initiator and executor of apoptosis, it seems reasonable to consider that impairment in caspase activity could reduce apoptosis and lead to carcinogenesis. Down-regulation of different caspases has been observed in different cancers^[20,82]. Strategies targeting caspase includes modification of intracellular caspase inhibitors, for example, the IAPs and FLICE (FADD-like ICE) inhibitory protein or c-FLIP which are intracellular inhibitors of extrinsic apoptosis signaling. In recent times, compounds that reduce the expression of c-FLIP and sensitize cells to Apo2L/TRAIL-induced apoptosis have been identified^[83].

Overexpression of IAPs has been associated with resistance of cancers to apoptosis^[84]. One of the encouraging anticancer approach comprises the use of small molecules that bind IAPs and stop their inhibitory effect on caspases. On the other hand, small molecule caspase activators that possessed arginine-glycine-aspartate motif decrease the activation threshold of caspase. This initiates auto-activation of procaspase-3, and finally, sensitizes cancer cells to chemotherapeutic agents^[20,85]. Several drugs have been designed to activate caspases. For example, apoptin is a caspase activator agent which employs a tumor-preferential apoptotic action and specifically initiate apoptosis in cancer but not healthy cells^[86]. Polyphenylurea XIAP chelators have been found to overcome the inhibitory effects of XIAP on caspase 3 and 7, promoting apoptosis in wide variety of malignant cells with minimal toxicity to ordinary cells^[11]. In addition, they sensitized tumor cells to apoptosis initiated through cancer drugs or *via* Apo2L/TRAIL. Alternatively, small molecule drugs simulate the effect of Smac by attaching to IAPs and stop their action. Particularly, Smac mimetics can synergize with Apo2L/TRAIL to initiate increased levels of caspase activation and apoptosis^[87].

An additional possibility to deplete IAP expression is through RNA interference (RNAi), which consists of producing and transferring small interfering RNA (siRNA) into tumor cells to avoid the overexpression of IAPs or similar other molecules. Treatment with XIAP siRNA

along with conventional chemotherapy can powerfully reduce XIAP synthesis and initiate cellular apoptosis^[38]. On the other hand, healthy cells are less dependent on IAPs, thus providing an significant advantage for these novel agents^[88].

CONCLUSION

Although non-steroidal anti-inflammatory drugs (NSAIDs) are currently the most widely used agents for chemo-prevention in CRC, the significant toxicity associated with long-term NSAID use, such as gastrointestinal bleeding, limits the complete advantage of NSAID-treatment in high-risk patients^[89]. This obliges the design of nontoxic agents that could initiate apoptosis with minimal toxicity and reduce the opportunity for acquired drug resistance. CRC treatments developed to stimulate apoptosis might play a critical effect in limiting the development and progression of CRC^[90]. As a result, a better understanding of the apoptotic signaling pathways and the instruments by which colorectal tumor cells escape apoptotic death could lead to potent treatment tactics to limit cancer cell growth. Currently, direct activation of the extrinsic pathway by pro-apoptotic receptors, inactivation of Bcl-2 proteins, caspase modification, and IAP inhibition are amongst the promising candidates. Identification of further chemotherapeutic targets that explicitly initiate apoptosis could provide new approaches to prevent colonic tumors. In the future, strategies that use apoptotic pathways will create new effective remedies with minimal toxicity.

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Regulation of CTNNB1 signaling in gastric cancer and stem cells

Shihori Tanabe, Kazuhiko Aoyagi, Hiroshi Yokozaki, Hiroki Sasaki

Shihori Tanabe, Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Science, Tokyo 158-8501, Japan

Kazuhiko Aoyagi, Hiroki Sasaki, Department of Translational Oncology, National Cancer Center Research Institute, Tokyo 104-0045, Japan

Hiroshi Yokozaki, Department of Pathology, Kobe University of Graduate School of Medicine, Kobe 650-0017, Japan

Author contributions: Tanabe S wrote the paper; Aoyagi K, Yokozaki H and Sasaki H contributed critical revisions of the manuscript for important intellectual content.

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Correspondence to: Shihori Tanabe, PhD, Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Science, 1-18-1, Kami-yoga, Setagaya-ku, Tokyo 158-8501, Japan. stanabe@nihs.go.jp
Telephone: +81-3-37001141
Fax: +81-3-37076950

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Abstract

Recent research has shown that the alteration of combinations in gene expression contributes to cellular phenotypic changes. Previously, it has been demonstrated that the combination of cadherin 1 and cadherin 2 expression can identify the diffuse-type and intestinal-type gastric cancers. Although the diffuse-type gastric cancer has been resistant to treatment, the precise mechanism and phenotypic involvement has not been revealed. It may be possible that stem cells transform into gastric cancer cells, possibly through the involvement of a molecule alteration and signaling mechanism. In this review article, we focus on the role of catenin beta 1 (CTNNB1 or β -catenin) and describe the regulation of CTNNB1 signaling in gastric cancer and stem cells.

Key words: CTNNB1 signaling; β -catenin; Epithelial-mesenchymal transition; Gastric cancer; Stem cell

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Core tip: CTNNB1 signaling is essential for revealing cancer mechanisms. The molecular dynamism in stem cells and cancer is illustrated with a pathway cascade. The CTNNB1 protein interacts with signaling molecules upon stimulation, leading to the transcription of genes related to cell proliferation. Mutations of signaling molecules are also important factors for cancer development. CTNNB1 signaling in stem cells and cancer are mainly described in the article.

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INTRODUCTION

Transformed cells have dynamic molecular alterations, which can be identified *via* gene profiling^[1]. Essential genes can be identified using advancing human genomic techniques, such as the clustered regularly interspaced short palindromic repeats (CRISPR) gene editing system^[2]. In gastrointestinal cancers, various molecules, including catenin beta 1 (CTNNB1 or β -catenin), have important roles in phenotypic transitions^[3-6]. Mutations in β -catenin or adenomatous polyposis coli (APC) induce β -catenin/T cell transcription factor (TCF) signaling in colon cancer^[7,8]. Meanwhile, Wnt/ β -catenin signaling has a role in stem cell signaling^[3,9]. The inhibition of glycogen synthase kinase 3 beta (GSK3 β) promotes v-myc avian myelocytomatosis viral oncogene homolog (c-Myc or MYC) and β -catenin activity toward endoderm identification *via* forkhead box A2 (FoxA2) expression^[9]. In cancer stem cells (CSCs), β -catenin signaling, which is downstream of the CSC marker prominin 1 (CD133 or PROM1), is required for CSC maintenance^[10]. Although CD133-induced β -catenin signaling activation is cancer cell-type specific, β -catenin binds to the proximal promoter regions of integrin subunit beta 6 (*ITGB6*) and *ITGB8* in gastric cancer cell lines^[10]. This β -catenin signaling may be regulated by specific target genes^[10].

Genomic rearrangements in the telomerase reverse transcriptase gene (*TERT*) and the up-regulation of *TERT* are important factors in high-risk neuroblastoma^[11]. The detection of causative mutations in cancers has been facilitated since the completion of the Human Genome Project^[12,13]. Because several individual mutations in gastrointestinal cancers have been identified as being targeted by tumor infiltrating lymphocytes, the mutations in cancer signaling cascades should be analyzed in the context of possible cancer immunotherapy^[14]. Signaling molecules, including β -catenin and signal direction switch caused by misregulation of expression, are the main focus of this article.

CTNNB1 AND WNT SIGNALING

The canonical Wnt signaling pathway includes Wnt-Frizzled, dishevelled (DVL), Axin, GSK3 inactivation, and β -catenin dephosphorylation, stabilization and translocation into the nucleus^[3]. The translocated β -catenin together with TCF transcriptionally regulates Wnt target genes, whereas the disruption of Wnt signaling caused by mutations in pathway genes can cause cancer^[3]. The interaction of β -catenin and E-cadherin may be involved in cell-cell communication and signal transduction^[15]. The disruption of VE-cadherin localization is involved in β -catenin phosphorylation and signaling *via* microparticles that are important for cell-cell communication in endothelial cells. However, this β -catenin activation is independent of Wnt/Frizzled^[16]. Tubeimoside-1, which has anti-tumor properties, has been shown to inhibit the growth and invasion of colorectal cancer cells through inhibiting the Wnt/ β -catenin signaling pathway^[17]. The Wnt/ β -catenin

pathway is essential to the epithelial-mesenchymal transition (EMT) in breast cancer cells over-expressing C-X-C motif chemokine ligand 12 (CXCL12, or stromal cell-derived factor-1; SDF-1)^[18]. The E6 region of high-risk human papillomavirus (HPV)-16, one of the possible causes of esophageal cancer, induces cell growth of esophageal cancer through activation of the Wnt/ β -catenin signaling pathway and downregulation of miR-125b^[19]. GSK3-mediated β -catenin phosphorylation is a key event in Wnt/ β -catenin signaling^[20]. GSK3 associates with AXIN to phosphorylate and regulate β -catenin^[20]. These studies indicate the importance of Wnt/ β -catenin signaling in tumorigenesis and EMT.

CTNNB1 SIGNALING IN GASTRIC CANCER

TERT activates Wnt/ β -catenin signaling and promotes MYC expression^[21]. The expression of TERT in gastric cancer is correlated with advanced TNM stages and lymphatic metastasis, which suggests TERT may be a therapeutic target for GC patients^[21]. GC invasion and metastasis are associated with molecular mechanisms for TERT^[21]. MYC expression is regulated by TERT *via* the Wnt/ β -catenin pathway^[21]. Dishevelled-Axin domain containing 1 (DIXDC1), a positive regulator of the Wnt pathway, is a significant prognostic indicator of intestinal-type gastric carcinoma^[22]. DIXDC1 contains a DIX domain that is involved in the formation of a complex along with Axin, Dvl, APC, GSK3 β , and β -catenin^[22,23]. It has been shown that GSK3 β -dependent phosphorylation of β -catenin is inhibited in the presence of Axin^[23]. Axin regulates Wnt signaling as scaffold for the APC-glycogen synthase kinase-3 β - β -catenin complex to down-regulate β -catenin, and Axin mutations in the DIX domain abolish JNK activity, whereas β -catenin signaling is not affected by Axin mutations^[24]. MicroRNA-1225-5p (miR-1225-5p) has been reported to function as a tumor suppressor for gastric carcinoma, acting through inhibition of the insulin receptor substrate-1 (IRS1) and β -catenin signaling pathways to suppress gastric carcinoma proliferation and metastasis^[25].

CTNNB1 SIGNALING IN STEM CELLS

Wnt/ β -catenin signaling plays an important role in stem cell maintenance. The self-renewing mesenchymal cells with stem cell characteristics inhabit a niche for maintaining their stemness^[26]. By modifying β -catenin in mouse osteoblasts, acute myeloid leukemias with common chromosomal alterations occur, and Notch signaling increases in hematopoietic cells^[26,27]. The stem cell niche may be regulated by Wnt signaling and the nuclear accumulation of β -catenin^[27]. Upon activation of β -catenin, the Notch ligand jagged 1 is up-regulated in osteoblasts, which leads to the activation of Notch signaling in hematopoietic stem cell progenitors, moving them towards malignant transformation^[27].

In glioma stem cells, interleukin 17 receptor (IL-17R)

expression is involved in self-renewal^[28]. IL-17 up-regulates the expression of stemness/mesenchymal markers, such as fibronectin, CD44 and SOX2, in glioma stem cells^[28]. IL-17 regulates signal transducer and activator of transcription 3 (STAT3), nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B), GSK3 β and β -catenin in glioma stem cells^[28].

In CXCL12 (or SDF-1)-overexpressed breast cancer cells, the Wnt/ β -catenin pathway is required for the EMT, which induces cancer stem cell-like phenotype formation toward proliferation and metastasis in breast cancer cells^[18,29]. Breast tumorigenesis can be suppressed by inhibition of β -catenin/LEF-1 signaling^[30]. A synthesized peptide (TAT-NLS-BLBD-6) inhibits the nuclear interaction of β -catenin and LEF-1 in human breast cancer cells, suppressing Wnt/ β -catenin signaling and resulting in inhibition of tumorigenesis^[30]. Considering that TAT-NLS-BLBD-6 inhibits β -catenin/LEF-1 downstream target genes, including *CDKN2A*, *CLDN1*, *ID6* and *SOX2*, Wnt/ β -catenin signaling is likely to promote oncogenesis *via* LEF-1-targeted gene expression^[30].

The Notch and Wnt/ β -catenin signaling pathways play important roles in maintaining and promoting liver cancer stem cells^[31]. Liver cancer stem cells expressing stemness markers, such as CD90, CD24, CD13 and CD133, with poor prognosis in patients are maintained by Notch and Wnt/ β -catenin signaling^[31]. Upon niche formation, WNT-SHH signaling modulates stem cell fates^[32]. While canonical Wnt signaling mediated by β -catenin and LEF-1 is essential for placode formation, the combination of SHH and Wnt signals may be crucial for stem cell niche formation^[32].

Interleukin-22 (IL-22) induces epithelial regeneration through intestinal stem cells (ISCs), whose niche provides Wnt, Notch and epidermal growth factor signals for normal epithelial maintenance^[33]. Wnt/ β -catenin signaling maintains these ISCs, whereas the IL-22 pathway may be involved in STAT3 signaling and cross-linked^[33].

CTNNB1 AND THE EPITHELIAL-MESENCHYMAL TRANSITION

SNAI1 and CTNNB1 pathway in EMT

The loss of E-cadherin and the transformation of cells to the mesenchymal phenotype are involved in Smad signaling and the formation of β -catenin/LEF-1 complexes^[34]. Transforming growth factor β 1 (TGF β 1) promotes the EMT *via* the Smad-independent Ras-Raf-MEK-ERK-AP-1 signaling pathway, which up-regulates the expression of the snail family zinc finger 1 (*SNAI1*) gene^[34]. IL-8 plays a role in the maintenance of the tumor EMT through both autocrine and paracrine pathways^[35]. The IL-8 pathway transduces the Ras-ERK and PI3K-AKT signals to induce IL-8 transcription through Snail and Twist, which activate the autocrine IL-8 pathway^[35]. Moreover, IL-8 promotes E-cadherin transcription through Brachyury^[35]. The paracrine IL-8 pathway consists of the recruitment of tumor-associated macrophages and neutrophils into tumor sites to promote EMT using

cytokines^[35].

The expression of EMT regulator SNAI1 is correlated with an increased risk of tumor relapse in breast cancer patients and the progression of colorectal cancer^[36]. E-cadherin loss promotes the expression of EMT regulators, including β -catenin and NF- κ B, which suggests that the pathway for SNAI1 and β -catenin may be crosslinked^[36-39]. The expression of cadherin 1 (CDH1 or E-cadherin) is a marker of tumor aggressiveness in routinely processed radical prostatectomy specimens^[40]. The disease progression in patients with high-stage category cancers can be predicted with the expression of CDH1^[40]. Decreased CDH1 may release β -catenin from the β -catenin complex, allowing it to translocate into the nucleus and activate transcription of target genes, such as MYC^[40,41]. The cancer metastatic process is mediated *via* circulating tumor cells expressing EMT markers such as *ETV5*, *NOTCH1*, *SNAI1*, *TGFB1*, *ZEB1* and *ZEB2*^[42]. Considering that the expression of CTNNB1 is up-regulated in endometrial circulating tumor cells in endometrial cancer, CTNNB1 is a potential therapeutic target for endometrial cancer^[42]. SNAI1, SNAI2, and SNAI3 expression in the ductal epithelium is up-regulated during development, and SNAI1 and SNAI2 are co-expressed with insulin^[43]. CDH1 expression decreases during the EMT process in β -cell differentiation into islets. The expression of β -catenin is altered in process of β -cell clustering formation in islets^[43].

TGF β and CTNNB1 signaling in EMT

The TGF β -induced EMT is regulated by phosphatase and tensin homologue deleted from chromosome 10 (PTEN), a tumor suppressor gene in lung cancer cells^[44]. Upon stimulation with TGF β , β -catenin translocates into nucleus. This activity is inhibited by the deletion of the phosphatase and C2 domains of unphosphorylated PTEN^[44]. The expression of E-cadherin is down-regulated with TGF β , which is inhibited by the phosphatase and C2 domains of unphosphorylated PTEN^[44]. The isoflavone calycosin-7-O- β -D-glucopyranoside induces osteogenic differentiation through the BMP and WNT/ β -catenin-signaling pathways^[45]. Osteogenic differentiation is regulated by TGF β signaling, which suggests some coordination of the β -catenin and TGF β signaling pathways^[45]. Jumoni domain-containing protein 2B (JMJD2B) may also be involved in TGF β 1-mediated β -catenin nuclear accumulation^[46]. Nuclear translocation of β -catenin may be regulated by JMJD2B in the EMT process^[46]. TGF β 1 down-regulates the canonical WNT signaling pathway and inhibits photoreceptor differentiation of adult human Müller stem cells^[47]. In human Müller stem cells, TGF β 1 down-regulates WNT2B, dickkopf WNT signaling pathway inhibitor 1 (DKK1) and active β -catenin and up-regulates WNT5B to inhibit canonical Wnt signaling^[47].

CTNNB1 IN CANCER STEM CELLS

Extrinsic factors are important when assessing cancer risk^[48]. Stem cell division is related to cancer development, emphasizing the importance of understanding the mole-

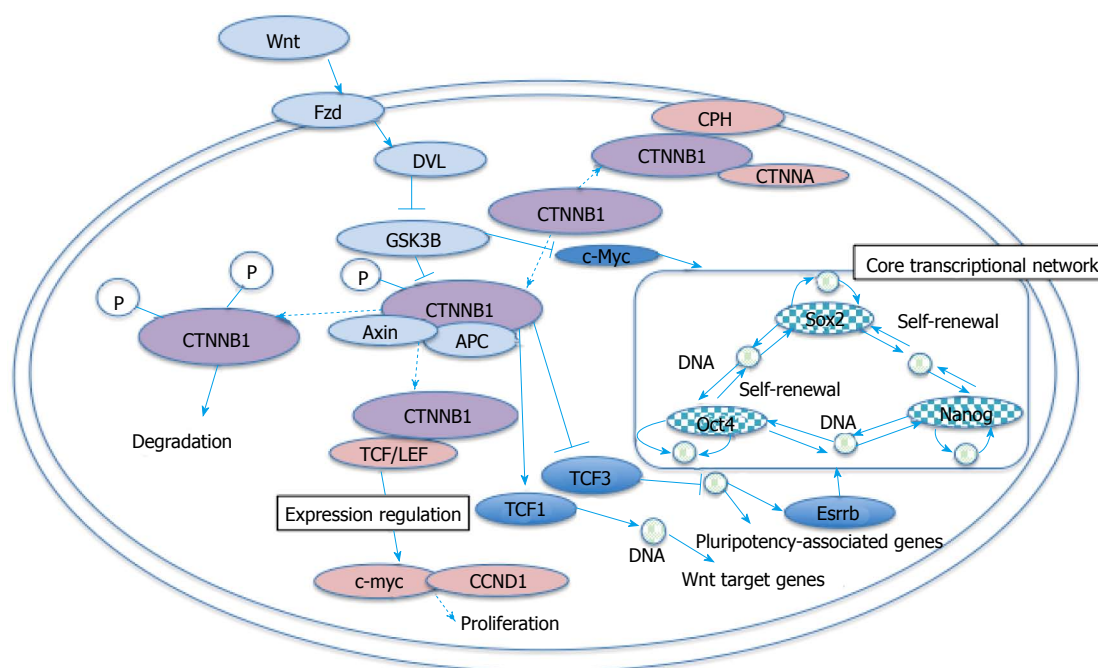


Figure 1 Main WNT/β-catenin pathway involved in cancer and stem cells. Wnt inhibits CTNNB1 phosphorylation by GSK3B, which leads to the transcription of pro-proliferation related genes via binding of CTNNB1 to TCF or LEF. P indicates phosphate. Red color shows molecules in pathways in cancer, whereas blue color shows molecules in signaling pathways regulating pluripotency of stem cells (KEGG). DVL: Dishevelled; TCF: T cell transcription factor; APC: Adenomatous polyposis coli.

cular pathways involved in stem cell maintenance, gastric cancer and cell proliferation^[48]. Ginsenoside Rh2, which inhibits growth of some types of cancer, decreases the number of CSC-like cells in hepatocellular carcinoma, possibly through β-catenin signaling^[49]. Furthermore, the CSC markers CD133 and Epithelial cell adhesion molecule (EpCAM) are decreased by ginsenoside Rh2^[49]. It is suggested that high levels of β-catenin are a signature of CSC-like cells^[49]. Wnt/β-catenin signaling leads to the translocation of β-catenin into nucleus and the transcription of *c-Myc*, *Axin2* and *Brachyury*^[50]. Axin is stabilized with GSK3β and inhibits β-catenin signaling^[50]. The proliferation of CSCs may also be regulated by Wnt/β-catenin signaling^[50]. The periprostatic adipose tissue-derived adipocytes regulate migration of prostate cancer cells^[51]. The chemokine CCL7 secretion of adipocytes stimulates the migration of CCR3-expressing prostate tumor cells^[51]. This signaling that is mediated by chemokines in CSCs could be a future target for investigation.

MUTATIONS IN CTNNB1-RELATED SIGNALING AND CANCER

Mutations of SMAD family member 4 (*Dpc4* or *Smad4*) and *Apc* in mice cause malignant intestinal tumors and stromal cell proliferation^[52]. The *DPC4* (*SMAD4*) gene is important for the TGFβ signaling pathway, which inhibits normal cell growth and promotes malignant cell growth^[52]. *APC* mutations in papillary thyroid carcinoma are associated with familial adenomatous polyposis^[53]. Through binding to 20-amino acid repeats, the APC/β-catenin signaling pathway is related to the development

of thyroid cancer in patients with familial adenomatous polyposis^[53]. Mutations in *AXIN2* cause colorectal cancer, in which the mutations stabilize β-catenin and activate β-catenin/TCF signaling^[54]. The *AXIN2* mutations result in accumulated nuclear β-catenin^[54]. *CTNNB1* and *APC* mutations also occur in colorectal cancer with defective DNA mismatch repair^[54]. For the treatment of cancer, identifying novel genome-wide therapeutic targets is essential, which suggests the importance of mutational studies in the cancer genome^[55]. Main WNT/β-catenin pathway involved in cancer and stem cells is shown in cartoon (Figure 1). The network information source is mainly from Kyoto Encyclopedia of Genes and Genomes (KEGG) (<http://www.genome.jp/kegg/>).

CONCLUSION

Our knowledge is increasing due to recent advances in bioinformatics and computational capacity. How to efficiently utilize this new data and knowledge is an important issue for future development of the big data era. The WNT/β-catenin pathway is involved in cancer and pluripotent stem cell signaling, which may suggest the mechanism underlying cancer stem cells. As cancer therapeutics has different effects in different genomic condition, individual medicine may be predicted with genetic variants. One useful direction for the use of genomic information may be the identification of targets for the treatment of diseases with appropriate predictions.

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Road map for pain management in pancreatic cancer: A review

Marie José Lahoud, Hampig Raphael Kourie, Joelle Antoun, Lana El Osta, Marwan Ghosn

Marie José Lahoud, Department of Anesthesiology and Critical Care, Faculty of Medicine, Saint Joseph University, Beirut 166830, Lebanon

Hampig Raphael Kourie, Joelle Antoun, Lana El Osta, Marwan Ghosn, Department of Oncology, Faculty of Medicine, Saint Joseph University, Beirut 166830, Lebanon

Author contributions: Lahoud MJ, Kourie HR and Ghosn M initiated the review; Lahoud MJ and Kourie HR performed the review, wrote and analyzed the data; Lahoud MJ, Kourie HR, Antoun J, El Osta L and Ghosn M reviewed the paper.

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Correspondence to: Marwan Ghosn, MD, Department of Oncology, Faculty of Medicine, Saint Joseph University, Monot St, PO Box, Beirut 166830, Lebanon. marwanghosnmd@yahoo.com
Telephone: +961-1-3226842
Fax: +961-1-1613397

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Abstract

Beside its poor prognosis and its late diagnosis, pancreatic cancer remains one of the most painful malignancies. Optimal management of pain in this cancer represents a real challenge for the oncologist whose objective is to ensure a better quality of life to his patients. We aimed in this paper to review all the treatment modalities incriminated in the management of pain in pancreatic cancer going from painkillers, chemotherapy, radiation therapy and interventional techniques to agents under investigation and alternative medicine. Although specific guidelines and recommendations for pain management in pancreatic cancer are still absent, we present all the possible pain treatments, with a progression from medical multimodal treatment to radiotherapy and chemotherapy then interventional techniques in case of resistance. In addition, alternative methods such as acupuncture and hypnosis can be added at any stage and seems to contribute to pain relief.

Key words: Pain management; Interventional; Medical; Treatment; Pancreatic cancer

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Core tip: This paper presents a road map for the pain management of pancreatic cancer. All treatment modalities incriminated in the management of pain in pancreatic cancer going from painkillers, chemotherapy, radiation therapy and interventional techniques to agents under investigation and alternative medicine are reviewed and their indications are discussed.

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INTRODUCTION

Pancreatic cancer (PC) is one of the most aggressive tumors with less than 5% survival at 5 years^[1]. More than 80% of these tumors are detected at an advanced stage because of the difficulty of diagnosis^[2]. At early stages, PC remains an asymptomatic disease. Many signs and symptoms appear gradually with advancing disease as jaundice, abdominal and back pain, weight loss and poor appetite, digestive problems, diabetes and blood clots. Abdominal and back pain has always been one of the major symptoms in patients with PC with more than 50% of patients with PC suffering from pain^[3]. There is no pathognomonic pain description, but often, over time, the pain of PC may radiate more through the abdomen to the back area. Due to the preponderance and the relevance of this symptom, in some clinical trials^[4,5], pain control was considered as one of the criteria of response to the treatment.

Many physiopathology mechanisms are incriminated in generating pain in this disease. Understanding the mechanism of pain will help the oncologists adapt the treatment panel to ensure a good quality of life for their patients.

We aimed in this review to describe first the different hypotheses of pain physiopathology in pancreatic cancer, to discuss medical and interventional options and finally to present a road map for oncologists leading to the optimum pain management in pancreatic cancer.

RESEARCH

Search strategy for the identification of studies

Electronic searches of the literature, published till September 2015, were conducted using PubMed database to identify articles reporting the pain management of pancreatic cancer.

Only trials describing pancreatic cancer pain management modalities were included. The year of publication of the article was not among the exclusion criteria. Search strategies employed included key words and Boolean operators described as follows: "Pancreatic cancer" and "pain management". This search was augmented by a hand search of the reference lists of relevant articles included in the literature review. Two different investigators performed the search ML and HK and the abstracts were independently reviewed for possible inclusion.

Types of studies: Review articles, peer review articles and some ongoing trials evaluating pancreatic cancer pain management modalities were included in the review. The language of publication of eligible studies was restricted to English.

Selection criteria

The articles to be included were first selected individually on the basis of their titles by 2 of the authors. Then, the abstracts of the available articles were examined. Our searches yielded 24 study reports from the PubMed database. These selected articles were then evaluated by 2 authors (ML and HK). Finally, 18 reports published till September 2015 were considered eligible by both authors and were retrieved for data extraction. Some of the remaining studies were used in the introduction and the discussion sections.

PHYSIOPATHOLOGY OF PAIN IN PC

Pain in PC may be the result of three components: Visceral, somatic, and neuropathic pain. Visceral nociceptive influxes are produced by ductal obstruction, damage and inflammation in the upper abdominal viscera. Somatic pain is caused by cancer extension into the peritoneum and bones. The signals reach the celiac plexus nerves, at T12-L1 vertebral levels, *via* the sympathetic system. Primary sensory neurons involved in pain sensation release predominantly substance P and glutamate in the dorsal horn of the spinal cord. From there, they synapse through the splanchnic nerves with T5-T12 dorsal root ganglia, then to the central nervous system^[6]. The most characteristic aspect of pancreatic neoplasia is an extrapancreatic nerve plexus invasion, which consists of the first route of metastasis. This may explain the neuropathic pain sensation. Pour *et al*^[7] highlighted the histogenetic similarity between neuronal cells and the pancreatic cancer cells. The two types of cells may share identical growth factor receptors and surface adhesion molecules and have an affinity to neural tissues. Although PC cells damage nerves^[8], several studies showed a harmony with cell-to-cell adhesion and migration of pancreatic cancer cells along the dorsal root ganglia neuritis inducing a mutual trophic nerve-cancer cell interaction^[9-11]. Nerve cells neurotrophic factors and chemokines are capable of boosting PC cell invasiveness, multiplication, and locomotion. On the other hand, PC cells secrete neuromodulatory agents, which cause neuroplasticity and neuropathic pain. An increase of density of calcitonin gene-related peptide (CGRP) and tyrosine hydroxylase was noted, connoting a proliferation of nerve growth factors^[12]. Studies have shown that growing sensory fibers are associated with necrotic damage of the nerve fiber endings and increased pain sensation^[13].

Another described mechanism of pain is the neo-vascularization and increased density of sensory and sympathetic nerve fibers within these newly formed vessels^[12]. Molecules secreted by tumor cells such as vascular endothelial growth factor, artemin, interleukin-1 and prostaglandins play a role in the interaction between the vascular system and the sensory neurons^[14,15].

In addition to the local nerve invasion, a high density of macrophages is noted within the tumor, which in-

duces excessive expression and secretion of nerve growth factors^[16]. The pancreas is innervated by sensory neurons that express CGRP and tyrosine kinase receptor A, which are the receptors of nerve growth factors. The high density of macrophage-secreted nerve growth factors activates the sensory nerves and generates pain afferents. Thus inflammation *via* macrophages and other inflammatory cells contributes to the pain sensation.

The NGF also activates the transient receptor potential vanilloid receptor subtype 1 (TRPV1)^[17]. The TRPV1 is a nonselective cation channel, which increases the permeability for sodium and calcium ions when activated and causes neuronal depolarization with burning sensation and release of substance P. In fact, an upregulation of substance P receptors, neurokinin receptors-1 (NK1-R), was described in pancreatic cancer. NK-1 antagonist MEN 11467 inhibited pancreatic cancer cell growth^[18].

Of interest, the presence of pain is a predictor of survival in pancreatic adenocarcinoma^[5,15,19]. This is not the case in other pancreatic malignancies where tumoral invasion mechanisms are different and do not imply neural invasion. In addition, the localization of the tumor plays a role in the pain pattern, pancreatic body presenting the highest incidence cancer localization in pancreas^[20]. This is probably due to its proximity to the celiac plexus.

In conclusion, pain in pancreatic cancer has a complex physiopathology. It eminently implies a neuronal invasion and a neurogenic inflammation.

PAIN MANAGEMENT MODALITIES IN PC

Medical approach

Opioids and derivatives: Most cancer related pain is controlled by pharmacological oral treatments. The optimal management is based on the World Health Organization (WHO)'s analgesic ladder, with a progressive administration of nonopioids (aspirin and paracetamol); then, as necessary, mild opioids (codeine); then strong opioids, until reaching pain relief. Morphine, the standard "step 3" opioid, has been widely used for the control of chronic cancer pain, especially in moderate to severe pain^[21], and is the first-line medical therapy for pancreatic cancer^[22]. Oral route is the gold standard with two types of molecules: Normal release with rapid onset for breakthrough pain and modified release marked by a long acting effect, used for maintenance treatment^[21]. The second route of administration, if patient is unable of oral intake is subcutaneous. The oral, sublingual and nebulized routes of administration of morphine are not common because there is no evident superiority over the oral route^[21].

Fentanyl is an alternative to morphine for cancer pain management in its different administration routes. Transdermal patches are suitable for patients whose opioids requirements are stable^[23,24]. Sublingual fentanyl tablets and nasal spray may be an option for breakthrough pain^[25,26].

Oxycodone showed a superior analgesic effect on visceral pain in a tissue-differentiated experimental pain model^[27]. This hypothesis was not clinically confirmed. Similar analgesia is provided with oral morphine and oxycodone^[28] and particularly in PC^[29].

Buprenorphine is another strong opioid available as a prescription choice. A systemic review by Schmidt-Hansen *et al.*^[30] ranked Buprenorphine as a "fourth-line option" after morphine, oxycodone and fentanyl in cancer pain management.

At the end-of-life stage in advanced PC, thoracic epidural opioid in combination with local anesthetics could be considered^[31].

Opioids must be employed with regards to their considerable potential for side effects including sedation and respiratory depression, pruritus, nausea and constipation. The prescription of a stool softener and laxatives for constipation, diphenhydramine for pruritus, and antiemetics for nausea are recommended.

Apart from the classically known opioids side effects, several experimental studies performed on mouse models showed that morphine can interfere with regulation of cancer cell growth by inhibiting apoptosis and by stimulating angiogenesis and metastasis^[32,33]. So far, the results of both *in vitro* and *in vivo* studies are contradictory and not conclusive. The effect appears to vary depending on the cancer cell type. Prospective randomized controlled trials are currently conducted to find an answer to this dilemma^[34]. In the meanwhile, opioids are still a central cornerstone in the management of pancreatic cancer pain.

Antiepileptics: Considering the multidimensional PC pain mechanisms, adjuvant medications and multimodal approach are recommended. Gabapentin and Pregabalin are anti-epileptic agents used as first line treatment in neuropathic pain, including diabetic neuropathy^[35], post herpetic pain^[36], and neuropathic pain of central origin^[37]. Their effectiveness is also demonstrated in cancer-related neuropathic pain^[38]. Their target is voltage dependent calcium channel. They block calcium influx into presynaptic nerve terminals, thus reducing the release of excitatory neurotransmitters on spinal neurons. Upstream transmission of neuropathic pain in the central nervous system and neuronal excitability are consecutively inhibited^[39]. These molecules may have beneficial effects as adjuvants and opioids - sparing for coeliac plexus pain^[40,41].

Corticosteroids: Steroids have been proved particularly useful as adjuvant therapy for visceral pain^[42]. Glucocorticoids inhibit prostaglandin synthesis, a precursor of inflammation cascade, and reduce vascular permeability decreasing tissue edema. Steroid receptors are also localized in the central and peripheral nervous systems and are responsible for neuron plasticity, decreasing discharge in an injured nerve and in neuropathic pain^[43]. Moreover, perioperative dexamethasone administration has been associated with improved survival after

Table 1 Potential molecules that were under investigation

Molecule	Mechanisms	Ref.
Capsaicin/resiniferatoxin	Activation of vanilloid receptors on cancer cells, inducing apoptosis	Hartel <i>et al</i> ^[17]
MEN 11467	NK-1R antagonists, inhibiting cell growth and neuronal invasion	Friess <i>et al</i> ^[18]
HSV-Enk viral vector	Increased met-enkephalin production in the peripheral nerve terminal endings and in dorsal root ganglion, reducing pain	Lu <i>et al</i> ^[45] Yang <i>et al</i> ^[46]
Phentolamine	Alpha-adrenergic blockade of sympathetic induced pain	Yasukawa ^[47]

NK-1R: Neurokinin-1 receptors; HSV-Enk: Herpes simplex virus carrying the human preproenkephalin.

pancreatic adenocarcinoma resection^[44].

Approaches under investigation

As mentioned before, neurokinin receptors-1 receptor and its ligand substance P are activated in PC, which contribute to cancer cell growth and neuronal invasion^[18]. A promising treatment approach is to target this pathway by NK-1R antagonists MEN 11467, inhibiting nerves alteration and neuropathic pain. Table 1 summarizes the molecules under investigation^[17,18,45-47].

Chemotherapy and radiation therapy

Pain control is largely implemented as secondary end point in the trials evaluating chemotherapeutic regimen in PC. In 1997, the approval of gemcitabine as the standard of care in the treatment of advanced PC was not only based on the small benefit in survival (less than 2 mo) but also on the better pain control. Twenty-three point eight percent of patients receiving Gemcitabine in advanced PC were classified as positive in pain category (pain intensity or/and analgesic use was reduced), while only 4.8% of patients receiving 5-FU were considered in the same category. FOLFIRINOX also showed a better quality of life and pain control in the management of metastatic PC^[4,5].

Radiation therapy is frequently used in PC for many purposes going from local control of the disease when associated to chemotherapy in adjuvant or locally advanced setting, to pain management and control in palliative and metastatic disease. Radiotherapy is particularly effective in controlling and relieving pain caused by large tumors compressing other organs or structures, such as nerves or the spine. The effect of radiation therapy is usually late, and it occurring many weeks after the initiation of treatment. The radiation therapy can shrink the tumor, which may help in relieving the pain. The radiation therapy can also be effective in targeting some metastatic lesions.

Interventional therapies

Celiac plexus block and neurolysis: Despite all the available analgesics and the possibility of combination of different molecules, treatment is often suboptimal, and many side effects are observed^[48,49]. Thus, more efficient forms of pain management are essential for such patients. An alternative approach is the use of celiac plexus block (CPB) or neurolysis (CPN).

The major component of pancreatic carcinoma pain is mediated by sympathetic fibers from the pancreas and is relayed through the celiac plexus to the splanchnic nerves. A local anesthetic, mainly bupivacaine, can be used in combination with steroids to temporary inhibit the celiac plexus^[50]. Celiac plexus neurolysis (CPN) represents the prolonged interruption of the plexus by the injection of alcohol or phenol^[51]. Formerly, it was managed by anesthesiologists and radiologists *via* a posterior approach. However, complications occurred in 1% of cases, secondary to displacement of the needle, causing paraplegia or pneumothorax when entering the spinal artery or diaphragm respectively^[52-54]. Anterior approach evolved lately *via* the guidance of transcutaneous ultrasound, X-ray fluoroscopy, computed tomography, or recently endoscopic ultrasonography^[55-57]. Endoscopic ultrasonography which permits direct access to the celiac plexus, is an attractive technique compared to percutaneous CPN, because complications can be avoided. Furthermore, it allows ultrasound guided fine needle aspiration sampling and tumor staging^[58-60].

Sympathetic inhibition can be as well done at the level of the splanchnic nerves. Splanchnicectomy consists in sectioning the roots of the splanchnic nerves from T5 to T10. The approach by thoracotomy was first described in 1942 by Mallet-Guy^[61]. Thoracoscopic splanchnicectomy, less invasive, was introduced in 1993^[62]. Good results were noted with thoracoscopic unilateral left splanchnicectomy^[63,64]. It can be repeated for right side in case of pain recurrence or done bilaterally from the beginning^[63]. With splanchnicectomy, analgesic effects last for approximately 2 mo, even for 3 mo of partial to complete relief^[52].

Intrathecal therapy

Intrathecal therapy can be proposed for end-of-life stages or in refractory pain^[30]. The implantable intrathecal drug delivery systems are an alternative with reduced drug toxicities and improvement of pain scores^[65]. Various molecules can be used including morphine, fentanyl, local anesthetics, baclofen and/or clonidine^[66,67].

Alternative medicine

Acupuncture: The developments of effective and safe therapeutic strategies that complement pharmaceutical treatment are interesting in the management of PC pain. Acupuncture analgesia has been widely used to

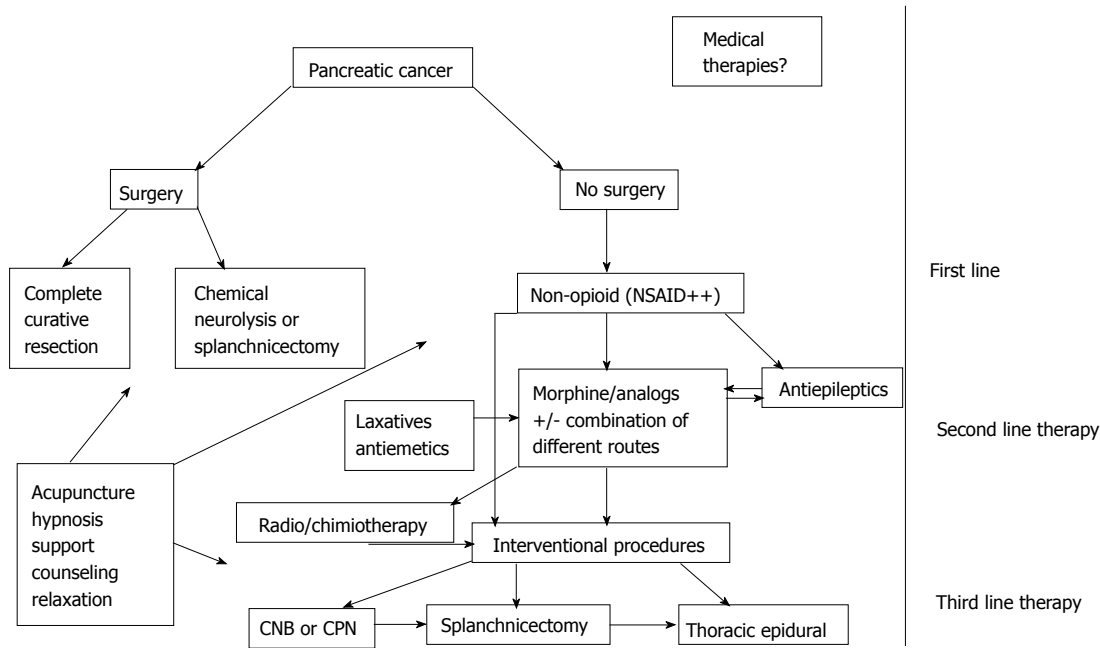


Figure 1 Algorithm of pancreatic cancer pain management. CPN: Celiac plexus neurolysis; NSAID: Non-steroidal anti-inflammatory drug.

treat cancer pain^[68]. A systemic review published in 2012 including a total of 15 randomized controlled trials, showed that acupuncture is an effective adjunctive method in cancer pain management and pain relief was superior compared to drug therapy alone^[69]. Chen *et al.*^[70] showed the effect of acupuncture on PC pain. Acupuncture's effects can be explained as viscerocutaneous, cutaneo-visceral, cutaneo-muscular, and visceromuscular reflexes^[71]. Jiaji points, which correlate to segmental dispersion of the sympathetic and parasympathetic systems, were chosen on the nerve segments of the pancreas.

Hypnosis

Hypnosis is an increasingly used approach to pain control in patients with cancer. There is significant evidence that hypnosis is useful at reducing cancer-related pain^[72,73]. Hypnosis modulates the pain sensation by a functional disconnection between the prefrontal cortex, center of decision-making and the anterior cingulate, zone playing a role in attention and motivation. This will make therapeutic suggestions easily integrated^[74].

DISCUSSION

With the presence of a large panel of strategies and modalities in the pain management of PC and the absence of clear recommendations and guidelines, every oncologist is treating pain in PC according to their own experience. The choice of pain management modality depends of the pain characteristics and physiopathology, the stage and the prognosis of the disease, the performance status of the patient, his comorbidities, his past medical history and his future therapeutic options (Figure 1).

Owing to the fact that PC pain involves visceral, somatic and neuropathic components, the main therapeutic approach is a multimodal analgesia. A combination of drugs and/or techniques with distinct mechanisms of action leads to a synergistic effect and allows a better analgesia with decreased consumption of opioids, and hence lesser opioid-related adverse events. A standardized multimodal analgesia protocol based on available evidence is hard to be proposed in PC patients. The essential points remain the addition of antiepileptics for neuropathic pain, the frequent use of corticosteroids and the referral to an interventional procedure after a pluridisciplinary case study. The patients with metastatic disease will benefit more from systemic therapies if the pain is multi-localized and generalized. However, local treatments such as radiation therapy can play a role in controlling pain by targeting some well-defined metastasis generating pain. Patient with a poor prognosis (survival less than three months) will not benefit from an interventional therapeutic approach as patients with better prognosis, because of the risk of the intervention, especially in patients with bad performance status, compared to the time of benefice.

The presence of comorbidities or the past medical history can guide the pain management in PC. Chronic use of corticoids should be careful because of the increased risk of gastric bleeding, especially when combined to non-steroidal anti-inflammatory drugs, which amplify the risk by 15-folds^[75]. Gastric protection should be considered. Patients presenting long-term constipation or ileus paralytic should receive morphine associated to laxatives with close surveillance. Patients with PC have a higher risk of developing thrombosis, and are usually under prophylactic or therapeutic anti-coagulants. Special considerations should be taken to discontinue anticoagulant therapy in

Table 2 Summarizing all treatment approaches, administration route or techniques and indications

Treatment approaches	Medications and modalities	Administration route/techniques	Indications
Medical treatment	Opioids and derivatives	Per os S/C Patch (fentanyl)	Moderate to severe pain Impossible oral intake (occlusion) Breakthrough pain
	Antiepileptics corticosteroids	Nasal Per os Per os or IV	Breakthrough pain Neuropathic pain Adjuvant, especially in metastatic bone pain
Interventional treatment	Celiac plexus block (LA) or neurolysis	Transcutaneous guidance ¹ or endoscopic ultrasonography	Refractory pain
Alternative medicine	Splanchnicectomy	Thoracotomy, thoracoscopy	Intractable pain in non resectable tumor
	Intrathecal therapy	Implantable intrathecal drug delivery systems	End-of-life stage
Chemotherapy and radiation therapy	Acupuncture	Jiaji points	adjuvant to drug therapy and/or interventional techniques
	Hypnosis	Sessions with an expertise	Any stage of pain
	FOLFIRINOX	IV	Locally advanced tumor
	Gemcitabine		

¹Ultrasound, X-ray fluoroscopy or computed tomography. S/C: Subcutaneous; IV: Intravenous; LA: Local anesthetics.

interventional therapeutic approach (celiac plexus block, intra-theal treatment), if the patient is under chronic thromboprophylactic prevention for primary or secondary risk. The discontinuation of anticoagulation is sometimes a reasonable cause to choose less invasive analgesic strategies in high-risk patients.

In PC patients who have not received chemotherapeutic agents, transitory painkillers and not a definitive pain management approach will be a logical option, while waiting for the response to the treatment.

Finally, the choice of pain management modality can be influenced by the team expertise and abilities. Celiac plexus neurolysis under endoscopic ultrasonography requires specialized equipment and expert specialists, which are not always available. CT scan or X-ray fluoroscopy guided posterior approach remains an equivalent technique with good results. Thoracotomic bilateral splanchnicectomy is mainly reserved for patients who require abdominal surgery (occlusion, biliary and/or intestinal bypass), making it a two-in-one procedure with no additional surgical risk for the patient^[76].

CONCLUSION

Developing pain management recommendations in pancreatic cancer seems to be an interesting and practical target for oncologists and palliative care physicians, since more than the half of these patients will develop severe pain without any detailed and structured road map. We exposed all the pain treatments that are available and proven efficient and safe, medical multimodal treatment, radiotherapy, chemotherapy and finally interventional techniques as a last resort (Table 2). Team expertise and competence remain the major factors in the treatment choice.

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Basic Study

Plasma chitinase 3-like 1 is persistently elevated during first month after minimally invasive colorectal cancer resection

HMC Shantha Kumara, David Gaita, Hiromichi Miyagaki, Xiaohong Yan, Sonali AC Hearth, Linda Njoh, Vesna Cekic, Richard L Whelan

HMC Shantha Kumara, David Gaita, Hiromichi Miyagaki, Xiaohong Yan, Sonali AC Hearth, Linda Njoh, Vesna Cekic, Richard L Whelan, Division of Colon and Rectal Surgery, Department of Surgery, Mount Sinai West Hospital, New York, NY 10019, United States

Hiromichi Miyagaki, Department of Gastroenterological Surgery, Osaka University, Osaka 565-0871, Japan,

Richard L Whelan, Icahn School of Medicine at Mount Sinai, New York, NY 10029, United States

Author contributions: Shantha Kumara HMC contributed to the conception, design, sample processing, analysis and interpretation of data, revision of the articles; Gaita D contributed to the manuscript writing, collection of human material and clinical data; Miyagaki H, Yan X and Hearth SAC contributed to human sample collection, processing, analysis and interpretation of data; Njoh L contributed to the statistical analysis, interpretation of data; Cekic V contributed to collection of human material and clinical data; Whelan RL contributed to the conception, design, interpretation of data, critical revision of the article; all authors drafted the article and made critical revisions and approved the final version of the article to be published.

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Correspondence to: Richard L Whelan, MD, Professor of Surgery, Chief, Division of Colon and Rectal Surgery, Department of Surgery, Mount Sinai West Hospital, Suite 7B, 425 West, 59th Street, New York, NY 10019, United States. rwhelan@chpnet.org
Telephone: +1-212-5238172
Fax: +1-212-5238857

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Abstract

AIM: To assess blood chitinase 3-like 1 (CHI3L1) levels for 2 mo after minimally invasive colorectal resection (MOCR) for colorectal cancer (CRC).

METHODS: CRC patients in an Institutional Review Board approved data/plasma bank who underwent elective MOCR for whom preoperative (PreOp), early post-operative (PostOp), and 1 or more late PostOp samples [postoperative day (POD) 7-27] available were included. Plasma CHI3L1 levels (ng/mL) were determined in

duplicate by enzyme linked immunosorbent assay.

RESULTS: PreOp and PostOp plasma sample were available for 80 MICR cancer patients for the study. The median PreOp CHI3L1 level was 56.8 CI: 41.9-78.6 ng/mL ($n = 80$). Significantly elevated ($P < 0.001$) median plasma levels (ng/mL) over PreOp levels were detected on POD1 (667.7 CI: 495.7, 771.7; $n = 79$), POD 3 (132.6 CI: 95.5, 173.7; $n = 76$), POD7-13 (96.4 CI: 67.7, 136.9; $n = 62$), POD14-20 (101.4 CI: 80.7, 287.4; $n = 22$), and POD 21-27 (98.1 CI: 66.8, 137.4; $n = 20$, $P = 0.001$). No significant difference in plasma levels were noted on POD27-41.

CONCLUSION: Plasma CHI3L1 levels were significantly elevated for one month after MICR. Persistently elevated plasma CHI3L1 may support the growth of residual tumor and metastasis.

Key words: Colorectal cancer; Recurrence; Minimally invasive colorectal resection; Chitinase 3-like 1; Metastasis

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Core tip: Colorectal cancer (CRC) resection surgery is well known to be associated with short lived immunosuppression and transient plasma protein changes. We have documented that a second set of blood protein alterations that last for 3 to 5 wk after CRC; interestingly, all of these proteins play a role in angiogenesis. This group of pro-angiogenic proteins includes vascular endothelial growth factor, placental growth factor, angiopoietin-2, monocyte chemo-attractant protein-1 and matrix metalloproteinase 2. Our published data further confirms that pro-angiogenic postoperative plasma from cancer patients stimulates *in vitro* endothelial cell proliferation, migration, and invasion. In this manuscript we are presenting data to demonstrate that a pro-angiogenic protein, chitinase 3-like 1, in CRC patients remain elevated for month after minimally invasive colorectal resection.

Shantha Kumara HMC, Gaita D, Miyagaki H, Yan X, Hearsh SAC, Njoh L, Cekic V, Whelan RL. Plasma chitinase 3-like 1 is persistently elevated during first month after minimally invasive colorectal cancer resection. *World J Gastrointest Oncol* 2016; 8(8): 607-614 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v8/i8/607.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v8.i8.607>

INTRODUCTION

Colorectal cancer (CRC) is the second most diagnosed cancer in the United States, with an expected 142820 cases and 50830 deaths in 2013^[1]. Surgical resection is the primary treatment for the 80% of CRC patients who present without metastatic disease. While this procedure is considered curative for these patients,

more than 40% who present with stage II or III disease will develop a recurrence^[1,2].

It has been hypothesized that surgical resection of tumors may paradoxically contribute to the development of cancer recurrences. Murine studies have shown those laparotomy and bowel resections are associated with increased tumor growth and establishment vs results in anesthesia control mice^[3-5]. In humans, numerous case reports have noted increased tumor growth soon after surgery in patients with residual cancer^[6-9]. A number of mechanisms, including surgery-related immunosuppression and the removal of primary tumor generated anti-angiogenic factors, have been proposed to account for accelerated tumor growth after surgery^[10,11]. Of interest, the last decade has seen the emergence of another possible mechanism, namely surgery-related proangiogenic plasma compositional changes that may support the establishment of metastases and the growth of already present tumor deposits during the early post-surgical period.

Angiogenesis or neo-vascularization is necessary for tumor growth greater than 1-2 mm^[3,12,13]. During a nascent tumor's initial avascular dormant stage, cells obtain nutrients by passive diffusion only. The activation of what has been called the "angiogenic switch" leads to the development of new vessels that infiltrate and extend beyond the tumor mass which permits growth and, later, metastasis. Similarly, further growth of established metastases requires new vessel formation.

It has been shown that colorectal resection is associated with continual elevations (2-5 wk) in the blood levels of a number of angiogenesis promoting proteins including vascular endothelial growth factor (VEGF), angiopoietin-2 (Ang-2), placental growth factor (PIGF), soluble vascular cell adhesion molecule-1 (sVCAM-1), monocyte chemotactic protein-1 (MCP-1), and matrix metalloproteinase-3 (MMP-3)^[14-18]. Furthermore, human plasma from the second and third weeks after surgery has been shown to promote endothelial cell (EC) proliferation, invasion, and migration, which are key steps in angiogenesis^[14,19]. It is possible, therefore, that the postoperative (PostOp) plasma composition may encourage the growth of residual tumor metastases after "curative" colorectal resection of a primary tumor. In an effort to better understand the impact of surgery the search goes on for other proteins with proangiogenic effects whose blood levels may be increased after surgery.

Chitinase 3-like 1 (CHI3L1), also named as YKL-40 and human cartilage glycoprotein-39 (HCgp-39), is a member of the glycosyl hydrolase 18 protein family^[20]. The substrate for this family of proteins is chitin, a polymer of N-acetyl-glucosamine. Importantly, chitins are not found in mammals yet these proteins are produced by a number of mammalian cell types including EC's neutrophils, macrophages, and vascular smooth muscle cells. CHI3L1 does not have any known enzymatic activity in mammals, however, it is believed that this protein binds to endogenous carbohydrates such as hyaluronic

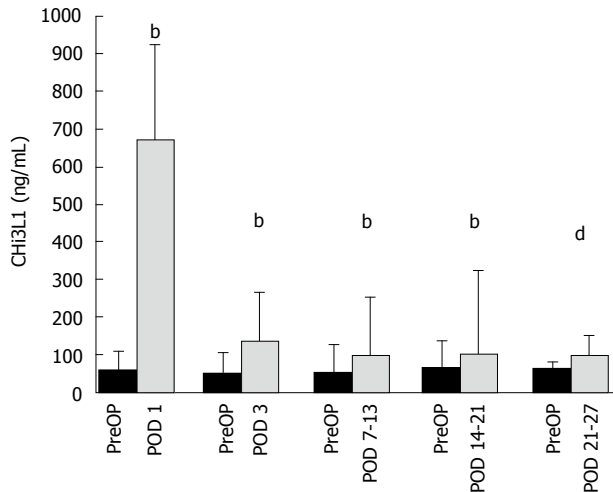


Figure 1 Enzyme linked immunosorbent assay determined preoperative and postoperative chitinase 3-like 1 levels of colorectal cancer patients. CHI3 L1 levels are expressed median and 75% quartile range: [PreOp vs POD1 ($n = 79$), PreOp vs POD3 ($n = 76$), PreOp vs POD7-13 ($n = 62$), PreOp vs POD14-20 ($n = 22$)] $^bP \leq 0.001$; PreOp vs POD 21-27 ($n = 20$), $^dP = 0.001$. PreOp: Preoperative; CHI3L1: Chitinase 3-like 1; POD: Postoperative day.

acid and heparin as well as specific receptors such as the interleukin (IL)-13 receptor $\alpha 2$ in macrophages^[21]. CHI3L1 and other members of the glycosyl hydrolase 18 protein families are thought to play a role in innate and specific immune function, tissue remodeling, and the proliferation of fibroblasts, epithelial, synovial, and other cell types. CHI3L1 has also been shown to limit inflammation related organ injury and apoptosis in some settings^[21].

There is also experimental evidence that CHI3L1 promotes tumor angiogenesis. Several investigators have demonstrated that CHI3L1, *in vitro*, increased EC (HUVEC) migration and tube formation which are essential initial steps in the process of angiogenesis^[22,23]. Macrophages in tumor stroma have also been shown to express CHI3L1 which likely also promotes tumor angiogenesis^[24].

Blood levels of CHI3L1 are negligible under normal physiologic conditions, however, elevated blood levels of CHI3L1 have been found in patients with chronic inflammatory conditions^[25] and a wide variety of cancers including colorectal^[22,26], breast^[27], prostate^[28], lung^[29,30], thyroid^[31], endometrial^[32], pancreatic^[33], hepatocellular^[34], ovarian^[35], gastric cancer^[36], and malignant melanoma^[37]. Additionally, for the majority of these malignancies, a correlation has been demonstrated between blood levels of CHI3L1 and a poor prognosis^[26,29-44]. Also, increased expression of CHI3L1 in CRC has been shown to be strongly associated with increased microvascular density^[22]. Although it has been shown that serum levels of CHI3L1 are elevated prior to surgery in CRC patients, the impact of surgical resection on blood levels after surgery are unknown. Because CHI3L1 involved in tissue remodeling and angiogenesis we hypothesized that following minimally invasive colorectal resection (MICR) for CRC blood levels of CHI3L1 might be increased. This

study was carried out to investigate plasma levels of CHI3L1 during the first 4 to 6 wk following MICR for CRC.

MATERIALS AND METHODS

Patient selection for study

Patient populations who underwent elective MICR were selected for this study from an Institutional Review Board (IRB) approved multi center plasma and data bank that was organized by Columbia University and that included the following institutions: New York Presbyterian Hospital, Columbia University; the Ferguson Clinic, Grand Rapids, Michigan and Mount Sinai West Hospital Center, New York. The broadly stated purpose of this effort is to study the physiologic, immunologic, and oncologic ramifications of open and minimally invasive surgical methods. Prospective data including demographic, operative, and short term recovery statistics were collected for all patients. Patients who were immunosuppressed or transfused perioperatively were excluded. Patients undergoing urgent or emergent surgery were, likewise, excluded.

Blood sampling and processing

To be eligible for entry into this study preoperative (PreOp) and, at least, several PostOp plasma samples had to be available for CRC patients who underwent MICR. Of note, blood samples after postoperative day (POD) 7 were obtained at follow up office appointments but were not scheduled on a specific POD. Late post-operative samples were not available on the same day from all patients so the late samples were bundled into 7 d (or longer) time periods (POD 7-13, POD 14-20, POD 21-27, and POD 28-41). Samples were collected in tubes containing heparin. The samples were processed within 6 h of collection, and the plasma fraction stored in aliquots at -80°C until the assay was performed.

Plasma CHI3L1 analysis

Plasma CHI3L1 levels were determined in duplicate using commercially available enzyme linked immunosorbent assay (ELISA) (R and D Systems, Minneapolis). CHI3L1 concentrations are reported as nanograms per milliliter (ng/mL).

Statistical analysis

For continuous variables, data are expressed as mean \pm SD. Frequencies and percentages were determined for categorical variables. In regards to the CRC Pre vs PostOp CHI3L1 comparisons, the results are reported as the median and 95% CIs and the Wilcoxon signed rank test was used to analyze the data. Correlation between PostOp CHI3L1 plasma levels and incision size and length of surgery was evaluated by the Spearman's rank correlation coefficient (r_s). All data analysis was performed utilizing SPSS version 15.0 (SPSS, Inc., Chicago, IL). Because the sample size varies for the POD 7-13, POD 14-20, POD 21-27 and POD 28-41 time points a separate Pre-Op bar is included for each time point in the Figure 1.

Table 1 Demographic and clinical characteristics of the study population

Age, years (mean \pm SD)	65.66 \pm 12.83
Sex, n (%)	
Male	42 (52.5)
Female	38 (47.5)
Incision length, cm (mean \pm SD)	7.78 \pm 3.61
Operative time, min (mean \pm SD)	308.3 \pm 124.2
Length of stay, d (mean \pm SD)	6.68 \pm 4.30
Type of resection, n (%)	
Right	24 (30.0)
Transverse	5 (6.2)
Left	6 (7.5)
Sigmoid/rectosigmoid	13 (16.3)
LAR/AR	26 (32.5)
APR	2 (2.5)
Subtotal/total	4 (5.0)
Surgical method, n (%)	
Laparoscopic-assisted	47 (59.0)
Hand-assisted/hybrid laparoscopic	33(41.0)

RESULTS

A total of 80 MICR patients with CRC (42 male/38 female, age 65.66 \pm 12.83 years) were included in the study. The majority of patients underwent right colectomy, lower anterior resection/anterior resection, or sigmoidectomy (Table 1). Laparoscopic-assisted methods were used in 59% while hand-assisted minimally invasive methods were utilized in 41% of the patients. There were 12 conversions (15%) to open methods (defined as final incision > 7 cm in laparoscopic-assisted cases and incision > 11 cm in hand-assisted cases). The mean surgical incision length was 7.8 \pm 3.6 cm for the entire population, the mean operative time was 308.3 \pm 124.2 min. The mean length of stay was 6.6 \pm 4.3 d. Seven complications (8.8%) were noted including small bowel obstruction (3), wound infection (1), hernia (1), hyperkalemia (1), and hematoma (1). There were no perioperative deaths. The cancer pathological stage breakdown of the study population was as follows: Stage I, 20 (25.0%); stage II, 25 (31.3%); stage III, 32 (40%); stage IV, 3 (3.7%).

The median PreOp CHI3L1 level was 56.8 CI: 41.9, 78.6 ng/mL (n = 80). Significantly elevated median plasma levels were noted on POD 1 (667.7 CI: 495.7, 771.7 ng/mL, n = 79, P < 0.001), POD 3 (132.6 CI: 95.5, 173.7 ng/mL, n = 76, P < 0.001), POD 7-13 (96.4 CI: 67.7, 136.9 ng/mL, n = 62, P < 0.001), POD 14-20 (101.4 CI: 80.7, 287.4 ng/mL, n = 22, P < 0.001), and POD 21-27 (98.1 CI: 66.8, 137.4 ng/mL, n = 20, P = 0.001) when compared to PreOp levels. The percent increase from median baseline at each time point was 1068% at POD 1, 157% at POD 3, 88% at POD 7-13, 50% at POD 14-20, and 64.0% at POD 21-27. No significant difference found at the POD 28-41 time point.

To determine whether there was a statistical correlation between incision length and post surgery plasma

CHI3L1 levels, the laparoscopic-assisted group (mean incision size 5.8 cm) and the hand-assisted group (mean incision, 10.7 \pm 3.5 cm) were compared. While the mean incision size for the hand assisted group was higher, there was no statistically significant difference in PostOp plasma CHI3L1 levels between the groups. Furthermore, at 5 of the 6 PostOp time points there was no correlation between CHI3L1 levels and incision length. There was a statistically significant correlation between incision length and CHI3L1 levels at the POD 14-20 time point. There was no significant correlation between cancer stage and PreOp CHI3L1 plasma level.

DISCUSSION

We have demonstrated in several studies that elective surgery for CRC is associated with persistent plasma protein changes that render the blood proangiogenic for 2-3 wk as judged by *in vitro* invasion, migration and EC proliferation analysis^[14-18]. This is in contradistinction to the vast majority of serum proteins that have been studied perioperatively whose levels are transiently elevated after major surgery (alterations resolving in < 1 wk such as IL-2, IL-6, CRP, etc). CHI3L1, which has been shown to promote EC proliferation and tube formation among its other effects, can now be added to the list of persistently elevated proangiogenic proteins.

In this study, mean plasma CHI3L1 levels of 80 CRC patients rose to a peak of 1068% over the PreOp baseline value on POD1, and remained significantly elevated at 50%-157% over baseline for four weeks after MICR until returning to baseline at the POD 28-34 time point. Of note, the percent changes in CHI3L1 levels over baseline were amongst the highest observed when compared to the results of the 9 other proteins previously shown to have long duration plasma increases post MICR.

Because CHI3L1 levels have been shown to be increased preoperatively in cancer patients when compared to tumor free patients, a fall in blood levels would be anticipated after the primary tumor is resected. Why then do plasma levels of CHI3L1 rise during the first month after surgery? Most likely there are several mechanisms. As regards the initial elevation during the first 3-4 d after MICR, although unproven, it is possible that macrophages and polymorphonuclear leukocytes (PMN's), which play a vital role in the acute inflammatory response that follows surgery, generate the added CHI3L1. This hypothesis is based on the following observations. Both PMN's and macrophages have been shown to generate CHI3L1 in the setting of chronic inflammation (e.g., IBD and rheumatoid arthritis) and blood levels of CHI3L1, known to be increased in these patients, have been shown to directly correlate with disease severity.

As regards the persistent plasma elevation noted during weeks 2 to 4, the authors believe, also admittedly without direct evidence, that the healing surgical wounds are the source of the added CHI3L1. Since CHI3L1 has

been shown to play key role in tissue remodeling as well as angiogenesis it is certainly possible that it is involved with the wound healing process^[9,26]. The hypothesis is that wound levels are very high such that the CHI3L1 spills over into the blood stream raising plasma levels. Support for this concept can be found in several human studies that measured VEGF levels in wound fluid and the blood of surgery patients.

In a Dutch study of MICR patients, on POD 4, wound VEGF fluid levels were found to be 7 times higher than serum concentrations which were elevated over PreOp baseline levels^[45]. The same research team, in a study of mastectomy patients noted that on POD 4 wound VEGF levels were between 23 and 32 times greater than serum levels^[46]. There is also strong preliminary unpublished data of the authors suggesting that wound levels of ANG2, and MMP2 are elevated and many times higher than plasma concentrations after MICR (plasma levels also elevated 3-4 wk). Further, the fact that the proteins shown to have markedly elevated wound levels play roles in neovascularization (like CHI3L1) indirectly suggests that the origin of the CHI3L1 during most of the first PostOp month is the wound. As mentioned above, the authors are currently conducting a study that is simultaneously assessing wound and plasma protein levels which will soon shed more light on this topic.

What are the potential clinical ramifications, if any, of the month long elevation in CHI3L1 levels? There is some research evidence that CHI3L1 may play a direct role in chronic inflammation related epithelial cancer development *via* up-regulation of β catenin^[25]. As mentioned earlier, in addition to promoting angiogenesis in general there is strong *in vivo* evidence that CHI3L1 promotes tumor angiogenesis in particular. Macrophages are known to promote cancer progression by producing a number of growth and proangiogenic factors^[47]. Tumor associated macrophages lack antigen presenting abilities and have been found to cluster in avascular areas of breast carcinomas^[47-49]. Kawda *et al.*^[22] revealed that the CHI3L1 increased the secretion of inflammatory chemokines, IL-8 and monocyte chemo-attractant protein-1 (MCP-1), from SW480 cells *via* mitogen-activated protein kinase (MAPK) pathway *in vitro*. Furthermore, CHI3L1 expressing colon cancer cells significantly increased macrophage recruitment in xenograft mice, as well as tumor growth and angiogenesis^[22]. Persistently elevated levels of plasma CHI3L1 levels together with IL8 and MCP-1^[17] may collectively enhance the blood angiogenic property after MICR.

The proangiogenic effects of CHI3L1 should be considered in the context of the other 9 proteins (all with proangiogenic effects) whose levels have also been shown to be persistently elevated after MICR. There is a concern that these plasma changes might promote tumor angiogenesis early after resection of the primary cancer in patients with established distant micro-metastases. Further, these compositional changes may encourage the establishment of new metastases

by circulating viable tumor cells present in the blood stream after surgery. Is there any evidence of increased tumor growth after surgery in humans?

The medical literature contains numerous case reports of rapid growth of residual metastases after major surgery^[7-9]. In particular, rapid growth of pre-existing metastatic cancer in the liver after resection of the primary colorectal tumor has been noted by several investigators^[6,50-52]. Of note, in one investigation the vascular density of metastases has been noted to be significantly increased over the pre-resection baseline several months after primary resection; this suggests that tumor angiogenesis is stimulated after surgery^[53]. Finally, there is strong experimental data showing that surgical trauma is associated with accelerated tumor growth^[3-5].

The data from this study and others mentioned above suggest that the first month after surgery may be a precarious time for cancer patients who harbor residual tumor since the plasma composition is proangiogenic for up to a month. The fear is that these conditions will foster blood vessel formation in the tumor and, in so doing, will promote tumor growth. Because conventional adjuvant or palliative PostOp chemotherapy is usually started 4 to 8 wk post operatively the patient is left to their own during this time period. It is logical to administer some type of anti-cancer treatment in this unused time window. The challenge is to find effective anti-cancer agents that do not interfere with the process of wound healing. Presently, a phase I clinical trial is underway that is assessing perioperative treatment with polyphenon E (a green tea extract) and a milk thistle plant component called siliphos. Both agents have been shown to inhibit tumor growth while not inhibiting wound healing which makes them safe for the early PostOp period^[54].

A weakness of this study was the relatively small number of late plasma samples collected. Most of the late samples were obtained during follow-up visits scheduled by the patients; unfortunately, many patients refused late PostOp blood draws. Thus, there were fewer late samples than at the PreOp, POD 1, and POD 3 time points. This made it necessary to bundle the late samples into 7 to 13 d blocks. Also, the timing of the late samples varied considerably. A larger study will allow a more meaningful correlation of PreOp CHI3L1 levels and cancer stage. Similarly, a follow up study with more uniform and comprehensive PostOp blood sampling should allow a better assessment of PostOp levels, in general, as well as the impact of surgical method, if any, on post surgery plasma levels.

The results of this study demonstrate that surgical stress, in particular MICR, is associated with notably increased plasma levels of CHI3L1 that persist for 1 mo after surgery. The source of this elevation is unclear. The clinical implications of these findings, if any, are uncertain. In theory, among other possible effects, this transient plasma alteration may promote tumor angiogenesis in patients with residual cancer after surgical resection of the primary tumor. The fact that plasma levels of 9 other

proteins with proven proangiogenic effects have been shown to be elevated for 2 to 4 wk after MICR lends support to this hypothesis. Further study of perioperative plasma CHI3L1 levels and of the clinical ramifications of the surgery-related long duration proangiogenic plasma compositional changes appear to be warranted.

COMMENTS

Backgrounds

Major abdominal surgery is well known to be associated with a brief period of immunosuppression and short lived plasma protein changes. Recently, another group of blood protein alterations that lasts at least 3 to 5 wk after colorectal cancer (CRC) resection have been noted. All of these proteins play a role in angiogenesis. This set of proangiogenic proteins includes vascular endothelial growth factor (VEGF), placental growth factor (PIGF), angiopoietin-2, soluble vascular adhesion molecule-1 (sVCAM-1), monocyte chemo-attractant protein-1 (MCP-1) and matrix metalloproteinase-3. Proangiogenic chitinase 3-like 1 (CHI3L) protein promotes *in vitro* human endothelial cell (EC) migration and tube formation, however, the impact of minimally invasive colorectal resection (MICR) for CRC on plasma levels of CHI3L1 is unknown.

Research frontiers

CHI3L1, is a member of the Chitinase family of proteins and has chemotactic and proangiogenic properties similar to those of MCP-1 and IL8 that are mediated, in part, by MCP-1. Colon, breast, and hepatocellular carcinomas have been shown to express higher levels of CHI3L1. It has been shown that CHI3L1 may utilize its chitin binding ability to communicate with other signal transduction pathways to modulate inflammation, apoptosis, tissue remodeling, cell growth and angiogenesis. CHI3L1 has been shown to promote *in vitro* cancer cell proliferation, macrophage recruitment, human EC migration and tube formation, and contributes to wound healing. The authors analyzed preoperative (PreOp) and post-MICR CHI3L1 levels in CRC patients. Significantly elevated blood levels of CHI3L1 may promote tumor angiogenesis and therefore, the growth of residual tumor during the first month after MICR.

Innovations and breakthroughs

Persistently elevated plasma levels of the proangiogenic proteins including VEGF, Ang-2, PIGF, sVCAM-1 and MMP3 have been noted for 3-5 wk after CRC resection. Plasma from the 2nd and 3rd weeks after CRC resection has been shown to stimulate EC proliferation and migration *in vitro* when compared to EC culture results with PreOp plasma. This study reports that plasma CHI3L1 levels are significantly elevated over PreOp levels for a month after MICR for CRC. These persistent plasma compositional changes may promote tumor angiogenesis and growth in patients with residual cancer deposits in the 1st postoperative (PostOp) month.

Applications

This study results further support that persistent plasma compositional changes may promote tumor angiogenesis and growth in patients with residual cancer deposits in the first PostOp month. Thus, it is logical to give anti-cancer therapy perioperatively, for safe use in this period, in addition to having anti-cancer effects, candidate agents must not impair wound healing.

Terminology

The significantly increased blood proangiogenic protein levels during the early PostOp period after MICR and open colorectal resection may be associated with the short lived acute inflammatory response that occurs after surgery and resolves in the first week. In contrast, the later and persistent elevation noted during weeks 2-4 after surgery may be related to wound healing. Persistently elevated plasma CHI3L1 levels shown in this study, together with the similarly increased levels of the other proangiogenic proteins such as VEGF, ANG2, PIGF, sVCAM-1 and MMP3 may collectively promote tumor angiogenesis and therefore, the growth of residual tumor during the first month after MICR.

Peer-review

The paper is written in a good language, the logic is clear and the subject and results are discussed graphically and meaningfully.

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Basic Study

Immunohistochemical analysis of the Wnt/ β -catenin signaling pathway in pancreatic neuroendocrine neoplasms

Vivian Weiss, Julie Dueber, Jesse P Wright, Justin Cates, Frank Revetta, Alexander A Parikh, Nipun B Merchant, Chanjuan Shi

Vivian Weiss, Julie Dueber, Justin Cates, Frank Revetta, Chanjuan Shi, Department of Pathology, Microbiology and Immunology, Vanderbilt University Medical Center, Nashville, TN 37232, United States

Jesse P Wright, Alexander A Parikh, Department of Surgery, Surgical Oncology, Vanderbilt University Medical Center, Nashville, TN 37232, United States

Nipun B Merchant, Department of Surgery, Surgical Oncology, University of Miami Hospital, Miami, FL 33165, United States

Author contributions: Weiss V and Dueber J collected the pathologic data and wrote the manuscript; Wright JP analyzed Ki67 and collected the clinical data; Cates J performed statistical analysis and edited the manuscript; Revetta F performed immunohistochemistry; Parikh AA, Merchant NB and Shi C designed the study and edited the manuscript.

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Correspondence to: Chanjuan Shi, MD, PhD, Associate Professor, Department of Pathology, Microbiology and Immunology, Vanderbilt University Medical Center, Medical Center North C-2310D, 1121 21st Ave. S, Nashville, TN 37232, United States. chanjuan.shi@vanderbilt.edu
Telephone: +1-615-9368342
Fax: +1-615-3437023

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Abstract

AIM: To investigate the role of the Wnt/ β -catenin pathway in pancreatic neuroendocrine neoplasms (PanNENs).

METHODS: Tissue microarrays containing 88 PanNENs were immunohistochemically labeled with antibodies to β -catenin, E-cadherin, adenomatous polyposis coli (APC), chromogranin and synaptophysin. One case had only metastatic tumors resected, whereas others ($n = 87$) received pancreatectomy with or without partial hepatectomy. Pathology slides, demographic, clinicopathologic, and follow up data were reviewed. Patients' demographics, clinicopathologic features, and immunohistochemical results from 87 primary tumors were compared between patients with low stage (stage I / II) and high stage (stage III/IV) tumors. In addition, correlation of immunohistochemical results from primary tumors with disease-specific survival (DSS) was evaluated.

RESULTS: Strong membranous β -catenin staining in the primary tumor was observed in all 13 stage III/IV PanNENs as compared to 47% (35/74) of stage I / II

tumors ($P < 0.01$). However, the strong membranous β -catenin staining was unassociated with tumor grade or DSS. Decreased membranous β -catenin staining was associated with decreased membranous E-cadherin labeling. Nuclear β -catenin staining was seen in 15% (2/13) of stage III/IV PanNENs as compared to 0% (0/74) of stage I/II tumors ($P = 0.02$). The case with metastasectomy only also showed nuclear β -catenin staining. Two of the three cases with nuclear β -catenin staining were familial adenomatous polyposis (FAP) patients. Lack of APC expression was seen in 70% (57/81) of the cases, including the 3 cases with nuclear β -catenin staining. Expression of E-cadherin and APC in primary tumor was not correlated with tumor grade, tumor stage, or disease specific survival.

CONCLUSION: The Wnt/ β -catenin pathway was altered in some PanNENs, but did not impact DSS. PanNENs in FAP patients demonstrated nuclear β -catenin accumulation and loss of APC.

Key words: β -catenin; Familial adenomatous polyposis; Pancreatic neuroendocrine neoplasm; Adenomatous polyposis coli; E-cadherin

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Core tip: Dysregulation of the Wnt/ β -catenin pathway is present in some pancreatic neuroendocrine neoplasms (PanNENs). However, compared to other malignancies, this signaling pathway may have different functions in PanNENs as strong membranous β -catenin expression is more frequently present in stage III/IV tumors and membranous expression of β -catenin and E-cadherin does not have a prognostic importance in this setting. Additionally, PanNENs arising in patients with familial adenomatous polyposis (FAP) demonstrate abnormal nuclear β -catenin accumulation, and PanNENs may be one of the extraintestinal manifestations of FAP.

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INTRODUCTION

Although rare, pancreatic neuroendocrine neoplasms (PanNENs) are the second most common malignancy in the pancreas, accounting for 1%-2% of pancreatic tumors^[1,2]. Most PanNENs are well-differentiated, with a 10 year survival rate of approximately 40%. Based on mitotic count and Ki67 labeling index, the World Health Organization (WHO) 2010 has classified PanNENs into three grades: Grade 1 with Ki67 $\leq 2.0\%$ and mitotic

rate < 2 mitoses/10 high power fields (HPFs), grade 2 with Ki67 3%-20% or mitotic rate 2-20 mitoses/HPFs, and grade 3 with Ki67 $> 20\%$ or mitotic rate > 20 mitoses/HPFs^[3]. Grade 1 and 2 tumors are considered as well-differentiated neuroendocrine tumors, while grade 3 PanNENs include well-differentiated tumors with Ki67 $> 20\%$ and poorly differentiated neuroendocrine carcinomas^[4].

PanNENs, like many other tumors, are thought to evolve following cellular dysregulation and proliferation. One of the pathways known to be involved in normal cell growth, proliferation, differentiation, and apoptosis is the Wnt/ β -catenin signaling pathway. It is then conceivable that dysregulation of this critical homeostatic pathway may lead to tumor growth and proliferation. In fact, most colonic adenocarcinomas arise from tubular adenomas through dysregulation of the Wnt/ β -catenin pathway^[5]. Gastrointestinal well-differentiated neuroendocrine tumors, colonic glandular-neuroendocrine mixed tumors, and pancreatic acinar cell tumors have all been reported to have mutations and/or altered expression in the Wnt/ β -catenin pathway^[6-10]. A subset of ileal neuroendocrine neoplasms have been shown to have adenomatous polyposis coli (APC) mutations or loss of heterozygosity^[11]. However, it is unclear whether this pathway plays a significant role in pancreatic endocrine tumorigenesis. In this study, we assessed the expression of β -catenin, E-cadherin and APC in PanNENs.

MATERIALS AND METHODS

Patient selection

This study was approved by the Vanderbilt Institutional Review Board. Tissue microarrays (TMAs) were constructed from the resection specimens of 88 PanNEN patients who underwent pancreatectomy only ($n = 84$), pancreatectomy with partial hepatectomy ($n = 3$), and metastasectomy (partial hepatectomy) only ($n = 1$) at Vanderbilt University Medical Center from 01/1998 to 08/2012. The TMAs included 2-6 tumor and 1-2 normal pancreatic tissue cores from each patient studied. Demographic and clinicopathologic information was abstracted from the electronic medical record. Pathologic data were also recorded upon reviewing of original hematoxylin and eosin-stained slides. The American Joint Committee on Cancer TNM staging system 7th edition was used to divide PanNENs into four stages: Stage I (T1-2N0M0), stage II (T3N0M0 or T1-3N1M0), stage III (T4N0-1M0), and stage IV (T1-4N0-1M1). Stage I and II PanNENs were grouped into low stage disease, whereas stage III and IV high stage disease. Patient outcomes were confirmed using the social security death index.

Immunohistochemistry

Immunohistochemical labeling for β -catenin, E-cadherin, APC, chromogranin and synaptophysin was performed. Details of the antibodies used in this study are provided in Table 1.

Table 1 List of antibodies used

Antibody	Source	Dilution
β-catenin	BD Bioscience CAT# 610154 Mouse monoclonal (San Jose, CA)	1:1000
E-cadherin	BD Bioscience CAT# 610182 Mouse Monoclonal (San Jose, CA)	1:800
APC	Abcam (ab15270) Rabbit Polyclonal (Cambridge, MA)	1:200
Chromogranin	Abcam (ab15160) Rabbit polyclonal (Cambridge, MA)	1:500
Synaptophysin	Abcam (ab32127) Rabbit monoclonal (Cambridge, MA)	1:500

APC: Adenomatous polyposis coli.

Unstained slides were first deparaffinized by routine methods. Antigen retrieval was performed as follows: Slides were heated in citrate buffer (pH 6.0) at 100 °C for 20 min or in EDTA (pH 9.0) at 98 °C for 20 min, followed by a 10-min cool down to room temperature. All slides were then quenched with 0.03% (v/v) H₂O₂ with NaN₃ for 5 min. Slides were blocked for 20 min with serum-free protein block (Dako, Carpinteria, CA), followed by application of primary antibodies. Slides were then incubated with Evison + HRP-labeled polymer (Dako) for 30 min, followed by 5 min incubation with DAB (Dako).

Immunohistochemical stains were reviewed by two pathologists (VW and CS). Membranous β-catenin and E-cadherin staining was scored as negative (0), weak (1), moderate (2) or strong (3). Scores from replicate cores were averaged. Those with an average score > 2 were considered strong labeling, and those with ≤ 2 as decreased. Nuclear β-catenin and cytoplasmic APC staining was scored as positive and negative (in at least 10% of the tumor cells). Immunohistochemical results from primary tumor were used for statistical analysis.

Statistical analysis

Immunohistochemical labeling for β-catenin, E-cadherin and APC was compared between PanNENs and normal pancreas using the Fisher's exact test. Immunohistochemical results were also correlated with clinicopathologic parameters using standard bivariate methods. Kaplan-Meier disease-specific survival (DSS) curves were plotted and compared by Cox proportional hazards regression using the Stata® software package (v13, StataCorp, College Station, TX). All reported *P* values were derived from two-tailed hypothesis tests.

RESULTS

The 88 patients included 46 females and 42 males. The demographics and clinicopathological features of the 87 cases with primary tumor resected are listed in Table 2. While 85% (74/87) of the tumors were WHO grade 1 or 2 well-differentiated tumors, 15% (13/87) were WHO grade 3 well-differentiated tumors with Ki67 > 20%. Poorly differentiated neuroendocrine carcinomas including

Table 2 Demographics, clinicopathologic features, Wnt/β-catenin expression and survival status of patients with high vs low stage pancreatic neuroendocrine neoplasms

	Low stage (<i>n</i> = 74)	High stage (<i>n</i> = 13)	Total (<i>n</i> = 87)
Average age (range)	56 (32-81 yr)	50 (35-77 yr)	55 (32-81 yr)
Male/female	34/40	7/6	41/46
Syndromic (%)	11/74 (15)	4/13 (31)	15/87 (17)
Functional (%)	15/74 (20)	0/13 (0)	15/87 (17)
Tumor size ^a (mean ± SEM)	2.8 ± 0.3 cm	4.4 ± 0.5 cm	3.0 ± 0.2 cm
Tumor grade ^a (%)			
Grade 1/2	67/74 (91)	7/13 (54)	74/87 (85)
Grade 3	7/74 (9)	6/13 (46)	13/87 (15)
Infiltrative growth pattern ^a	21/74 (28)	8/13 (58)	29/87 (33)
LVI ^b	20/74 (28)	12/13 (92)	32/87 (37)
PNI	9/74 (12)	3/13 (25)	12/87 (14)
Necrosis	8/74 (11)	3/13 (25)	11/87 (13)
Strong membranous β-catenin ^b	35/74 (47)	12/13 (92)	48/87 (55)
Nuclear β-catenin ^a	0/74 (0)	2/13 (15)	3/87 (3)
Strong membranous E-cadherin (%)	30/74 (41)	8/13 (62)	38/87 (44)
APC	22/67 (33)	2/13 (15)	24/80 (30)
Death from disease ^b	7/74 (10)	6/13 (50)	13/87 (15)

^a*P* < 0.05; ^b*P* < 0.01. LVI: Lymphovascular invasion; PNI: Perineural invasion; APC: Adenomatous polyposis coli.

small cell carcinomas and large cell neuroendocrine carcinomas were excluded from this study as they are known to have very different molecular and biologic features from well-differentiated tumors^[3,4]. Most patients (74/87, 85%) had low stage tumors (stage I / II). High stage tumors were associated with large tumor size, high tumor grade, infiltrative growth pattern and lymphovascular invasion, but did not show associations with age, gender, syndrome, tumor functionality, perineural invasion and tumor necrosis (Table 2).

To explore whether the Wnt/β-catenin pathway was altered in PanNENs, TMAs were labeled with antibodies to β-catenin, E-cadherin and APC. Membranous β-catenin labeling was strong in the exocrine pancreas, but was weak in normal pancreatic islets (Figure 1A). Over half of the PanNENs studied (48/87, 55%) displayed strong membranous staining for β-catenin in the primary tumor (Figure 1B and C, Table 2). Interestingly, all 13 stage III /IV PanNENs strongly expressed membranous β-catenin in primary tumor, compared to only 47% (35/74) of stage I / II tumors (Table 2, *P* < 0.01). There was no correlation between membranous β-catenin staining and tumor grade.

As expected, most primary tumors (85/87, 98%) were negative for nuclear β-catenin labeling (Table 2). However, when stratified by tumor stage, nuclear β-catenin staining was more often present in tumors of high stage (2/13, 15% vs 0/74, 0% of stage I / II tumors; *P* = 0.02). In addition, the case with metastasectomy only (stage IV) also showed nuclear β-catenin staining. Histologically, each of the 3 tumors with nuclear β-catenin labeling showed the typical morphologic

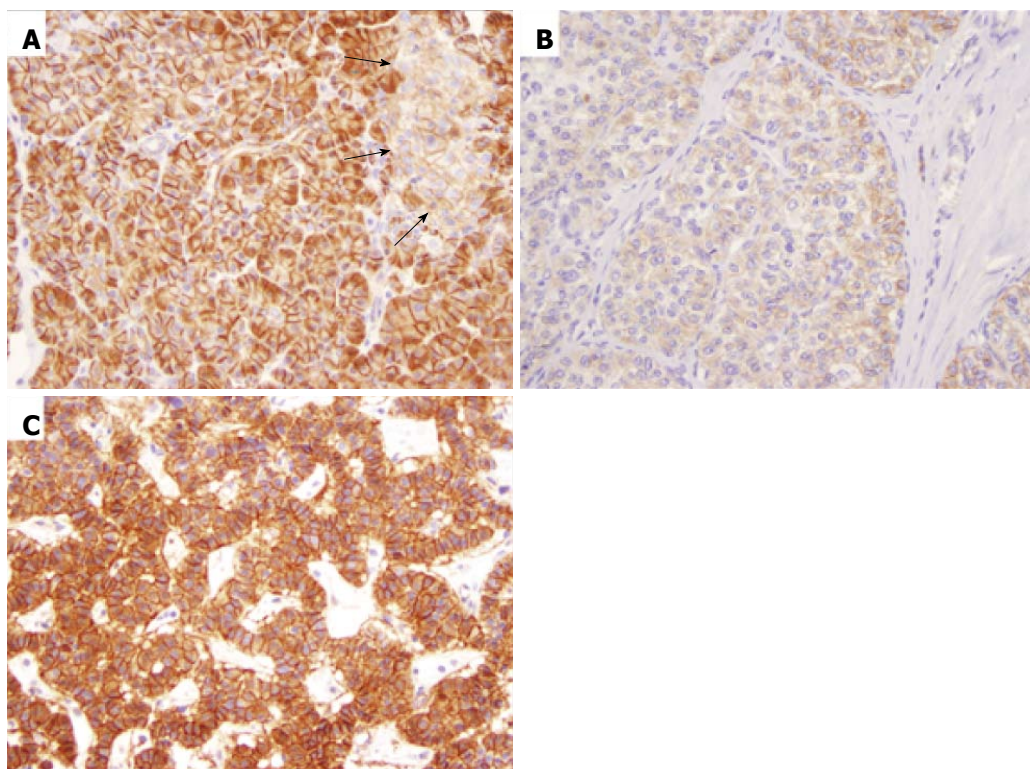


Figure 1 β -catenin expression in the pancreas and pancreatic neuroendocrine neoplasms (original magnification 200 \times). A: Strong membranous β -catenin staining in the exocrine pancreas and weak membranous staining in pancreatic islets (black arrows); B: Weak membranous β -catenin staining in one pancreatic neuroendocrine neoplasm; C: Strong membranous β -catenin staining in another pancreatic neuroendocrine neoplasm.

features of well-differentiated neuroendocrine tumors (Figure 2A and D). Additional immunohistochemical labeling was subsequently performed to rule out solid-pseudopapillary neoplasms (SPNs), a pancreatic tumor characterized by nuclear accumulation of β -catenin and loss of membranous E-cadherin. Each expressed both neuroendocrine markers (synaptophysin and chromogranin, Figure 2B and C) and E-cadherin (membranous, Figure 2E), consistent with neuroendocrine neoplasms.

Careful review of the clinical history disclosed that two of the three cases with nuclear β -catenin expression were from FAP patients. One was a fifty-year-old male who presented with a stage IV PanNEN and multiple colonic adenocarcinomas arising in the setting of polyposis at the initial diagnosis. Sequencing the *APC* gene demonstrated a germline 853delT mutation. The other was a forty-year-old female who presented with a stage IV PanNEN. She had undergone total colectomy for FAP at 22 years of age. *APC* gene sequencing demonstrated a germline R564X (1690C < T) mutation. Immunohistochemical staining of the PanNENs from both patients showed loss of APC expression (Figure 2F).

Similar to β -catenin, membranous E-cadherin labeling was frequently strong in pancreatic acinar cells, but weak in pancreatic islets (Figure 3A). Strong membranous E-cadherin expression in the primary tumor was observed in 44% (38/87) of PanNENs (Figure 2E), whereas decreased membranous E-cadherin expression

was present in more than half of the cases (Figure 3B). Complete loss of membranous E-cadherin expression was only observed in one case (1/87, 11%), which also showed decreased membranous β -catenin labeling and complete loss of APC expression. However, nuclear β -catenin accumulation was not seen in the tumor. No associations between membranous E-cadherin expression and tumor grade or stage were observed in this series. Noteworthy is that tumors with decreased membranous β -catenin also showed decreased membranous E-cadherin staining compared to cases with strong membranous β -catenin staining ($P < 0.01$).

Cytoplasmic APC labeling was not seen in the exocrine pancreas, but was detected in most of the pancreatic islets (Figure 3C). While 86% (30/35) of normal pancreatic islets in this study clearly expressed APC, only 30% (24/80) of the primary tumors retained APC expression (Figure 3D, $P < 0.01$). Of note, all 3 cases with nuclear β -catenin staining demonstrated loss of cytoplasmic APC expression (Figure 2F). There was no correlation between APC and β -catenin expression ($P > 0.05$). APC expression was not correlated with tumor stage or grade ($P > 0.05$).

Median follow-up for the cases with resected primary tumor was 47 mo (range, 0.3-163 mo) during which 13 patients (15%) died of progressive disease (Table 2). Univariate analysis showed that high tumor stage, tumor functionality, large tumor size, high tumor grade, infiltrative growth pattern, and lymphovascular and

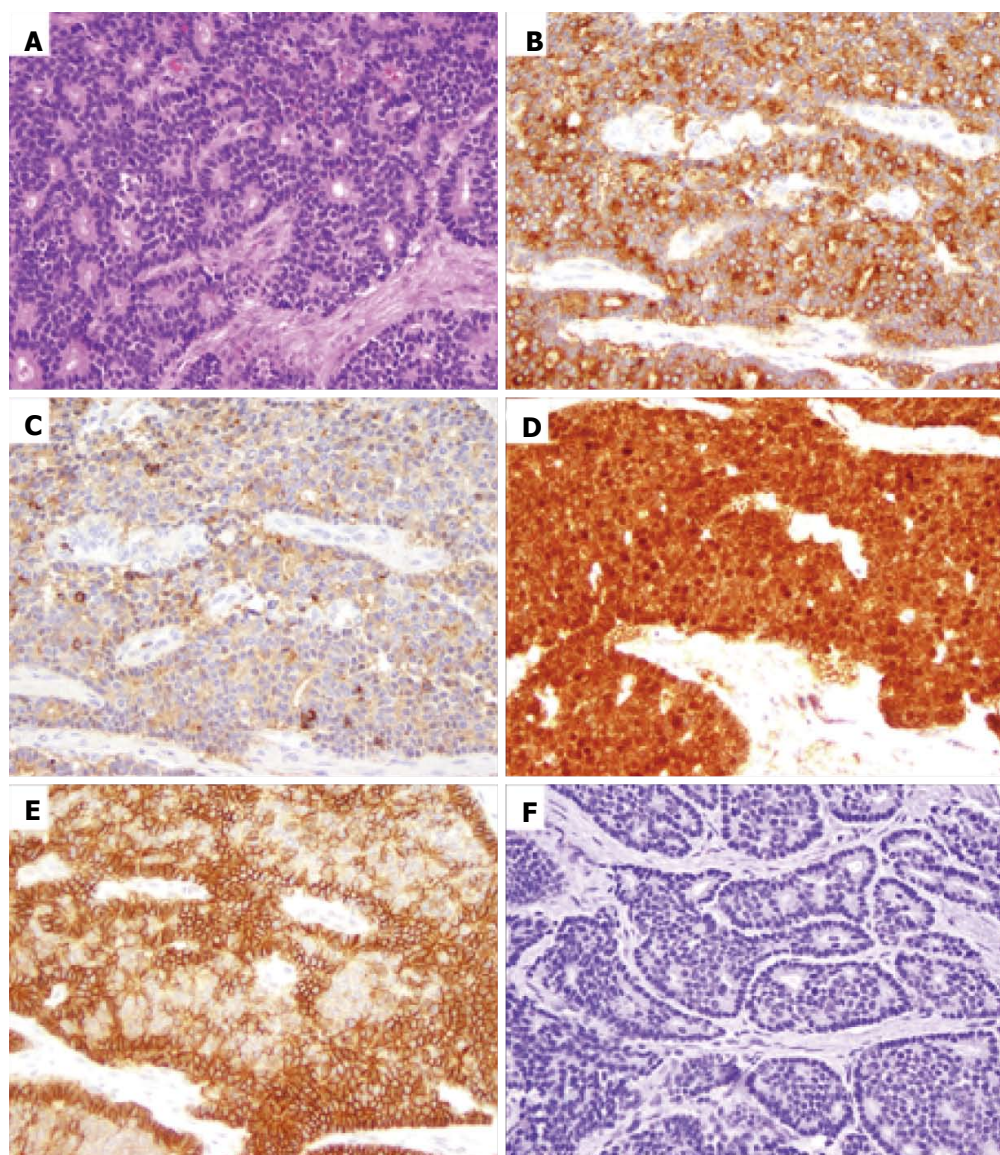


Figure 2 A pancreatic neuroendocrine neoplasm from a familial adenomatous polyposis patient (original magnification 200 ×). A: Hematoxylin and eosin stain showing a well differentiated neuroendocrine tumor composed of monomorphic cells with "salt and pepper" chromatin; B and C: Immunohistochemical labeling for synaptophysin (B) and chromogranin (C) showing positive staining; D: Immunohistochemical labeling with β catenin showing β catenin nuclear accumulation and loss of membrane staining; E: Immunohistochemical staining for E-cadherin showing strong E-cadherin membranous expression; F: Immunohistochemical labeling for APC showing loss of APC expression. APC: Adenomatous polyposis coli.

perineural invasion were all associated with decreased DSS. In multivariate survival analysis, stage III/IV disease demonstrated a significantly worse DSS (hazard ratio = 10.4, $P < 0.05$) compared to stage I / II disease. When controlling for tumor stage and WHO grade, none of the immunohistochemical markers showed prognostic significance.

DISCUSSION

In the current study we investigated expression of select Wnt/ β -catenin pathway components in PanNENs. Approximately half of PanNENs displayed strong expression of β -catenin at the plasma membrane. As expected, strong membranous expression of β -catenin correlated with strong membranous E-cadherin. In many

other malignancies, decreased membranous β -catenin and E-cadherin expression predicts poor prognosis. We observed that in PanNENs, strong membranous β -catenin expression was frequently present in stage III / IV tumors and that membranous expression of β -catenin and E-cadherin did not have a prognostic importance in this setting. These data suggest that the role of the Wnt/ β -catenin signaling pathway in PanNENs is different than in other neoplasms such as gastric and colon adenocarcinomas^[5,12-15].

APC mutations have been implicated in the development of enterochromaffin cell neuroendocrine neoplasms, and are seen in 23% of these types of tumors arising in the midgut^[11]. However, alterations in APC have not been well characterized in neuroendocrine neoplasms of the foregut. We report that most PanNENs show loss of

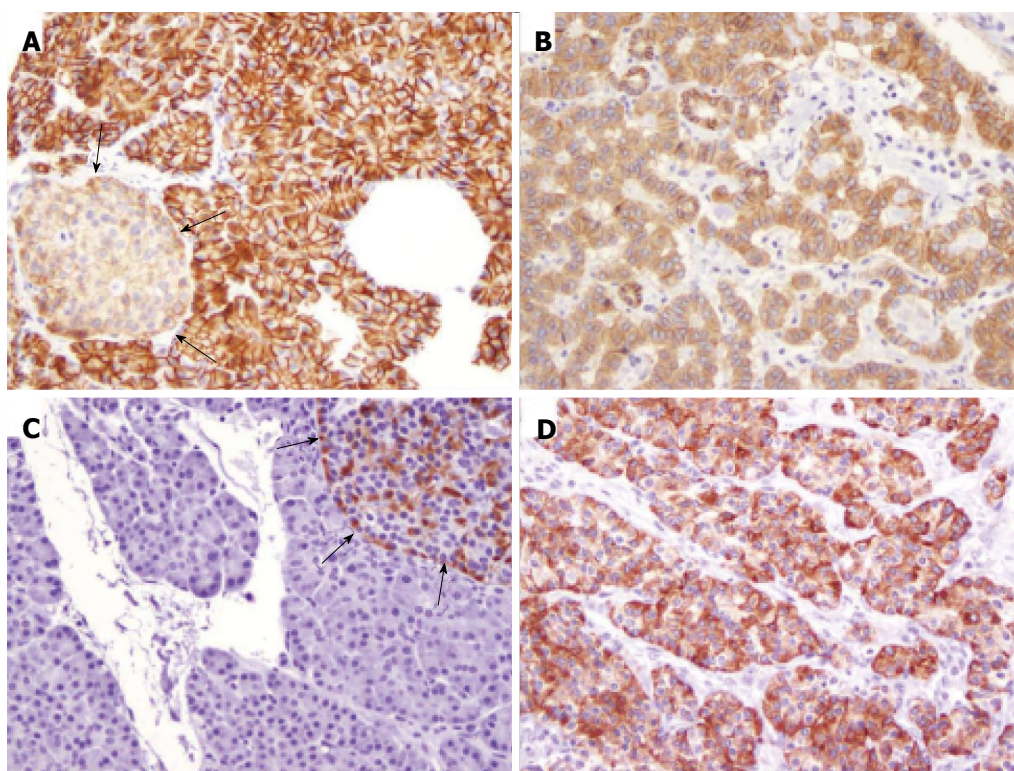


Figure 3 Expression of E-cadherin and adenomatous polyposis coli in the pancreas and pancreatic neuroendocrine neoplasms (original magnification 200 ×). A: Strong membranous E-cadherin labeling in the exocrine pancreas and weak membranous E-cadherin labeling in pancreatic islets (black arrows); B: Weak membranous E-cadherin in one pancreatic neuroendocrine neoplasm; C: Expression of cytoplasmic APC by pancreatic islets (black arrows) but not by the exocrine pancreas; D: Cytoplasmic APC labeling in one pancreatic neuroendocrine neoplasm. APC: Adenomatous polyposis coli.

APC expression, which may increase β -catenin signaling capacity for processes such as cell-cycle, apoptosis, and differentiation. Other described mutations in PanNENs may also lead to increased β -catenin signaling. For example, studies of MEN-1 deficient PanNENs (sporadic and familial) and pulmonary neuroendocrine tumors suggest that *MEN-1* mutations lead to increased β -catenin signaling^[16,17]. Canonical β -catenin signaling is characterized by β -catenin accumulation within nuclei, where it regulates gene expression. However, we detected nuclear β -catenin in only 3 of 88 tumors and did not observe differences in β -catenin expression between non-syndromic and MEN-1-associated PanNENs (data not shown). Nevertheless, strong membranous β -catenin expression was observed in 55% of PanNENs, which was not present in normal pancreatic islets. In a mouse model, knockout of β -catenin expression has been shown to suppress tumorigenesis and growth of *Men1*-deficient PNENs^[17]. Therefore, β -catenin may represent a potential therapeutic target in these tumors.

The three tumors in our study with strong nuclear β -catenin expression also showed loss of APC expression (two of these tumors were from FAP patients). FAP is defined by mutations in the *APC* gene on chromosome 5q21^[18]. APC binds β -catenin, targeting it for degradation. Therefore, when APC is mutated, β -catenin accumulates in the nucleus and activates gene transcription. Loss of APC expression, as well as nuclear accumulation of β -catenin, suggest that the *APC* gene is biallelically

inactivated in these two FAP-associated tumors, and that PanNENs may be another extra-intestinal manifestation in patients with FAP. Prior to this investigation, an association between PanNENs and FAP had not been described.

Abnormal nuclear β -catenin accumulation has been demonstrated in other FAP-associated tumors, such as desmoid-type fibromatosis. Nuclear β -catenin expression was also recently described in intestinal neuroendocrine tumors from FAP patients^[19]. Abnormal nuclear β -catenin accumulation has also been demonstrated in Sertoli cell tumors of the testis^[20] and hepatoblastomas^[21] arising in FAP patients. Pancreatoblastoma^[22] and adrenocortical carcinoma^[23] also show nuclear and cytoplasmic staining for β -catenin, while papillary thyroid carcinoma^[24] has been found to have cytoplasmic labeling in FAP patients.

Abnormal immunolabeling for β -catenin in PanNENs in patients with FAP also has important implications in the pathological diagnosis of these neoplasms. In the pancreas, SPNs almost always harbor β -catenin gene mutations, and these neoplasms characteristically show nuclear β -catenin expression. SPN is one of the primary differential diagnoses for PanNENs, since these tumors can have a solid growth pattern; conversely, PanNENs can occasionally show pseudopapillary areas. Additionally, the cells of both can be round-to-oval, with uniform nuclei^[25]. PanNENs and SPNs of the pancreas both express CD56 and synaptophysin. Differentiating these tumors is often based on the presence of nuclear β -catenin in

SPN. Our results suggest that, at least for patients with FAP, nuclear β -catenin labeling should not be used as a primary diagnostic criterion for differentiating these lesions, as both neoplasms will have nuclear staining. Instead, other immunostains should be performed. For example, SPNs, but not PanNENs, will express CD10, show loss of membranous E-cadherin, and be negative for chromogranin^[26].

In summary, dysregulation of the Wnt/ β -catenin pathway is present in some PanNENs. However, this signaling pathway may have different functions in PanNENs as compared to other malignancies. Additionally, PanNENs arising in patients with FAP demonstrate abnormal nuclear β -catenin accumulation, and PanNENs may be one of extraintestinal manifestations of FAP.

COMMENTS

Background

Alterations in the Wnt/ β -catenin signaling pathway may lead to tumor growth and proliferation. For example, most colonic adenocarcinomas arise from tubular adenomas through dysregulation of the Wnt/ β -catenin pathway. In addition, reduced membranous expression of β -catenin and E-cadherin is associated with a poor prognosis in most malignancies.

Research frontiers

Dysregulation of the Wnt/ β -catenin signaling pathway is seen in some neuroendocrine tumors of the gut.

Innovations and breakthroughs

Dysregulation of the Wnt/ β -catenin pathway is present in some pancreatic neuroendocrine neoplasms (PanNENs). However, compared to other malignancies, this signaling pathway may have different functions in PanNENs as membranous expression of β -catenin and E-cadherin does not have a prognostic importance in this setting. Additionally, PanNENs arising in patients with familial adenomatous polyposis (FAP) demonstrate abnormal nuclear β -catenin accumulation, and PanNENs may be one of the extraintestinal manifestations of FAP.

Applications

β -catenin may represent a potential therapeutic target in PanNENs as strong membranous β -catenin expression was observed in 55% of PanNENs and knockout of β -catenin expression has been shown to suppress tumorigenesis and growth in a mouse model. Nuclear β -catenin accumulation is seen in some PanNENs, especially those from FAP patients, which should be differentiated from solid pseudopapillary neoplasm, a low-malignant pancreatic tumor with similar morphology and expressing nuclear β -catenin. In addition, screening for PanNEN may be needed in patients with FAP.

Peer-review

This is a nice study.

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Observational Study

Activated systemic inflammatory response at diagnosis reduces lymph node count in colonic carcinoma

Rory P Kennelly, Brenda Murphy, John O Larkin, Brian J Mehigan, Paul H McCormick

Rory P Kennelly, Brenda Murphy, John O Larkin, Brian J Mehigan, Paul H McCormick, Department of Colorectal Surgery, St James Hospital, Dublin 8, Ireland

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Correspondence to: Dr. Rory P Kennelly, Department of Colorectal Surgery, St. James Hospital, James's Street, Dublin 8, Ireland. rorykennelly@rcsi.ie
Telephone: +353-87-9458963
Fax: +353-1-4103400

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Abstract

AIM: To investigate a link between lymph node yield and systemic inflammatory response in colon cancer.

METHODS: A prospectively maintained database was interrogated. All patients undergoing curative colonic resection were included. Neutrophil lymphocyte ratio (NLR) and albumin were used as markers of SIR. In keeping with previously studies, $NLR \geq 4$, albumin < 35 was used as cut off points for SIR. Statistical analysis was performed using 2 sample *t*-test and χ^2 tests where appropriate.

RESULTS: Three hundred and two patients were included for analysis. One hundred and ninety-five patients had $NLR < 4$ and 107 had $NLR \geq 4$. There was no difference in age or sex between groups. Patients with $NLR \geq 4$ had lower mean lymph node yields than patients with $NLR < 4$ [17.6 ± 7.1 vs 19.2 ± 7.9 ($P = 0.036$)]. More patients with an elevated NLR had node positive disease and an increased lymph node ratio (≥ 0.25 , $P = 0.044$).

CONCLUSION: Prognosis in colon cancer is intimately linked to the patient's immune response. Assuming standardised surgical technique and sub specialty pathology, lymph node count is reduced when systemic inflammatory response is activated.

Key words: Systemic inflammatory response; Lymph node yield; Lymph node count; Colon cancer; Colonic cancer; Neutrophil-lymphocyte ratio; Neutrophil to lymphocyte ratio; Lymph node ratio

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Core tip: A fascinating field of research is the relationship between systemic inflammatory response and loco-regional inflammatory response in colorectal cancer. This manuscript examines this relationship in a large cohort of patients from a tertiary referral centre. We measured systemic response by assessing serum markers at diagnosis and we measured local response by looking at pathological lymph node counts in the post operative surgical specimen. This is the first report to show that patients with evidence of an activated systemic inflammatory response at diagnosis have a reduced nodal harvest at time of surgery. This finding sheds light on the complex interaction between cancer and the patient. This host-tumour response forms the basis for the most advanced cancer research today.

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INTRODUCTION

Prognosis in colon cancer is largely predicted by clinico-pathological criteria, namely the TNM system which divides patients into groups based on tumour invasion, local nodal involvement and distant metastatic spread. Since its inception over 50 years ago^[1], TNM has been used to predict patient prognosis following surgery. While easy to apply and reproducible, recent research has highlighted deficiencies in the TNM system. Higher absolute lymph node counts improve patient prognosis regardless of stage^[2] and an increased lymph node ratio in node positive disease reduces prognosis^[3], two variables not measured by TNM.

Tumour-host immunological response is an important factor in prognosis at both a local and systemic level^[4,5], another feature not measured by TNM. Local response has been assessed by looking at lymphocytic infiltration of colonic tumour and an improved survival is seen in patients who have mounted a local lymphocytic response^[6]. Readily available markers of systemic inflammatory response (SIR) have prognostic value in patients undergoing surgery for colon cancer^[7]. A combination of C-reactive protein and albumin (the modified Glasgow prognostic score) and the neutrophil-lymphocyte ratio (NLR) have been proposed as surrogate markers of patient systemic immune response to cancer. A high modified Glasgow Prognostic Score (mGPS) and/or an elevated NLR (≥ 4) infer a poorer prognosis. A combination of an activated local response and the lack of an activated SIR imparts an improved prognosis^[7], strengthening the link between cancer survival and immune response at both a local and systemic level.

To date, the information gleaned from lymph node

harvest has revolved around absolute number and the presence or absence of cancer spread. Improved prognosis from higher lymph node counts has been explained as essentially a sampling error in patients with sub optimal surgical resection^[8]. This explanation cannot account for the wide variance in yields experienced even in centres where radical excisions are performed as a matter of routine^[9].

Lymph nodes are the first line of defence in the loco-regional immune response to cancer. It is possible that an increased local response to cancer results in lymph node hypertrophy resulting in an increased lymph node yield. Failure of a local response may result in less lymph node activation and allow for systemic "escape" resulting in activation of the systemic immune response. If this is the case, assuming standardised surgical and pathology techniques, a lower lymph node yield could be expected in patients with signs of an activated SIR in the preoperative period.

The aim of this study was to investigate a link between lymph node yields and systemic inflammatory response in patients undergoing surgery for colonic carcinoma.

MATERIALS AND METHODS

Patients who had undergone elective surgery for colon cancer between January 2007 and July 2013 were identified from a prospectively maintained database. Due to the known effect of radiotherapy on lymph node counts^[10], rectal cancer patients were excluded. All surgeries were performed by the same subspecialty trained consultant surgeons and all samples analyzed by the same pathology department. Laparoscopic and open procedures were included.

Using a hospital-wide electronic patient record computer system, the results of pre-operative bloods drawn either on the day prior to or morning of surgery were recorded.

Neutrophil-lymphocyte ratio was derived from a standard white cell differential and in keeping with previous studies, an NLR of ≥ 4 and albumin < 35 were used as cut off levels indicating an activated SIR.

Statistical analysis was performed using Minitab v16 (Minitab Ltd. Coventry, United Kingdom). Continuous and categorical variables were analysed with 2 sample student's *t*-test and χ^2 tests where appropriate. Statistical analysis was performed by author RPK who has training in statistical methodology.

RESULTS

Three hundred and sixteen patients were identified for inclusion in the study. Fourteen patients were excluded due to incomplete data and analysis of CRP was ultimately abandoned as it was not routinely measured pre-operatively. The majority of the patients were male (168 vs 124) and the median age was 71. One hundred and seventy-eight patients had a laparoscopic

Table 1 Demographic data of study cohort

n = 302	
Age median (range)	71 (30-99)
Sex (M/F)	168/134
Stage	
I	42
II	137
III	114
IV	9
Lymph node count	17 (3-47)
Median (range)	

M: Male; F: Female.

Table 2 Analysis of impact of operative technique and patient factors on lymph node counts

n = 302	n	Lymph node yield	P value
Surgical approach			
Lap/lap assisted	178	18.7 ± 7.3	0.828
Open/lap converted to open	124	18.5 ± 8.3	
Age			
< 70 yr	152	18 ± 7.1	0.171
≥ 70 yr	150	19.3 ± 8.2	
Sex			
M	168	18.7 ± 8.2	0.894
F	134	18.6 ± 7.1	

M: Male; F: Female.

or lap-assisted operation. One hundred and twenty-four patients had either a planned open operation or a lap to open conversion. Most patients were either stage II (45%) or stage III (37%) (Table 1). Lymph node count did not vary significantly based on operative approach, age or gender (Table 2).

Of the 302 patients included for analysis. One hundred and ninety-five patients had NLR < 4 and 107 had NLR ≥ 4. Patients with an NLR ≥ 4, (*i.e.*, activated systemic inflammatory response) had a reduced total nodal count (17.6) compared to patients with an NLR of < 4 (19.2). Hypoalbuminaemia did not impact on lymph node count. Patients with low albumin and an elevated NLR were not more likely to have a reduced lymph node count than patients with elevated NLR alone (Table 3). NLR ≥ 4 correlated with an elevated lymph node ratio. A higher proportion of patients with NLR ≥ 4 had lymph node positive disease (Table 4).

DISCUSSION

The host-tumour response is at the forefront of cancer research and exploration of the relationship between the patient and their cancer has yielded success in areas previously resistant to traditional chemotherapy^[11]. Higher lymph node counts correspond with improved prognosis in colon cancer^[8]. The relationship is preserved within cancer stages^[2]. An activated systemic inflammatory response, in the preoperative period,

Table 3 Analysis of impact of pre-operative markers of systemic inflammatory response on lymph node counts

n = 302	n	Lymph node count	P value
NLR ≥ 4	107	17.6 ± 7.1	0.036
NLR < 4	195	19.2 ± 7.9	
Albumin < 35	66	18.8 ± 7.9	0.951
Albumin ≥ 35	226	18.7 ± 7.7	
NLR ≥ 4 and albumin < 35	39	17.9 ± 8.0	0.381
NLR < 4	195	19.2 ± 7.9	

NLR: Neutrophil lymphocyte ratio.

Table 4 Analysis of association between neutrophil/lymphocyte ratio and previously described predictors of poor prognosis

n = 302	NLR < 4	NLR ≥ 4	P value
n	195	107	
Lymph node positivity	72/195 (36.9%)	50/107 (46.7%)	0.085
Lymph node ratio > 0.25	13/72 (18%)	17/50 (34%)	0.044

NLR: Neutrophil lymphocyte ratio.

correlates with poor prognosis in colon cancer^[12]. While the reason for this finding is not clearly defined it is possible that an activated SIR represents a loss of local control and is an early marker of systemic awareness of a heretofore localised cancer process. The data presented here propose a unifying explanation for these separately defined phenomena.

Previous work has shown that neutrophil lymphocyte ratios can predict survival in many cancer types^[13]. Different ratios have been tested to find a reliable measure of activated systemic inflammatory response^[14,15]. The ratio that carries the greatest level of evidence is NLR ≥ 4^[16,17]. In our patient cohort, a preoperative elevated NLR correlated with a reduced lymph node yield (19 vs 17).

The clinical significance of a two-node difference may be questioned. While the finding is statistically significant it is worth asking whether there is any useful information to be gained. Cserni *et al*^[18] examined a large cohort of colon patients in the SEER database looking at the impact of nodal yield on survival. An improvement in prognosis was observed with increasing lymph node harvest. This finding was maintained in node negative and node positive patients. Indeed it was shown that with each additional node resected there is an associated increase in survival. Given this information, the findings in the current study may well provide important prognostic information.

Many studies have examined lymph node number and oncological outcome^[19]. Lymph node counts below 12 correlate with poor outcome, a finding largely explained by variances in surgical quality^[20]. However, even in good surgical specimens, low yields are sometimes encountered and many centres have questioned the validity of a binary marker of surgical quality in colon cancer^[21,22]. Regardless of this on-running controversy,

Table 5 Description of modified glasgow prognostic score

mGPS	Score
Crp \leq 10, albumin \geq 35	0
Crp \leq 10, albumin $<$ 35	0
Crp $>$ 10	1
Crp $>$ 10 and albumin $<$ 35	2

mGPS: Modified glasgow prognostic score.

the lymph node count in both categories in the present study was well above 12 therefore surgical quality is not in question.

Other factors can impact on lymph node yield. Age, gender and laparoscopic surgery can affect lymph node count however these factors were not found to influence yields in the present study. Lymph node counts are highly dependent on quality of pathological examination and this may represent the single greatest factor that influences inter-institutional variability in nodal yields^[23]. A single sub-specialty pathology department analysed all specimens in the present study limiting the impact of the pathologist on lymph node harvest.

NLR \geq 4 was compared to established measures of patient prognosis. Positive to negative lymph node ratios are a strong prognostic indicator in colonic carcinoma^[3]. Prognosis falls as ratios increase and a cut off of 0.25 (1 in 4 nodes positive) has particular prognostic significance^[24]. In the current patient cohort, a significant link is apparent between NLR and LNR of 0.25 and above. Lymph node ratios are dependent on absolute lymph node count and it is possible that previous findings regarding lymph node ratios are in fact a surrogate for the relationship between local and systemic inflammatory responses to cancer.

How systemic inflammatory response and cancer interact is a matter of debate. One theory suggests suppression of anti-tumour immunity by recruiting regulatory T cells and activating chemokines^[25] resulting in tumour growth and spread. It has been proposed that SIR acts as a marker of pre-existing comorbid disease and high risk pathological tumour characteristics (*i.e.*, lymphovascular invasion, peritoneal involvement.) Interestingly, studies designed to explore this question have not shown a link^[7].

A significant relationship was not shown for albumin and lymph node yield in our study. This was not an unexpected finding given previous work by the Glasgow group. The original Glasgow Prognostic Score assigned a score of 1 for hypoalbuminaemia. Further work showed no prognostic significance for hypoalbuminaemia alone^[26] (Table 5). The modified score only attributes a score for a low albumin if there is a concomitant elevated C reactive protein. As CRP was not measured in the present study, albumin levels did not provide any added benefit.

Our study has limitations. Due to the nature of the institutional database, patient information is not available regarding co-morbid conditions. However, all

included patients represent elective surgical candidates, deemed fit for surgery with no acute illness precluding an operation. The exclusion of emergency procedures lends some security regarding confounding causes of elevated markers of systemic inflammatory response. We were unable to complete our analysis of CRP due to levels not being available on the majority of our patients. While a pre-operative CRP is desirable, it does not form part of the standard pre-operative work up for our patients and is unlikely to be a routine test performed in most institutions. All patients undergo a full blood count prior to surgery therefore neutrophil lymphocyte ratio should be a readily available measure in all hospitals.

The primary focus of our study is on the peri-operative period but there is much scope for further research in this field. We have not reported long term outcomes and survival data in our patient cohort as the median follow-up time period in the study group was not of sufficient length. These patients will be followed prospectively and outcome will be reported in future planned analyses.

This study, albeit preliminary, may yield important prognostic value for our patients. Pre-operative identification of SIR could potentially alter treatment decisions, *i.e.*, serve as an indication for adjuvant chemotherapy, determine frequency of clinical and radiological surveillance as well as confer additional prognostic information.

Although the mechanism remains poorly understood, it is evident that there is an intrinsic link between the host immune response and patient outcome in colon cancer. The results of this study indicate that lymph node count is reduced where systemic inflammatory response is activated in colon cancer. We propose that neutrophil-lymphocyte ratio can therefore be used to predict nodal yield and provide additional valuable information regarding prognosis.

COMMENTS

Background

A host systemic inflammatory response to a tumour has been shown to negatively affect prognosis in patients undergoing surgery for colonic carcinoma. Surrogate markers of systemic inflammatory response researched to date have included routine laboratory investigations such as serum albumin, C-reactive protein and the neutrophil-lymphocyte ratio which together comprise the modified glasgow prognostic score. A more pronounced systemic inflammatory response as shown by a high glasgow prognostic score infers a poor prognosis in colon cancer. Lymph node count in colon cancer has long been established as a surgical quality indicator. Research has shown that a higher lymph node yield confers improved prognosis, a phenomenon which appears to be independent of nodal disease burden. This has previously been attributed to selection bias with lower nodal yield thought to be associated with the quality of the operating surgeon and examining histopathologist. However, this does not explain the large variation in nodal yields that sub-specialist centres encounter, where departments of highly specialised surgeons and histopathologists are routinely involved in multi-disciplinary cancer care.

Research frontiers

The host-tumour immune response is a fascinating field of cancer research

that promises success in offering new treatment modalities in areas previously resistant to more conventional therapies. Current research supports that neutrophil lymphocyte ratios and similar markers of systemic inflammatory response can predict survival in many cancer types, likewise with local inflammatory response as characterised by tumour-infiltrating lymphocytes. These phenomena are at present not incorporated into any staging or classification system for colon cancer, despite the overwhelming evidence that they provide invaluable prognostic information. Considering this wealth of information, current research is exploring the exciting realm of immunotherapy, for which many clinical trials are underway.

Innovations and breakthroughs

The relationship of prognosis in colon cancer with systemic inflammatory response has been researched to date, as has that with lymph node yield. To the authors' knowledge however, this is the first study to examine a link between these two phenomena. The study supports that poor prognosis in colon cancer is due in part to failed or impeded local immune response to a tumour and subsequent systemic loss of control. The findings may also explain why lymph node counts are often highly variable, even in sub-speciality tertiary referral centres where variance in surgical and histopathological quality is unlikely to be a major confounding factor. This research suggests that the current focus on an absolute number of nodes as an indicator of quality is perhaps flawed, and a change in perspective with regards to lower-than-expected nodal yields should be employed.

Applications

The results of this study may offer important prognostic value for the patients. In identifying SIR pre-operatively, the authors can identify patients in whom lower nodal yield and a poorer prognosis is anticipated. This may potentially alter post-operative course of treatment, *i.e.*, serve as an indication for adjuvant chemotherapy, determine frequency of clinical and radiological surveillance, as well as pave the way for the development of novel pre-operative immunotherapeutic interventions.

Terminology

Systemic inflammatory response (SIR): Reaction to an infectious or non-infectious stimulant by activation of whole body inflammatory cascade following failure of local immunological homeostasis; lymph node yield: Number of lymph nodes identified in pathological specimen following oncological resection; neutrophil-lymphocyte ratio (NLR): Ratio of circulating neutrophils to lymphocytes. A circulating neutrophilia relative to a lymphopaenia is a surrogate marker of systemic inflammatory response. In keeping with previous studies, we chose a NLR of $\geq 4:1$ as the cut-off for SIR; lymph node ratio: Ratio of positive to negative nodes in a specimen.

Peer-review

The manuscript describes findings of statistical-analysis to assess a link between lymph node yields and systemic inflammatory response in patients undergoing for colon carcinoma. Authors suggest an intrinsic link between the host immune-response and patient outcome in colon cancer, and propose that neutrophil-lymphocyte ration can be used to predict nodal yield and provide additional valuable information regarding prognosis. This article is concisely written, and contains interesting findings.

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Observational Study

Academic hospital staff compliance with a fecal immunochemical test-based colorectal cancer screening program

Georgia Vlachonikolou, Paraskevas Gkolfakis, Athanasios D Sioulas, Ioannis S Papanikolaou, Anastasia Melissaritou, Giannis-Aimant Moustafa, Eleni Xanthopoulou, Gerasimos Tsilimidos, Ioanna Tsironi, Paraskevas Filippidis, Chrysoula Malli, George D Dimitriadis, Konstantinos Triantafyllou

Georgia Vlachonikolou, Paraskevas Gkolfakis, Athanasios D Sioulas, Ioannis S Papanikolaou, Anastasia Melissaritou, Giannis-Aimant Moustafa, Eleni Xanthopoulou, Gerasimos Tsilimidos, Ioanna Tsironi, Paraskevas Filippidis, Chrysoula Malli, George D Dimitriadis, Konstantinos Triantafyllou, Hepatogastroenterology Unit, Second Department of Internal Medicine and Research Institute, Attikon University General Hospital, Medical School, University of Athens, 12462 Athens, Greece

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Correspondence to: Athanasios D Sioulas, MD, PhD, Hepatogastroenterology Unit, Second Department of Internal Medicine and Research Institute, Attikon University General Hospital, Medical School, University of Athens, 1 Rimini Street, 12462 Athens, Greece. athsioulas@yahoo.gr
Telephone: +30-210-5832090
Fax: +30-210-5326422

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Abstract

AIM: To measure the compliance of an Academic Hospital staff with a colorectal cancer (CRC) screening program using fecal immunochemical test (FIT).

METHODS: All employees of "Attikon" University General Hospital aged over 50 years were thoroughly informed by a team of physicians and medical students about the study aims and they were invited to undergo CRC screening using two rounds of FIT (DyoniFOB® Combo H, DyonMed SA, Athens, Greece). The tests were provided for free and subjects tested positive were subsequently

referred for colonoscopy. One year after completing the two rounds, participants were asked to be re-screened by means of the same test.

RESULTS: Among our target population consisted of 211 employees, 59 (27.9%) consented to participate, but only 41 (19.4%) and 24 (11.4%) completed the first and the second FIT round, respectively. Female gender was significantly associated with higher initial participation ($P = 0.005$) and test completion - first and second round - ($P = 0.004$ and $P = 0.05$) rates, respectively. Physician's (13.5% *vs* 70.2%, $P < 0.0001$) participation and test completion rates (7.5% *vs* 57.6%, $P < 0.0001$ for the first and 2.3% *vs* 34%, $P < 0.0001$ for the second round) were significantly lower compared to those of the administrative/technical staff. Similarly, nurses participated (25.8% *vs* 70.2%, $P = 0.0002$) and completed the first test round (19.3% *vs* 57.6%, $P = 0.004$) in a significant lower rate than the administrative/technical staff. One test proved false positive. No participant repeated the test one year later.

CONCLUSION: Despite the well-organized, guided and supervised provision of the service, the compliance of the Academic Hospital personnel with a FIT-based CRC screening program was suboptimal, especially among physicians.

Key words: Colorectal cancer; Screening; Academic hospital staff; Fecal immunochemical test; Compliance

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Core tip: The fecal immunochemical test (FIT) for hemoglobin represents an attractive alternative to colonoscopy for population colorectal cancer (CRC) screening programs, since it combines high diagnostic effectiveness and wide acceptance, probably in relation to its non-invasive nature. Accordingly, we assessed the compliance of the staff of an Academic Hospital with a CRC screening program by means of FIT. Despite the well-organized, guided and supervised provision of the service, our results indicated very low overall uptake rate of the test and, interestingly, significantly less compliance of physicians and nurses as compared to the rest Hospital personnel.

Vlachonikolou G, Gkolfakis P, Sioulas AD, Papanikolaou IS, Melissaritou A, Moustafa GA, Xanthopoulou E, Tsilimidos G, Tsironi I, Filippidis P, Malli C, Dimitriadis GD, Triantafyllou K. Academic hospital staff compliance with a fecal immunochemical test-based colorectal cancer screening program. *World J Gastrointest Oncol* 2016; 8(8): 629-634 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v8/i8/629.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v8.i8.629>

INTRODUCTION

Colorectal cancer (CRC) screening using colonoscopy

in average-risk adults aged more than 50 years (or in younger subjects with a family history of CRC) decreases the incidence and mortality rates for CRC through detection and removal of premalignant lesions^[1]. Given that the compliance of the general population in colonoscopy screening programs is low, other screening modalities have been implemented as well^[2]. The high diagnostic performance of the fecal immunochemical test (FIT) as compared to other CRC screening tests combined with its superior acceptability as a non-invasive screening measure^[3,4] result in being widely recommended for CRC screening. In addition, a positive FIT-test could represent a stronger motivation for patients to participate in colonoscopy-based CRC screening programs. However, there are no studies to confirm or reject the latter assumption, especially among health care providers.

The aim of this study was to evaluate the compliance of the staff of an Academic Hospital with a CRC screening program using FIT.

MATERIALS AND METHODS

During an initiation event at the Conference Auditorium of our Hospital, expert Gastroenterologists of the Hepatogastroenterology Unit of the Second Department of Internal Medicine and Research Institute, Medical School, Athens University presented the study aims and outlines to "Attikon" University General Hospital employees. Employees aged 50 years or older were identified through the Human Resources Department of the Hospital. They were thoroughly informed by a team of physicians and medical students and they were invited to be screened for CRC by means of two rounds of FIT (DyoniFOB® Combo H, DyoniMed SA, Athens, Greece).

Candidates received directly as well as by mail a letter explaining the rationale of the program, an informed consent form to sign before the procedure and a questionnaire that covered demographic data of the participants and family history for CRC or colonic polyps. In addition, a FIT kit was provided for free with instructions for collecting the stool sample and returning the kit for processing at the Hepatogastroenterology Unit of our Hospital. Upon the return of the first kit, participants were requested to repeat the procedure with a second kit. Participants were informed on their results and those who tested positive were offered colonoscopy. One year after completing the two rounds, all participants were contacted by telephone in order to be reminded of the need to repeat screening.

The study outcome was to evaluate the compliance of the Hospital personnel with a FIT-based screening program by measuring the personnel participation rates.

The study protocol and the participants' informed consent form had been approved by the Attikon University General Hospital Ethics Committee.

Statistical analysis

Categorical variables presented as value (%) were

Table 1 Baseline characteristics of study participants and non-participants

Characteristics	Participants <i>n</i> (%)	Non-participants <i>n</i> (%)
Gender		
Male	23 (39)	93 (61)
Female	36 (61)	59 (39)
Employee status		
Physicians	18 (30)	115 (76)
Nurses	8 (14)	23 (15)
Administrative and technical staff	33 (56)	14 (9)
Educational level		
High	20 (34)	
Intermediate	20 (34)	
Basic	19 (32)	
Family history of colonic polyps		
Yes	14 (24)	
No	45 (76)	

analyzed using a χ^2 test or Fisher's exact test with the Yates correction, as needed. *P* value < 0.05 indicated statistical significance.

The statistical methods used in the present study were reviewed by Professor Konstantinos Triantafyllou, Medical School, National and Kapodistrian University, Athens, Greece who has been trained in biostatistics.

RESULTS

The study was carried out from September 2013 to March 2015.

Target population - initial willingness to participate

The target population - 116 males and 95 females - consisted of 133 physicians, 31 nurses and 47 administrative and technical staff members.

Fifty-nine (27.9%) employees agreed to participate and signed the informed consent form; 23 (19.8%) males vs 36 (37.9%) females (*P* = 0.005). All of them were asymptomatic, none had family history of CRC but 14 had a family history of colonic polyps. Participants as well as non-participants baseline characteristics are presented in Table 1.

Physicians showed significantly lower willingness to participate (18 of 133 or 13.5%) in comparison to the administrative and technical staff group (33 of 47 or 70.2%, *P* < 0.0001) and the nurses group (8 of 31 or 25.8%, *P* = 0.03).

1st FIT round completion

Among the participants, only 41 returned the first kit for evaluation indicating an overall completion rate of 19.4%; 15% and 54% of the participants with and without a family history of colonic polyps, respectively (*P* = 0.74). The first FIT round was completed by significantly more female in comparison to male participants (27 of 95 or 28.4% vs 14 of 116 or 12.1%, *P* = 0.004).

As shown in Figure 1, there was higher test com-

pletion rate in the administrative and technical staff group (57.6%) in comparison to the physicians' and nurses groups (7.5% and 19.3%; *P* < 0.0001 and *P* = 0.004, respectively).

2nd FIT round completion

The overall completion rate for the second FIT test was 11.4% (Figure 1). Only 24 of the 40 employees who received the second test kit returned it for evaluation; 5% with family history of colonic polyps and 34% without (*P* = 0.06).

Similarly to the first round, significantly more female participants completed both first and second FIT rounds in comparison to male participants (16 of 95 or 16.8% vs 8 of 116 or 6.8%, *P* = 0.05).

Figure 1 shows that the physicians' group second FIT round completion rate (2.3%) was the lowest in comparison to the nurses and the administrative/technical staff groups (16.1% and 34%; *P* = 0.007 and *P* < 0.0001, respectively). No difference (*P* = 0.11) was detected between nurses and administrative/technical staff groups in second FIT round completion rates.

Out of the 65 performed FIT examinations, a single test was proved false positive based on the colonoscopy that followed. One year later, we informed by telephone the 41 study participants who completed at least the first FIT round for the need to repeat screening. Twenty-two of them denied further screening since they believed that the negative results of their past tests would last forever, while 18 were willing to undergo screening annually. Only one of the participants accepted to be screened, but even this subject did not finally proceed to being tested.

DISCUSSION

A fundamental aspect of all screening programs and a prerequisite for their success is participation and compliance of the target population. Even though several tests for CRC screening exist, their participation rates remain suboptimal^[5,6]. Population screening programs include endoscopic procedures (*i.e.*, colonoscopy, flexible sigmoidoscopy or colon video capsule endoscopy), radiologic tests, including barium enema and computed tomography-colonography and fecal tests, such as the fecal occult blood test (FOBT) and FIT. FIT, being a non-invasive screening test could serve as an attractive alternative to colonoscopy for CRC screening, since it combines effectiveness and wide acceptance, probably due to its non-invasive nature. This has resulted in its widespread adoption in countries with organized CRC screening programs^[3,7]. Recently, a CRC screening program using biannual FIT that was conducted in Italy revealed not only a reduction by 30% in CRC mortality of the general population but also a 10% decrease in CRC incidence after 8 years of study^[8].

In the present study we evaluated for the first time, the compliance of the staff of an Academic Hospital to a non-invasive CRC screening program, using FIT.

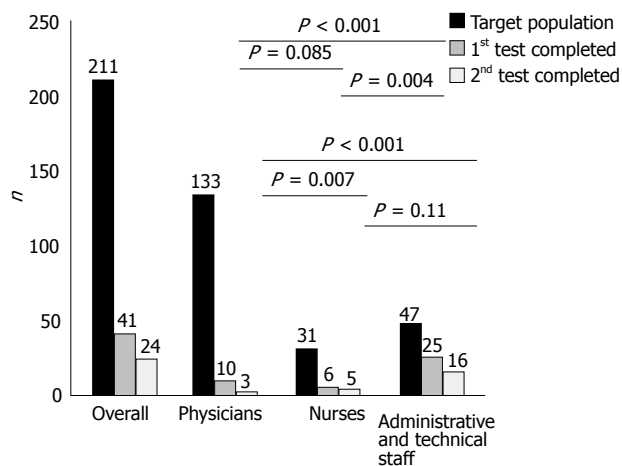


Figure 1 Significantly less physicians and nurses completed the two rounds of the fecal immunohistochemical test compared to the hospital administrative and technical personnel.

We proceeded with two rounds of FIT testing in order to obtain better test sensitivity without compromising overall test uptake, based on the results of a study that showed that the participation rate was similar when completing one or two samples of the FIT test^[9].

Three randomized trials have indicated a participation rate for FIT approximately 60%, higher than that of the gFOBT^[9-11], while the reported overall participation rate in the FIT studies ranges widely from 17%-77%^[8-12].

Our results showed that: (1) the overall uptake of the FIT was within the lower reported rates worldwide, even in subjects with positive family history for colonic polyps; (2) significantly less physicians and nurses completed the FIT screening program compared to the hospital administrative and technical personnel; (3) female individuals showed both a higher willingness to participate and a higher participation rate after the two FIT rounds; and (4) participants were reluctant to continue screening annually.

The screening completion rate of our population was deemed lower - 19.4% for the first and 11.4% for the second round, respectively - than expected for a well organized, controlled and supervised in the participants' occupational environment program. As shown in Figure 1 the low overall screening completion rate is a consequence of physicians - mainly - and nurses' low completion rates. These disappointing rates, reaching the lowest 2.25% among physicians for the second test round, may indicate a major study limitation. Issues like embarrassment and discomfort may have eventually influenced the compliance of the medical and nursing staff, since there was an interaction between the providers of the screening test and the participants in the same practice. Low participation rates among hospital employees were shown during a CRC screening program that consisted of a questionnaire, followed by an endoscopic procedure among the personnel of an Academic Medical Center in Israel. The program yielded

similar results to ours, as merely 24.7% of the staff that was invited to participate completed the questionnaire; 16.2% of them agreed to be further tested^[13].

Providing the tests by dedicated unrelated to the participants services, *e.g.*, committees organized by the Ministry of Health, although more complex, time consuming and expensive, could eventually, overcome this obstacle.

On the other hand, in a cancer screening program for cervical, breast, colorectal and prostate cancers in health care workers in Italy, the participation rate was higher than that of the general population with an overall compliance of 83.8%^[14]. Moreover, it has been suggested that compliance to other cancer screening programs, such as screening for prostate cancer in men and breast cancer in women has been positively associated with compliance to CRC screening^[15]. Therefore, regional and site specific issues may also be important for CRC screening program successful implementation.

Several additional factors influence CRC-screening uptake; awareness for CRC, different screening modalities - invasive and non-invasive - as well as the way in which the latter are offered to the population. Interestingly, almost half of our screening subjects who completed at least the first test round denied further screening due to ignorance for repeating CRC screening annually. Similarly, in a recent study among Greek medical students it was shown that there is lack of information about CRC screening^[16]. Awareness of the risk factors for CRC, signs and symptoms as well as the prognosis of the disease are all factors that can increase screening uptake^[17,18]. European studies have reported low awareness about CRC compared to other cancers such as lung cancer and prostate in men and breast cancer in women^[19].

Low educational level has been associated with low participation rates in CRC screening programs^[17], whereas it has also been demonstrated that there is a positive association between low socio-economic status with low screening compliance rates^[20]. Interestingly, the majority (55%) of employees who initially consent to be screened were at the intermediate of basic educational level (four nurses and 28 administrative/technical staff members) compared to those with high educational level (18 physicians, 4 nurses and 5 administrative/technical staff members). This discordance with the existing literature could, at least partially, be explained by the small number of participants and the aforementioned issues of embarrassment and discomfort.

In a recent investigation regarding screening programs for breast and cervical cancers at a University Hospital in Turkey among female health care personnel, it was concluded that female physicians and nurses don't pay the adequate attention to these screening programs^[21]. Nevertheless, in our study the completion rate of both FIT rounds was higher among the female compared to that of the male hospital staff (16.8% vs 6.8%).

Our study is limited by the small, very selected (emplo-

yeas of an academic health care provision) sample, which is not representative of the general population, and our results might not be directly extrapolated to other medical facilities worldwide. Another study caveat is the limited test candidate's baseline information provided by the Human Resources Department of the Hospital that precluded the identification of predictors of participation in the program beyond that of female gender. Finally, we acknowledge as limitations of our study the fact that no data was captured regarding possible participants' CRC screening in the past or current bowel symptoms. Identification of the reasons for non-compliance would have also been of great interest, although not available in the present study.

In conclusion, the compliance of the Attikon University General Hospital personnel with a FIT-based CRC screening program was suboptimal, especially among physicians, despite the well-organized, guided and supervised provision of the service.

ACKNOWLEDGMENTS

DyonMed SA, Athens, Greece kindly provided the fecal immunochemical test (DyoniFOB® ComboH) for the study purposes.

COMMENTS

Background

Colorectal cancer (CRC) screening by means of colonoscopy decreases the incidence and mortality rates for CRC through early detection and removal of premalignant lesions. Fecal immunochemical test (FIT) is also implemented as a CRC screening modality and has the advantage of being a non-invasive tool. The authors aimed to assess the compliance of an Academic Hospital staff with a CRC screening program using FIT.

Research frontiers

According to the results, the compliance of the Academic Hospital personnel with a FIT-based CRC screening program was suboptimal, especially among physicians.

Innovations and breakthroughs

Although it is established that low educational level is associated with low participation rates in CRC screening programs, the results of this study indicate that those with high educational level exhibited less compliance as compared to those of low-intermediate one.

Applications

The results of the present study may enhance the efforts for better population awareness regarding CRC screening.

Terminology

The FIT is a non-invasive test that detects human hemoglobin in stool and represents an efficacious means of CRC screening. Compliance refers to the individual's adherence to a recommended diagnostic or therapeutic procedure.

Peer-review

It is an interesting manuscript on important topic. The paper is well-written.

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Randomized Clinical Trial

Utility of different serum fibrosis markers in diagnosing patients with chronic pancreatitis and pancreatic adenocarcinoma

Anna Kozak, Renata Talar-Wojnarowska, Aleksandra Kaczka, Anna Borkowska, Leszek Czupryniak, Ewa Małecka-Panas, Anita Gąsiorowska

Anna Kozak, Renata Talar-Wojnarowska, Aleksandra Kaczka, Ewa Małecka-Panas, Anita Gąsiorowska, Department of Digestive Tract Diseases, Medical University of Lodz, 90-153 Lodz, Poland

Anna Borkowska, Leszek Czupryniak, Department of Diabetology and Metabolic Diseases, Medical University of Lodz, 92-213 Lodz, Poland

Author contributions: Kozak A performed the systematic search of the literature, conducted statistical analysis and wrote the manuscript; Talar-Wojnarowska R, Kaczka A and Czupryniak L contributed equally to the acquisition and analysis of data; Borkowska A carried out the laboratory part of the project; Małecka-Panas E revised the article critically; Gąsiorowska A designed the study and reviewed the final version of the text.

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Correspondence to: Kozak Anna, MD, Department of Digestive Tract Diseases, Medical University of Lodz, Kopcinskiego 22, 90-153 Lodz, Poland. annak.kozak@gmail.com
Telephone: +48-426-776667
Fax: +48-678-6480

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Abstract

AIM: To estimate the levels of serum cytokines in chronic pancreatitis (CP) and pancreatic ductal adenocarcinoma (PDAC) patients in order to evaluate their usefulness as possible biomarkers.

METHODS: The study included 167 Caucasian patients: 74 with PDAC (28 men and 42 women, aged 30-88 years), 78 with CP (50 men and 21 women, aged 20-79 years) and 15 age-matched healthy controls hospitalized in the Department of Digestive Tract Diseases, Medical University of Lodz, Poland between 2006 and 2013. Serum MCP-1, transforming growth factor (TGF)- β 1, HA and s-Fr were measured in patients with CP ($n = 78$), PDAC ($n = 74$) and healthy controls ($n = 15$) using ELISA (Corgenix United Kingdom Ltd R and D Systems). The severity of CP was assessed according to the Cambridge classification.

RESULTS: Both patients with CP and PDAC had a significantly higher mean TGF- β 1 serum level (1066 ± 582

and 888 ± 356 vs 264 ± 93 , $P < 0.0001$), mean s-Fr (2.42 ± 1.385 and 2.41 ± 1.275 vs 0.6 ± 0.370 , $P < 0.0001$) and mean HA (199 ± 254 and 270 ± 358 vs 40 ± 26 , $P < 0.0001$) compared to controls. There was no difference in mean MCP-1 between all the groups. There were no significant differences in any cytokine levels between the PC and PDAC groups. No significant differences between serum cytokines depending on age, gender or smoking status were found in CP patients. Mean s-Fr concentration was significantly higher in CP, lasting longer than 5 years compared to those with a shorter disease clinical course (2.639 ± 1.125 vs 1.870 ± 0.970 , $P < 0.03$). There was no correlation between tumor size, localization or TNM classification and serum TGF- β 1, MCP-1, s-Fr and HA levels in patients with PDAC. No significant differences between cytokines depending on diabetes presence in CP were found. Nevertheless, mean serum TGF- β 1 concentration in PDAC patients was higher in those with diabetes compared to the remaining group (986 vs 839 , $P = 0.043$).

CONCLUSION: Serum TGF- β 1, s-Fr and HA may be considered additional diagnostic markers of CP and PDAC. TGF- β 1 may be useful to predict endocrine insufficiency in PDAC.

Key words: Fibrosis; Chronic pancreatitis; Soluble type fractalkine; Pancreatic adenocarcinoma; Transforming growth factor beta-1; Hyaluronic acid; Monocyte chemoattractant protein-1

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Core tip: Fibrosis begins in an early stage of the disease in chronic pancreatitis and in pancreatic ductal adenocarcinoma. Cytokines such as transforming growth factor beta-1, hyaluronic acid, soluble fractalkine and monocyte chemoattractant protein-1 take part in connective tissue proliferation. It is essential to diagnose pancreatic diseases in the early stage in order to start an appropriate therapy. Cytokines can be useful clinical biomarkers.

Kozak A, Talar-Wojnarowska R, Kaczka A, Borkowska A, Czupryniak L, Małecka-Panas E, Gąsiorowska A. Utility of different serum fibrosis markers in diagnosing patients with chronic pancreatitis and pancreatic adenocarcinoma. *World J Gastrointest Oncol* 2016; 8(8): 635-641 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v8/i8/635.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v8.i8.635>

INTRODUCTION

Pancreatic fibrosis is the characteristic feature of chronic pancreatitis (CP) and pancreatic ductal adenocarcinoma (PDAC)^[1]. The inflammation in CP leads to irreversible destruction of pancreatic parenchyma, interstitial fibr-

osis and the following loss of exocrine and endocrine tissue^[2]. Consequently, CP significantly impairs quality of life^[3]. The mechanisms which induce fibrosis in PDAC and CP are not completely elucidated but involve persistent activation of pancreatic stellate cells (PSC). The fibrosis process starts in the early stage of CP and progresses with the duration of the disease^[4]. Accurate biological and functional markers of early stage CP are strongly needed for early treatment and prevention of complications. Furthermore, as patients with longstanding CP are at a markedly increased risk of developing pancreatic cancer, diagnosing CP at an early stage may result in cancer prevention^[5]. According to Whitcomb *et al*^[6], pancreatic fibrosis itself in chronic CP is a major risk factor for pancreatic cancer. Interstitial fibrosis markers could play a potential role as early stage CP diagnostic markers.

PDAC coexists with CP in 20%-35% of cases. In these circumstances, diagnostic tools such as US, EUS and CT are insufficient because their sensitivity and specificity is unsatisfactory^[7]. Several early stage markers have been proposed in CP and PDAC^[8]. Transforming growth factor beta-1 (TGF- β 1) and extracellular matrix proteins, especially hyaluronic acid (HA) and pro-collagen III peptide (P-III-P), are suggested to play a pivotal role in early stage pancreatitis^[7,9]. TGF- β 1 expression was found in adjacent areas to pancreatic fibrosis in PSC and acinar cells. TGF- β 1 is thought to regulate the synthesis of collagen in PSC^[10,11]. TGF- β 1 contributes to healing, acts as monocyte and fibroblast chemoattractant factors and increases synthesis and secretion of extracellular matrix^[12]. In addition, TGF- β 1 plays a role in wound healing, stimulating growth of connective tissue and scar formation^[13,14]. Basically, the same process takes place in pancreatic tissue when areas of focal necrosis are replaced by fibrosis. High expression of TGF- β 1 is observed in the fibrosis area in models of chronic pancreatitis in rats^[15]. TGF- β 1 up-regulates collagens at the same time as down-regulating collagenases and stromelysins in fibroblasts^[16]. Inhibition of the TGF- β 1 signaling pathway significantly reduced fibrosis in experimental pancreatitis models^[17,18].

Soluble fractalkine (s-Fr) was also proposed as a marker of early stage pancreatic fibrosis. S-Fr activates multiple signaling cascades in PSCs. This cytokine directly induces PSC proliferation without increasing the release of inflammatory mediators^[19]. S-Fr is secreted from PSC in response to various stimuli and contributes to the CP progression, also enhancing the migration of inflammatory cells, inducing vascular smooth muscle and endothelial cell proliferation^[20].

Monocyte chemoattractant protein-1 (MCP-1) acts like a strong chemoattractant factor for monocytes, macrophages and lymphocytes. Recent studies show that blocking MCP-1 activity suppresses the progression of experimental CP induced with dibutyltin dichloride in rats^[21]. Decreasing MCP-1 and TGF- β levels with anti-inflammatory drugs resulted in a reduced severity of CP, including the extent of inflammatory cell infiltration

and stromal fibrosis in a caerulein-induced CP mouse model^[22].

In addition to CP, pancreatic fibrosis also plays a crucial role in PDAC. Progressive fibrosis associated with PDAC may have diagnostic and prognostic relevance. Fibrotic degeneration is considered one of the major causes of chemotherapy resistance. New diagnostic markers are strongly needed in order to diagnose PDAC at an early stage.

Diabetes develops in more than half of patients with chronic pancreatitis^[23]. Most patients with pancreatic tumors have impaired glucose tolerance^[24]. Endocrine and exocrine insufficiency are both consequences of advanced fibrosis.

The aim of this study was to determine the potential diagnostic value of early stage fibrosis markers such as chemokines (s-Fr and MCP-1), cytokines (TGF- β 1) and markers of extracellular matrix (HA) in patients with CP and PDAC. Moreover, the usefulness of these cytokines as possible biomarkers of endocrine pancreatic function in CP and PDAC was considered.

MATERIALS AND METHODS

Patient selection

The study included 167 Caucasian patients: 74 with PDAC (28 men and 42 women, aged 30-88 years), 78 with CP (50 men and 21 women, aged 20-79 years) and 15 age-matched healthy controls hospitalized in the Department of Digestive Tract Diseases, Medical University of Lodz, Poland between 2006 and 2013. Written informed consent was obtained from all patients. The study protocol was approved by the ethics committee of Lodz Medical University.

Study design

The diagnosis of pancreatic tumors was established by ultrasound, computed tomography or endoscopic methods (EUS, ECPW). Histological samples were obtained by different methods: Endoscopic ultrasound-guided fine needle aspiration, brushing at ECPW or surgical sampling. The size of the tumor ranged from 1.5 to 6.0 cm in PDAC patients. At the time of diagnosis most tumors were over 3 cm in size (55.5%) and localized in the head of pancreas (86%). PDAC patients were classified according to the TNM classification: T1 = 21 patients (28%); T2 = 31 patients (42%); T3 = 10 patients (13%); T4 = 12 patients (16%); N0 = 55 patients (74%); N1 = 19 patients (26%); M0 = 47 patients (64%); and M1 = 27 patients (36%). Twenty-eight (38%) of PDAC patients had coexisting CP. Twenty-four PDAC patients (56%) had an average weight (BMI 18.5-24.99; mean 23.1 kg/m²), 11 patients were obese, 2 were overweight and 6 had a BMI under 18.5. Diabetes mellitus, if not diagnosed earlier, was detected with an abnormal glucose tolerance test (OGTT) in 24 PDAC patients.

The diagnosis of CP was determined by standard imaging criteria and clinical course. The diagnosis of

CP was based on the following findings: Calcifications, pancreatic stones, dilatation, stenosis or cyst formation of the main pancreatic duct and its branches shown by ultrasound, computed tomography or endoscopic retrograde pancreatography. Most CP patients had a history of alcohol abuse (42 patients, 59.2%). CP patients were classified into two groups according to Cambridge classification^[25]: 23 patients with mild CP (normal + equivocal + mild) and 43 with severe CP (moderate + marked). Among the studied CP patients, most (31 patients, 77%) had an average weight (BMI 18.5-24.99, mean 21.1 kg/m²), 4 were obese, none were overweight and 5 had a BMI under 18.5. Mean CP duration was 5.9 years (range 1-19). Diabetes mellitus coexisted in 30 patients (42.5%). All cases were DM type 2. We also evaluated the potential prognostic value of tested substances in PDAC, correlating survival with TGF- β 1, MCP-1, s-Fr and HA serum levels.

Peripheral venous blood samples were obtained from all analyzed patients at the time of hospital admission. Serum TGF- β 1, MCP-1, s-Fr and HA concentrations were determined with ELISA (Corgenix United Kingdom Ltd R and D Systems). Correlation of chemokines, cytokines and clinical data at the time of diagnosis in CP and PDAC patients was evaluated.

Statistical analysis

Statistical analysis and graphical data presentation were performed using PANDAS (Python Data Analysis Library) and Matplotlib in the IPython Notebook environment. The significance of differences between the groups was assessed using non-parametric methods (Mann-Whitney test). Two-sided *P* values were computed and a difference was considered statistically significant at *P* \leq 0.05.

RESULTS

Patients with CP and PDAC had a significantly higher plasma HA level (199 ± 254 and 270 ± 358 vs 40 ± 26 ng/mL, *P* < 0.0001) (Figure 1) compared to the controls. In CP patients, the TGF- β 1 level was 1066 ± 582 pg/mL and in PDAC patients, 888 ± 356 pg/mL, which was significantly higher than in healthy controls, 264 ± 93 pg/mL, *P* < 0.0001 (Figure 2). Our study showed a significant difference between s-Fr level in CP and PDAC patients compared to controls (2.42 ± 1.385 and 2.41 ± 1.275 vs 0.6 ± 0.370 ng/mL, *P* < 0.0001) (Figure 3).

MCP-1 level did not significantly differ between CP (313 ± 453 pg/mL) and PDAC (318 ± 481 pg/mL) patients or controls (252 ± 288 pg/mL), (*P* = NS) (Figure 4).

There was no significant differences between s-Fr, HA, TGF- β 1 and MCP-1 levels in CP and PDAC patients.

In CP patients, only the s-Fr level depended on the duration of the disease (Figure 5). In patients with a CP duration of more than 5 years, serum level of s-Fr

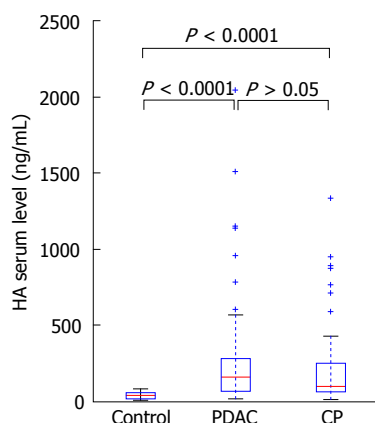


Figure 1 Serum hyaluronic acid in pancreatic adenocarcinoma and chronic pancreatitis patients compared to healthy controls. HA: Hyaluronic acid; PDAC: Pancreatic adenocarcinoma; CP: Chronic pancreatitis.

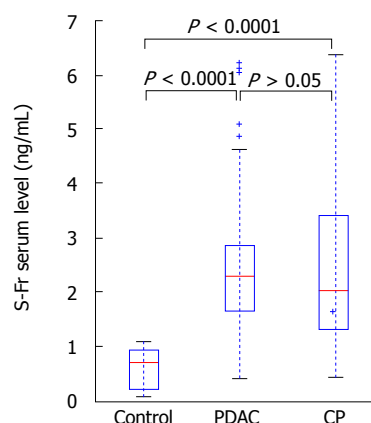


Figure 3 Serum soluble fractalkine in pancreatic adenocarcinoma and chronic pancreatitis patients compared to healthy controls. s-Fr: Serum soluble fractalkine; PDAC: Pancreatic adenocarcinoma; CP: Chronic pancreatitis.

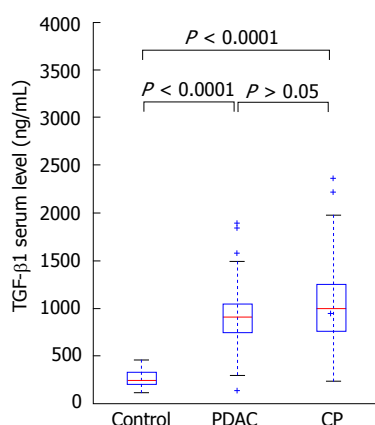


Figure 2 Serum transforming growth factor-β1 in pancreatic adenocarcinoma and chronic pancreatitis patients compared to healthy controls. PDAC: Pancreatic adenocarcinoma; TGF-β1: Transforming growth factor-β1; CP: Chronic pancreatitis.

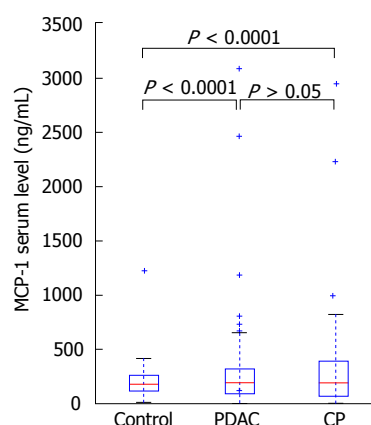


Figure 4 Serum monocyte chemoattractant protein-1 in pancreatic adenocarcinoma and chronic pancreatitis patients compared to healthy controls. MCP-1: Monocyte chemoattractant protein - 1; PDAC: Pancreatic adenocarcinoma; CP: Chronic pancreatitis.

was significantly higher compared to CP patients with a shorter disease clinical course (2.639 ± 1.125 vs 1.87 ± 0.970 , $P < 0.03$).

In patients with PDAC and diabetes, serum level of TGF-β1 was significantly higher than in PDAC without endocrine insufficiency (986 ± 341 vs 844 ± 364 , $P < 0.05$) (Figure 6).

No significant differences between serum level of TGF-β1, s-Fr, HA or MCP-1 depending on etiology, age, gender, endocrine insufficiency or Cambridge score in CP patients were found.

There was no correlation between tumor size, localization, TNM classification or coexisting CP and serum TGF-β1, MCP-1, s-Fr and HA levels in patients with PDAC. No tested substances in PDAC patients was related to survival.

The levels of tested substances also did not correlate with each other in both pancreatic diseases. The relationship of the levels of fibrosis markers and desmoplasia in PDAC should be examined in patients who undergo resection. However, in our study only 9 PDAC patients had surgery and statistical analysis

requires a larger study group.

DISCUSSION

In this study, we showed that CP patients had elevated serum levels of HA, TGF-β1, s-Fr but not MCP-1 compared to the healthy controls. Similarly, Yasuda *et al.*^[26] showed elevation of TGF-β1 and s-Fr but not MCP-1 levels in patients with CP. In the Pedersen *et al.*^[27] study, serum MCP-1 levels were also not different in patients with CP and controls. However, in experimental rat CP models, serum and pancreatic MCP-1 concentration significantly increased with the progression of fibrosis^[28]. It is known that migration of monocytes is one of the earliest events in the formation of pancreatic fibrosis. Thus, MCP-1 is considered to be a profibrogenic factor in the progression of chronic pancreatitis and is thought to be a marker of early stage pancreatitis^[29]. It may be assumed that the level of MCP-1 is normalized in patients with a longer disease clinical course.

Our results showed a significantly higher serum level of s-Fr in CP with a duration over 5 years compared

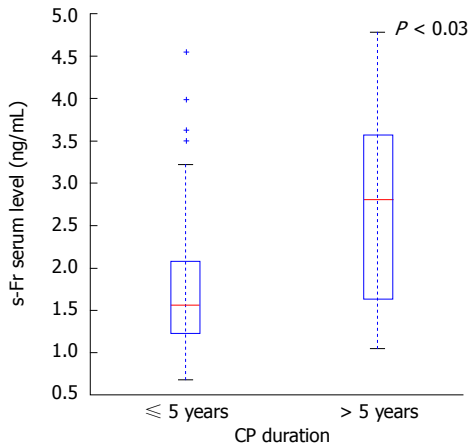


Figure 5 Serum soluble fractalkine level depends on the duration of chronic pancreatitis. s-Fr: Serum soluble fractalkine; CP: Chronic pancreatitis.

to those with a shorter clinical course. Other studies confirmed that the s-Fr serum level increases with the progression of CP^[30]. Ceyhan *et al.*^[30] assessed the s-Fr and its receptor (CX3CR1) levels in CP and normal pancreas by QRT-PCR, Western Blot and immunohistochemistry analyses. Their expression correlated with the severity of fibrosis and CP duration.

The observations of D'Heese *et al.*^[31] also suggest that s-Fr level correlates noticeably with severe fibrosis and the presence of pain in CP. Ceyhan *et al.*^[30] showed that fractalkine mRNA expression was associated with the severity of fibrosis in CP and CP duration.

Evaluation of serum in CP patients revealed significantly higher TGF- β 1 levels compared to healthy controls. This corresponds with the findings of Detlefsen *et al.*^[32] who studied material obtained after surgical resection. They found TGF- β receptors expressed on most myofibroblasts in the pancreatic tissue from CP patients. Moreover, receptors were mainly expressed in the early to moderate stages of pancreatic fibrosis.

In cases with more advanced tissue damage, the expression of TGF- β receptor appeared to be less intense than during the early stages^[32]. This finding may suggest that TGF- β 1 should be taken into consideration as an early stage fibrosis marker.

Other authors suggested that the expression of TGF- β 1 in CP tissue (detected by PCR assay and confirmed by Western blot assay) rises with the severity of CP assessed by the presence of exocrine and endocrine insufficiency, pain (scored 0-4) and complications associated with CP^[26]. However, no correlation between CP advancement and TGF- β 1 levels was found in our study.

We found that serum levels of HA, TGF- β 1, s-Fr were elevated in PDAC patients compared to healthy controls. Similar results were obtained by Erkan *et al.*^[33] who described elevated levels of extracellular matrix proteins, laminin, procollagen III peptide (P-III-P) and HA in patients with PDAC. The levels of tested substances increased with the fibrosis advancement. In our study,

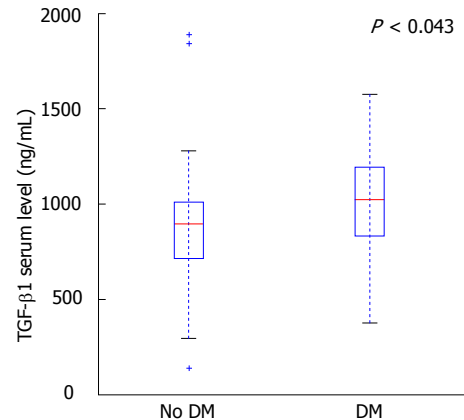


Figure 6 In pancreatic adenocarcinoma patients with coexisting diabetes mellitus, transforming growth factor- β 1 serum level was significantly higher than in pancreatic adenocarcinoma patients without endocrine insufficiency. TGF- β 1: Transforming growth factor- β 1.

we found that plasma TGF- β 1 levels were elevated in PDAC patients with coexisting diabetes compared to patients without endocrine insufficiency. This finding corresponds with the findings that TGF- β signaling stimulation inhibits proliferation of the pancreatic β -cells *in vitro*^[34]. Furthermore, overexpression of TGF- β 1 in the pancreatic β -cells of transgenic mice (which express TGF- β -inducible early response gene on acinar cells) induces islet cell development from ductular-like structures^[35]. We did not find any studies analyzing the correlation between TGF- β 1 levels in pancreatic disorders and co-existing diabetes in humans in the current literature.

In our study, there was no significant difference between the concentration of MCP-1 in PDAC patients and healthy controls. Some authors have hypothesized that serum MCP-1 levels are elevated in obese PDAC patients. In our study, MCP-1 level was not significantly higher in PDAC patients with a BMI above 25 compared to those with a normal weight. Other findings show a correlation of high circulating MCP-1 levels in obese individuals in general^[36]. This may be the reason why mostly cachectic or average weight PDAC and CP patients do not show elevated MCP-1 levels.

Other investigators have suggested that MCP-1 may be the differentiation marker for benign and malignant lesions (comparing PDAC and IPMN)^[37]. However, our results showed no differences between MCP-1 levels in PDAC and CP patients. We were also looking for a correlation between various clinical features and levels of tested markers in CP patients. No significant differences between serum level of TGF- β 1, s-Fr, HA and MCP-1 depending on etiology, age, gender, endocrine insufficiency or Cambridge score in CP patients were found. In our study, the level of s-Fr was not significantly higher in those with alcohol-related CP compared to CP of other etiologies. In the CP rats study, Uchida *et al.*^[38] proved a positive correlation of fractalkine level and alcohol intake. They used Wistar/Bonn Kobori (WBN/Kob) rats, widely accepted as a rodent model of CP.

We also looked for the correlation between various clinical features and levels of tested markers in CP patients. No correlation was found between tumor size, localization and serum TGF- β 1, MCP-1, s-Fr and HA levels.

Our results reveal that the levels of the markers did not correlate with each other. Similar results were published by Ito^[29] who showed that serum s-Fr did not correlate with MCP-1 or TGF- β 1 in CP patients. Our results were confirmed in the Adrych *et al.*^[39] study in which no association between serum TGF- β 1 and serum hyaluronic acid in CP patients was found. Our study confirmed that levels of fibrosis markers are elevated in CP and PDAC patients compared to healthy controls. However, tested substances do not distinguish CP from PDAC so they are not useful in a differential diagnosis. We did not find a correlation between marker levels and coexisting CP in PDAC patients.

Despite the considerable progress that has been made recently, the PDAC and CP prognosis is still poor and there are no specific treatments that can alter the course of these diseases.

The present study reveals that serum TGF- β 1, s-Fr and HA may be considered additional diagnostic markers of CP and PDAC. This study showed a relationship between the duration of CP and serum concentration of s-Fr. Elevated s-Fr levels in CP patients with a longer disease clinical course suggest a correlation of the severity of CP and the advancement of fibrosis. Thus, s-Fr may be considered a biological marker of CP and may be useful to assess the progression of CP.

Our results confirm results from other studies, suggesting that s-Fr could be used as an early stage PDAC marker before the invasive stage begins^[40]. Moreover, TGF- β 1 level was elevated in patients with concomitant diabetes. Therefore, assessing TGF- β 1 level may be useful to predict endocrine insufficiency in PDAC patients.

COMMENTS

Background

Late diagnosis of chronic pancreatitis or pancreatic adenocarcinoma limits treatment and results in a worse prognosis. Despite worldwide investigation, suitable diagnostic markers have not been proposed yet.

Research frontiers

Different serum fibrosis markers are taken into consideration as diagnostic and prognostic markers.

Innovations and breakthroughs

This is the first study to evaluate different fibrosis markers in the serum of chronic pancreatitis (CP) and pancreatic ductal adenocarcinoma (PDAC) patients compared to healthy controls. Significantly higher s-Fr, HA and transforming growth factor- β 1 serum levels compared to healthy controls suggest the potential utility of tested substances as diagnostic markers.

Applications

The presented results require further investigations in a larger study group.

Terminology

Pancreatic fibrosis is the characteristic feature of chronic pancreatitis and

pancreatic ductal adenocarcinoma. Revealing desmoplasia at an early stage can be a useful diagnostic tool.

Peer-review

This study presents a topic of interest in a diagnostic field. The methods and study population are adequate and the conclusions are reasonable and of possible practical use. This article presents an important issue and is the first study to evaluate different fibrosis markers in the serum of CP and PDAC patients compared to healthy controls.

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REVIEW

- 642 Role of targeted therapy in metastatic colorectal cancer
Ohhara Y, Fukuda N, Takeuchi S, Honma R, Shimizu Y, Kinoshita I, Dosaka-Akita H
- 656 Role of the preoperative usefulness of the pathological diagnosis of pancreatic diseases
Matsumoto K, Takeda Y, Onoyama T, Kawata S, Kurumi H, Ueki M, Miura N, Isomoto H
- 663 Pathogenesis and risk factors for gastric cancer after *Helicobacter pylori* eradication
Ohba R, Iijima K
- 673 Molecular mechanisms of chemoresistance in gastric cancer
Shi WJ, Gao JB

MINIREVIEWS

- 682 Pancreatic cancer: New hopes after first line treatment
Aroldi F, Bertocchi P, Savelli G, Rosso E, Zaniboni A

ORIGINAL ARTICLE

Retrospective Cohort Study

- 688 Association between serum vitamin D levels and gastric cancer: A retrospective chart analysis
Vyas N, Companioni RC, Tiba M, Alkhawam H, Catalano C, Sogomonian R, Baum J, Walfish A

Retrospective Study

- 695 Delaying surgery after neoadjuvant chemoradiotherapy improves prognosis of rectal cancer
Mihmanlı M, Kabul Gürbulak E, Akgün İE, Celayir MF, Yazıcı P, Tunçel D, Bek TT, Öz A, Ömeroğlu S
- 707 Questionnaire survey regarding the current status of super-extended lymph node dissection in Japan
Morita S, Fukagawa T, Fujiwara H, Katai H

Contents

World Journal of Gastrointestinal Oncology
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Role of targeted therapy in metastatic colorectal cancer

Yoshihito Ohhara, Naoki Fukuda, Satoshi Takeuchi, Rio Honma, Yasushi Shimizu, Ichiro Kinoshita, Hirotoshi Dosaka-Akita

Yoshihito Ohhara, Naoki Fukuda, Satoshi Takeuchi, Rio Honma, Yasushi Shimizu, Ichiro Kinoshita, Hirotoshi Dosaka-Akita, Department of Medical Oncology, Hokkaido University Graduate School of Medicine, Sapporo, Hokkaido 060-8638, Japan

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Correspondence to: Yoshihito Ohhara, MD, Department of Medical Oncology, Hokkaido University Graduate School of Medicine, North 15, West 7, Kita-ku, Sapporo, Hokkaido 060-8638, Japan. yoshihito-ohhara@kkr-smc.com
Telephone: +81-11-7065551
Fax: +81-11-7065077

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Abstract

Colorectal cancer (CRC) is a significant cause of cancer-related morbidity and mortality all over the world. Improvements of cytotoxic and biologic agents have

prolonged the survival in metastatic CRC (mCRC), with a median overall survival of approximately 2 years and more in the past two decades. The biologic agents that have proven clinical benefits in mCRC mainly target vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR). In particular, bevacizumab targeting VEGF and cetuximab and panitumumab targeting EGFR have demonstrated significant survival benefits in combination with cytotoxic chemotherapy in the first-line, second-line, or salvage setting. Aflibercept, ramucirumab, and regorafenib are also used in second-line or salvage therapy. Recent retrospective analyses have shown that *KRAS* or *NRAS* mutations were negative predictive markers for anti-EGFR therapy. Based on the evidence from large randomized clinical trials, personalized therapy is necessary for patients with mCRC according to their tumor biology and characteristics. The aim of this paper was to summarize the results of the major randomized clinical trials and highlight the benefits of the molecular targeted agents in patients with mCRC.

Key words: Metastatic colorectal cancer; Aflibercept; Ramucirumab; Regorafenib; Cetuximab; Panitumumab; Targeted therapy; Bevacizumab

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Core tip: The development of molecular targeted agents contributes to prolonging survival of patients with metastatic colorectal cancer (mCRC). One anti-vascular endothelial growth factor agent, bevacizumab, and two anti-epidermal growth factor receptor (EGFR) agents, cetuximab and panitumumab, have demonstrated clinical benefits in first-line, second-line, or salvage therapy in combination with cytotoxic chemotherapy. Moreover, *RAS* mutation has been proven to be a negative biomarker for anti-EGFR therapy in recent retrospective analyses. This article summarizes the evidence from large clinical trials and highlights the benefit of the molecular targeted agents in patients with mCRC.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common causes of cancer-related mortality^[1]. Earlier diagnosis through screening colonoscopy and improvements of treatment techniques have contributed to prolonged survival in the curable stage of CRC^[2]. Nevertheless, metastases are present in about 25% of patients with CRC at the time of diagnosis, and almost 50% of patients with CRC in total will develop metastases. Unfortunately, although the prognosis is usually limited in metastatic CRC (mCRC), systemic chemotherapy can control the disease, alleviate the symptoms related to cancer, and prolong survival^[3]. Systemic chemotherapy for mCRC consists mainly of fluoropyrimidines [intravenous 5-fluorouracil (5-FU) and oral capecitabine], irinotecan, and oxaliplatin. The most common treatment regimens for mCRC are FOLFIRI [bolus and infusional 5-FU/leucovorin (LV) plus irinotecan], FOLFOX (bolus and infusional 5-FU/LV plus oxaliplatin), and CapeOX (oral capecitabine plus oxaliplatin). These combination therapies have contributed to improving the response rate (RR) and prolonging survival in patients with mCRC^[4-6].

Since the Mid 2000s, biologic agents have been developed and demonstrated further clinical benefit in combination with cytotoxic chemotherapy. The biologic agents used for mCRC target angiogenesis (bevacizumab, aflibercept, ramucirumab, and regorafenib) and the epidermal growth factor receptor (EGFR) (cetuximab and panitumumab)^[7]. Bevacizumab has shown clinical benefit with both irinotecan-based and oxaliplatin-based regimens^[8-11]. Moreover, the continuation of bevacizumab after failure of first-line bevacizumab-containing chemotherapy was found to contribute to prolonging the survival of patients with mCRC^[12]. Anti-EGFR antibody agents, cetuximab and panitumumab, demonstrated a survival benefit in mCRC patients^[13,14]. At first, these agents were used in all mCRC patients, and then, no benefit of anti-EGFR agents was observed in mCRC tumors with activating mutation of *KRAS* exon 2^[15-17]. In addition, several recent studies have shown that all-*RAS* mutations in exon 2, 3, or 4 of *KRAS* or *NRAS* were negative predictive factors for anti-EGFR treatment^[18-20]. From these results, cetuximab and panitumumab have been used only in mCRC patients with *RAS* wild type.

The results of the major randomized clinical trials are summarized, and the benefits of the molecular targeted agents in patients with mCRC are highlighted.

ANTI-ANGIOGENIC AGENTS

Angiogenesis is a constitutional process to form a new

vascular network, through budding from host vascular endothelial cells and inserting into the pre-existing blood vessels. Especially in malignant tumors, angiogenesis plays important roles in tumor progression, invasion, and metastasis to distant organs^[21]. Vascular endothelial growth factor (VEGF) is one of the important factors that regulate tumor angiogenesis. VEGF is a family of secreted polypeptides that consists of five members [VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF)]^[22,23]. The members of the VEGF family bind to three variants of receptors, VEGFR-1 (FLT-1), VEGFR-2 (FLK-1/KDR), and VEGFR-3 (FLT-4)^[24,25]. VEGFR-2 is mainly responsible for the angiogenic pathway, whereas VEGFR-1 can act as a soluble circulating form that regulates VEGF binding to cell surface receptor^[26].

Anti-angiogenic agents exert their anti-neoplastic activities not only by inhibiting tumor angiogenesis but also by normalizing the tumor blood vessels. Vessel normalization ensures drug delivery to the tumor, which can increase the efficacy of cytotoxic agents^[27]. Thus, inhibition of angiogenesis has become a key strategy in cancer treatment^[28,29].

Bevacizumab

Bevacizumab is a recombinant humanized monoclonal IgG antibody that selectively binds to VEGF-A, and it demonstrates anti-tumor activity by blocking VEGFR2^[30,31]. It was first approved in 2004 by the United States Food and Drug Administration (FDA) for CRC in combination with other cytotoxic agents. Because of its functional activity, adverse events are mainly related to blood vessels. In several large trials, vascular-related adverse events such as hypertension, arterial/venous thromboembolic events, bleeding, gastrointestinal perforation, wound healing complications, fistula/intra-abdominal abscess, and proteinuria were reported^[32,33]. Rarely, reversible posterior leukoencephalopathy, which can cause various neurological symptoms, has been reported^[34]. Most of these adverse events are manageable by appropriate medication and withdrawal of bevacizumab.

First-line treatment: The benefit of bevacizumab added to chemotherapy was first reported in the AVF2107g trial^[8], in which 813 previously untreated mCRC patients were randomly assigned to either IFL (irinotecan, fluorouracil, and leucovorin) plus bevacizumab or IFL plus placebo. The addition of bevacizumab showed significant improvements in overall survival [OS, 20.3 mo vs 15.6 mo, hazard ratio (HR) = 0.66, $P < 0.001$], progression-free survival (PFS, 10.6 mo vs 6.2 mo, HR = 0.54, $P < 0.001$), and RR (44.8% vs 34.8%, $P = 0.004$). From this result, the FDA first approved bevacizumab in combination with 5-FU-based first-line chemotherapy in mCRC. Later, FOLFIRI plus bevacizumab showed a further survival benefit compared with modified IFL (mIFL) plus bevacizumab in the phase III BICC-C trial^[35]. The FOLFIRI arm had

a trend to longer PFS (11.2 mo vs 8.3 mo, $P = 0.037$) and OS (28.0 mo vs 19.2 mo, $P = 0.28$) compared with the mIFL arm. The RR was not significantly different between the two arms (57.9% vs 53.3%). Based on these results, FOLFIRI plus bevacizumab also became one of the standard regimens in the first-line treatment of mCRC.

Oxaliplatin-based chemotherapy combined with bevacizumab was evaluated in the NO16966 trial^[9,36]. In this pivotal 2x2 factorial randomized phase III trial, 1400 mCRC patients were assigned to oxaliplatin-based first-line chemotherapy (FOLFOX4/CapeOX) with or without bevacizumab. Although the median OS (21.3 mo vs 19.9 mo, HR = 0.89, $P = 0.0769$) and RR [38% vs 38%, odds ratio (OR) = 1.00, $P = 0.99$] were not significantly different between the two arms, the median PFS was significantly improved in the bevacizumab-containing arm compared with the placebo arm (9.4 mo vs 8.0 mo, HR = 0.83, $P = 0.0023$). Similar results were shown in the randomized phase II TREE-1/2 trial, which evaluated the safety and efficacy of bevacizumab added to chemotherapy (mFOLFOX6/bFOL/CapeOX)^[37]. Moreover, the benefit of cetuximab added to chemotherapy plus bevacizumab was evaluated in the CAIRO2 trial^[38]. However, in this phase III trial, the combination of CapeOX plus cetuximab and bevacizumab resulted in a significant decrease in PFS and no difference in OS and RR compared to CapeOX plus bevacizumab alone. In the *KRAS* wild type population, there were no significant differences in PFS (10.5 mo vs 10.4 mo, $P = 0.30$) and OS (21.8 mo vs 22.4 mo, $P = 0.64$), while RR was higher in chemotherapy with cetuximab than in chemotherapy without cetuximab. In the CAIRO2 trial, although patients with *KRAS* mutant type might have had a worse outcome for PFS and OS, no survival benefit of adding cetuximab onto bevacizumab plus chemotherapy was observed even in the *KRAS* wild population. Moreover, a meta-analysis also showed the poor prognosis of the combination of anti-EGFR agents and bevacizumab^[39].

Tegafur/gimeracil/oteracil (S-1) plus oxaliplatin (SOX regimen) for mCRC patients showed efficacy and safety in several trials from Asia^[40]. The phase III SOFT trial investigated the non-inferiority of SOX plus bevacizumab in comparison with mFOLFOX6 plus bevacizumab^[41]. The median PFS was 11.7 mo in the SOX plus bevacizumab arm compared with 11.5 mo in the mFOLFOX6 plus bevacizumab arm [HR = 1.04, $P = 0.015$ (non-inferiority)], and OS was 29.6 mo vs 29.7 mo [HR = 1.018, $P = 0.0133$ (non-inferiority)].

Recently, a combination of all cytotoxic agents (5-FU, oxaliplatin, and irinotecan) was developed to maximize tumor response as the FOLFOXIRI regimen in the treatment of mCRC^[42]. Based on this strategy, the efficacy and safety of FOLFOXIRI plus bevacizumab were evaluated in the TRIBE trial^[43]. In this trial, 508 mCRC patients were randomly assigned to receive FOLFOXIRI plus bevacizumab or FOLFIRI plus bevacizumab. Although FOLFOXIRI plus bevacizumab did not improve

OS (31.0 mo vs 25.8 mo, HR = 0.83, $P = 0.125$), a prolonged PFS (12.1 mo vs 9.7 mo, HR = 0.77, $P = 0.006$) and a higher RR (65% vs 53%, OR = 1.64, $P = 0.006$) were observed in the FOLFOXIRI plus bevacizumab arm compared with the FOLFIRI plus bevacizumab arm. The incidence of grade 3/4 adverse events, especially neutropenia, diarrhea, and stomatitis, was significantly higher in the FOLFOXIRI plus bevacizumab arm than in the FOLFIRI plus bevacizumab arm.

In elderly patients, there is no clear evidence of safety with the combination of bevacizumab with oxaliplatin or irinotecan-based chemotherapy. The AVEX trial was designed to evaluate the efficacy and safety of bevacizumab plus capecitabine in mCRC patients aged 70 years and older^[44]. In this trial, 280 elderly patients with a median age of 76 years (range 70-87 years) were randomized to bevacizumab plus capecitabine or capecitabine alone. PFS was improved with the addition of bevacizumab compared to capecitabine alone (9.1 mo vs 5.1 mo, HR = 0.53, $P < 0.0001$). Improved RR was also observed with bevacizumab plus capecitabine (19.3% vs 10.0%, $P = 0.042$), though OS was not significantly different (20.7 mo vs 16.8 mo, HR = 0.79, $P = 0.182$). Although the incidence of grade 3 or worse adverse events related to chemotherapy was slightly higher in the combination group (40% vs 22%), the combination of bevacizumab and capecitabine was a well-tolerated regimen (Table 1).

Second-line and salvage treatment or beyond progression:

In the E3200 trial, FOLFOX plus bevacizumab as second-line therapy in patients with mCRC after first-line irinotecan-based therapy without bevacizumab demonstrated significantly longer PFS and OS compared with the control arm of FOLFOX alone (PFS 7.3 mo vs 4.7 mo, HR = 0.61, $P < 0.0001$; OS 12.9 mo vs 10.8 mo, HR = 0.75, $P = 0.0011$)^[45]. It should be noted that bevacizumab was not administered in first-line therapy, and the dose of bevacizumab was higher (10 mg/kg) in this trial.

Furthermore, continuation of bevacizumab after disease progression in patients previously treated with bevacizumab seemed to have benefit in the large observational BRIT study^[46]. Based on this result, an open-label, phase III, ML18147 trial was conducted to evaluate the survival benefit of continuing bevacizumab as second-line chemotherapy^[12]. The use of bevacizumab beyond progression showed better OS (11.2 mo vs 9.8 mo, HR = 0.81, $P = 0.0062$) and PFS (5.7 mo vs 4.1 mo, HR = 0.68, $P < 0.0001$) compared with chemotherapy alone.

TAS-102 is a novel oral cytotoxic agent that contains the thymidine-based nucleic acid analogue, trifluridine, and a thymidine phosphorylase inhibitor, tipiracil hydrochloride. In salvage line treatment of mCRC, it was reported that TAS-102 could significantly improve OS compared with placebo in the RECURSE trial^[47]. Recently, the phase I/II C-TASK FORCE trial was conducted to investigate the efficacy and safety of

Table 1 Clinical trials of anti-angiogenic therapies in metastatic colorectal cancer

Trial name	Regimens	<i>n</i>	ORR	PFS (mo)	OS (mo)
First-line chemotherapy					
AVF2017g	IFL + Bevacizumab	402	44.8%	10.6	20.3
BICC-C	FOLFIRI + Bevacizumab	57	57.9%	11.2	28.0
NO16966	FOLFOX/CapeOX + Bevacizumab	699	38%	9.4	21.3
TREE-1/2	FOLFOX + Bevacizumab	71	52%	9.9	26.1
CAIRO2	CapeOX + Bevacizumab	378	50%	10.7	20.3
SOFT	SOX + Bevacizumab	256	61.5%	11.7	29.6
TRIBE	FOLFOXIRI + Bevacizumab	252	65.1%	12.1	31.0
AVEX ¹	Capecitabine + Bevacizumab	140	19.3%	9.1	20.7
Second-line, salvage-line chemotherapy, or beyond progression					
E3200	FOLFOX + Bevacizumab	286	22.7%	7.3	12.9
ML18147	Chemotherapy + Bevacizumab	410	5.4%	5.7	11.2
C-TASK FORCE	TAS-102 + Bevacizumab	25	4.0%	5.6	11.2
VELOUR	FOLFIRI + Afibercept	612	19.8%	6.9	13.5
RAISE	FOLFIRI + Ramucirumab	536	13.4%	5.7	13.3
CORRECT	Regorafenib	505	1.0%	1.9	6.4

¹AVEX trial enrolled mCRC patients aged 70 years and older. ORR: Objective response rate; PFS: Progression-free survival; IFL: Bolus 5-fluorouracil/leucovorin/irinotecan; FOLFIRI: Bolus and infusional 5-fluorouracil/leucovorin/irinotecan; FOLFOX: Bolus and infusional 5-fluorouracil/leucovorin/oxaliplatin; CapeOX: Capecitabine/oxaliplatin; SOX: S-1 (tegafur/gimeracil/oteracil)/oxaliplatin; FOLFOXIRI: Infusional 5-fluorouracil/leucovorin/irinotecan/oxaliplatin; OS: Overall survival; mCRC: Metastatic colorectal cancer.

TAS-102 plus bevacizumab in the salvage line setting. Median OS was 11.2 mo and PFS was 5.6 mo. In addition, although the RR was only 4.0%, the disease control rate was 72% with tolerable toxicity. However, the sample size was quite small ($n = 25$)^[48] (Table 1).

Aflibercept

Aflibercept is a recombinant fusion protein that can bind to VEGF-A, VEGF-B, and PlGF. It can act as a soluble decoy receptor, preventing these ligands from binding to their receptors and inhibiting the VEGF pathway. In the VELOUR study, aflibercept plus FOLFIRI demonstrated significant improvements in OS (13.5 mo vs 12.1 mo, HR = 0.82, $P = 0.032$) and PFS (6.9 mo vs 4.7 mo, HR = 0.76, $P < 0.0001$) compared with placebo plus FOLFIRI in previously treated mCRC patients^[49]. The RR was 19.8% in the aflibercept plus FOLFIRI arm and 11.1% in the FOLFIRI alone arm ($P = 0.0001$). The profile of adverse events was similar to that previously reported with bevacizumab, but some adverse events associated with cytotoxic agents were reported at a higher incidence in the aflibercept arm (Table 1).

Ramucirumab

Ramucirumab is a human IgG-1 monoclonal antibody targeting the extracellular domain of VEGFR-2, which is the primary mediator of the VEGF pathway. By binding to VEGFR-2, ramucirumab prevents all VEGF ligands from binding to VEGFR-2 and inhibits the VEGF pathway. In the phase III RAISE study, 1072 patients with disease progression on bevacizumab, oxaliplatin, and fluoropyrimidine were randomized to ramucirumab plus FOLFIRI or FOLFIRI alone as second-line treatment^[50]. Ramucirumab plus FOLFIRI demonstrated better OS (13.3 mo vs 11.7 mo, HR = 0.84, $P = 0.0219$) and PFS (5.7 mo vs 4.5 mo, HR = 0.79, $P < 0.0005$)

than FOLFIRI alone. The RR was similar in the two arms (13.4% in ramucirumab plus FOLFIRI vs 12.5% in FOLFIRI alone), as was the frequency of serious adverse events (36% in ramucirumab plus FOLFIRI vs 31% in FOLFIRI alone). From this result of RAISE, ramucirumab was approved in 2015 by FDA for mCRC in combination with FOLFIRI (Table 1).

Regorafenib

Regorafenib is an oral multi-kinase blocker that inhibits the activity of several protein kinases related to the angiogenic pathway (VEGFR-1, VEGFR-2, VEGFR-3, TIE-2), the oncogenic pathway (KIT, RET, RAF1, BRAF), and the tumor microenvironment (PDGFR and FGFR)^[51]. The CORRECT trial was conducted to evaluate the efficacy and safety of regorafenib in patients with mCRC who had progressed after all approved standard therapies^[52]. Patients treated with regorafenib had slightly prolonged OS (6.4 mo vs 5.0 mo, HR = 0.77, $P = 0.0052$) and PFS (1.9 mo vs 1.7 mo, HR = 0.49, $P < 0.0001$) compared with placebo. The most frequent adverse events of grade 3 or higher were hand-foot skin reaction, fatigue, diarrhea, hypertension, and rash or desquamation. Of note, fatal drug-induced liver injury was observed (Table 1).

ANTI-EGFR AGENTS

EGFR is a transmembrane glycoprotein that belongs to the human epidermal growth factor receptor (HER)-erbB family of tyrosine kinase receptors^[53]. Ligand binding to EGFR leads to the autophosphorylation of the intracellular domain and activates the downstream signaling pathway, including RAS/RAF/MAPK, STAT, and PI3K/AKT. The activation of the signaling pathway modulates cell proliferation, adhesion, angiogenesis,

migration, and metastasis^[54,55].

Cetuximab and panitumumab are anti-EGFR monoclonal antibodies used for mCRC in daily practice. The mechanisms of cetuximab and panitumumab are described below. At present, the use of cetuximab or panitumumab is restricted only to mCRC patients with *KRAS* and *NRAS* wild type because it was found that cetuximab or panitumumab had no effect in mCRC patients with the activating mutation of *KRAS* and *NRAS* oncogene^[20,56].

Cetuximab

Cetuximab is a chimeric, anti-EGFR monoclonal antibody of the IgG1 class targeted against the extracellular domain of the EGFR. By binding to the EGFR, cetuximab blocks intracellular EGFR signaling and modulates tumor cell growth by inhibiting proliferation, angiogenesis, and differentiation, stimulating apoptosis, and preventing metastasis^[57,58]. Cetuximab was first approved in 2004 in combination with irinotecan for mCRC patients with irinotecan-refractory disease. After that, several experimental analyses showed that the activating mutation of *KRAS* exon 2 was associated with intrinsic resistance to cetuximab. Given these findings, cetuximab was used only in mCRC patients with *KRAS* wild type^[15,56]. Moreover, recently, some reports revealed that use of anti-EGFR drugs for mCRC contributed to acquisition of a *KRAS* mutation^[59,60]. Misale *et al.*^[59] offered two possible explanations for the discordant results of *KRAS*: Heterogeneity of *KRAS* status within the primary tumor; and clonal selection during the process of metastasis. In this report, among 10 patients with *KRAS* wild type who acquired resistance to anti-EGFR therapy, 6 patients had the *KRAS* mutation after progression on anti-EGFR therapy. In the six patients for whom sufficient pre-treatment tumor samples were available for *KRAS* testing, *KRAS* mutations were found to be absent at pre-treatment. Similarly, Diaz *et al.*^[60] showed that emergence of mutant *KRAS* from wild type *KRAS* was a mediator of acquired resistance to anti-EGFR antibodies. These results indicate that treatment with anti-EGFR antibodies is associated with the acquisition of secondary *KRAS* mutations.

The most common toxicities are skin rash and hypomagnesemia^[61,62]. To prevent severe skin toxicity, preventive skin treatments are often performed for patients treated with cetuximab.

First-line treatment in *KRAS*-WT mCRC: The efficacy of cetuximab combined with chemotherapy in the first-line setting for mCRC was evaluated in two pivotal clinical trials: The phase III CRYSTAL study and the phase II OPUS study^[63-66].

In the CRYSTAL study, 1198 mCRC patients were randomly assigned to two treatment groups: FOLFIRI plus cetuximab or FOLFIRI alone. Tumor samples from 1063 patients were used for *KRAS* mutation analysis, and 397 patients (37%) had *KRAS* codon 12 and 13 mutations. Of 666 patients (63%) with *KRAS* wild type,

the benefit of addition of cetuximab to FOLFIRI was demonstrated as significantly improved RR (57.3% vs 39.7%, OR = 2.07, $P < 0.001$), PFS (9.9 mo vs 8.4 mo; HR = 0.70, $P = 0.0012$), and OS (23.5 mo vs 20.0 mo; HR = 0.80, $P = 0.0093$) compared with FOLFIRI alone. In the OPUS study, 337 mCRC patients received either FOLFOX-4 alone or FOLFOX-4 plus cetuximab. *KRAS* analysis was performed in 315 of the 337 cases. Among these patients, 179 (57%) were *KRAS* wild type. In the *KRAS* wild type population, patients treated with cetuximab in combination with FOLFOX-4 demonstrated a higher RR (57% vs 34%; OR = 2.551, $P = 0.0027$) and a better PFS (8.3 mo vs 7.2 mo; HR = 0.567, $P = 0.0064$) compared with those treated with FOLFOX-4 alone. No benefit in terms of OS was observed (22.8 mo vs 18.5 mo; HR = 0.855, $P = 0.39$).

In contrast, the phase III COIN trial including 1630 patients with mCRC who were randomized to an oxaliplatin-based regimen (FOLFOX or CapeOX) with or without cetuximab did not show any benefit with the addition of cetuximab to chemotherapy in terms of PFS (8.6 mo vs 8.6 mo; HR = 0.96, $P = 0.60$) and OS (17.0 mo vs 17.9 mo; HR = 1.04, $P = 0.67$) compared with chemotherapy alone, even in the *KRAS* wild type population^[67,68]. However, exploratory subgroup analyses demonstrated that the cohort of patients treated with FOLFOX plus cetuximab showed improved PFS (HR = 0.72, $P = 0.037$), while the cohort of CapeOX plus cetuximab had no significant difference in PFS compared with chemotherapy alone (HR = 1.02, $P = 0.88$). The RR improved from 57% to 64% with the addition of cetuximab to the oxaliplatin-based regimen. In the COIN trial, exploratory analyses were conducted in order to identify somatic molecular profile of the EGFR pathway, and its relationship to the site of the primary and metastases^[69]. *KRAS* mutations were more common in the right colon as compared to those from the left colon, and *BRAF* mutations were more common from the transverse and right colon as compared to those from the left colon. *KRAS* mutations were associated with lung-only metastases, *BRAF* mutations with peritoneal and nodal-only metastases, and microsatellite instability was associated with nodal-only metastases. At the point of differences between primary sites, other study reported that hepatic and pulmonary metastases were more frequently found in left-sided carcinomas, and peritoneal metastasis in right-sided carcinomas in the analyses based on 17641 patients with mCRC^[70]. Moreover, NORDIC VII was conducted to investigate the efficacy of cetuximab combined with the FLOX regimen^[71]. Patients were randomized to the following three arms: FLOX alone, cetuximab and FLOX, or cetuximab combined with intermittent FLOX. Even in patients with *KRAS* wild type, there was no evidence that cetuximab adds a significant benefit to NORDIC FLOX in first-line treatment of mCRC. From these negative results of the COIN and NORDIC VII studies, it seems that neither CapeOX nor FLOX is suitable for combination therapy with cetuximab.

Table 2 Clinical trials of anti-epidermal growth factor receptor therapies in metastatic colorectal cancer with *KRAS* wild type

Trial name	Regimens	n	ORR	PFS (mo)	OS (mo)
First-line chemotherapy					
CRYSTAL	FOLFIRI + Cetuximab	316	57.3%	9.9	23.5
OPUS	FOLFOX + Cetuximab	159	57%	8.3	22.8
COIN	FOLFOX/CapeOX + Cetuximab	362	64%	8.6	17.0
NORDIC-VII	FLOX + Cetuximab	97	46%	7.9	20.1
PRIME	FOLFOX + Panitumumab	325	55%	9.6	23.9
Second-line, salvage-line chemotherapy, or beyond progression					
20050181 trial	FOLFIRI + Panitumumab	303	36%	6.7	14.5
PICCOLO	Irinotecan + Panitumumab	230	34%	5.5	10.4
CO.17	Cetuximab	117	13%	3.7	9.5
20020408 trial	Panitumumab	124	17%	12.3 wk	8.1

ORR: Objective response rate; PFS: Progression-free survival; OS: Overall survival; FOLFIRI: Bolus and infusional 5-fluorouracil/leucovorin/irinotecan; FOLFOX: Bolus and infusional 5-fluorouracil/leucovorin/oxaliplatin; CapeOX: Capecitabine/oxaliplatin; FLOX: Bolus 5-fluorouracil/leucovorin/oxaliplatin.

In adding cetuximab to cytotoxic chemotherapy, the FOLFOX or FOLFIRI regimen is considered the best partner in mCRC patients with *KRAS* wild type. Thus, based on the positive results of the CRYSTAL and OPUS studies, the addition of cetuximab to FOLFOX or FOLFIRI was established as a gold standard in mCRC patients with *KRAS* wild type (Table 2).

Second-line and salvage treatment in *KRAS*-WT mCRC: Cetuximab monotherapy was compared with best supportive care (BSC) in heavily pretreated patients with mCRC after failure of fluoropyrimidines, irinotecan, and oxaliplatin (NCIC CO.17 trial)^[72]. A total of 572 mCRC patients were randomized to cetuximab plus BSC or BSC alone. Cetuximab improved OS and PFS and preserved quality of life measures. After the CO.17 trial, a retrospective analysis was performed to determine whether *KRAS* mutation status was associated with survival in the cetuximab and BSC groups^[16]. A total of 69% (394/572) of the cases were examined for *KRAS* mutation status. A *KRAS* mutation was detected in 40.9% of the cetuximab group and in 42.3% of the BSC group. For patients with *KRAS* wild tumors, treatment with cetuximab compared with supportive care alone significantly improved OS (9.5 mo vs 4.8 mo; HR = 0.55; $P < 0.001$) and PFS (3.7 mo vs 1.9 mo; HR = 0.40; $P < 0.001$).

The efficacy of cetuximab in combination with chemotherapy in the salvage setting was evaluated in two randomized clinical trials: The BOND-1 trial and the EPIC trial^[13,14]. The BOND-1 trial was a randomized phase III study that enrolled 329 patients with irinotecan-resistant mCRC. The superiority of cetuximab plus irinotecan in terms of RR and PFS was demonstrated compared with cetuximab alone. In the phase III EPIC trial, 1298 mCRC patients who experienced first-line fluoropyrimidine and oxaliplatin treatment failure were randomly assigned to either irinotecan plus cetuximab or irinotecan alone. The addition of cetuximab to irinotecan improved RR and PFS compared with irinotecan alone. However, both trials did not show the

benefit of cetuximab in combination with chemotherapy with respect to OS compared with monotherapy. So far, the detailed results of *KRAS* status in the BOND and EPIC trials have not been published (Table 2).

Panitumumab

Panitumumab is a fully human, monoclonal antibody targeting the EGFR with high affinity. The mechanism of inhibiting EGFR signaling pathway is similar to that of cetuximab, as described above^[73]. Panitumumab was first approved in 2006 by the United States FDA for the treatment of EGFR-expressing mCRC with disease progression despite prior treatment. The most common toxicities are skin rash and hypomagnesemia, like cetuximab. The utility of preventive skin treatment in panitumumab therapy has been reported in prospective studies^[74,75].

First-line treatment in *KRAS*-WT mCRC: The phase III PRIME study was conducted in chemo-naïve mCRC patients to evaluate the efficacy of panitumumab in combination with the FOLFOX-4 regimen in the first-line setting^[76]. In the PRIME study, 1183 mCRC patients were randomly assigned to receive FOLFOX-4 with or without panitumumab. The *KRAS* status of the tumors was available in 1096 of these patients (93%), and 440 patients (40%) had a mutation of *KRAS* status. In the *KRAS* wild type population, the FOLFOX-4 plus panitumumab arm had significantly improved PFS compared with the FOLFOX-4 arm (9.6 mo vs 8.0 mo; HR = 0.80, $P = 0.002$). There was no significant difference between FOLFOX-4 plus panitumumab and FOLFOX-4 alone in terms of OS and RR (OS 23.9 mo vs 19.7 mo, HR = 0.83, $P = 0.072$; RR 55% vs 48%, OR = 1.35, $P = 0.068$). This result of the PRIME study was similar to that of the OPUS trial. From the results of these two studies (phase II OPUS and phase III PRIME), the efficacy of the addition of anti-EGFR agents to oxaliplatin-based chemotherapy was demonstrated (Table 2).

Second-line and salvage treatment in KRAS-WT mCRC:

The role of panitumumab in combination with chemotherapy in the second-line or salvage setting for mCRC was evaluated in the following two trials. First, in the phase III 20050181 trial, 1186 patients were enrolled and randomized to two treatment arms: FOLFIRI plus panitumumab and FOLFIRI alone^[77,78]. The *KRAS* status of the tumors was investigated in 1083 cases (91%), and *KRAS* mutation was found in 45% (486/1083). In the wild type *KRAS* population, addition of panitumumab to the FOLFIRI regimen led to a significant improvement in PFS compared with FOLFIRI alone (6.7 mo vs 4.9 mo; HR = 0.82, *P* = 0.023). However, addition of panitumumab to chemotherapy did not show a significant difference in OS; and the FOLFIRI plus panitumumab arm had a trend to better OS than the FOLFIRI arm (14.5 mo vs 12.5 mo; HR = 0.92, *P* = 0.37). The RR was significantly higher in the panitumumab-containing regimen (36% vs 10%; OR = 5.50, *P* < 0.0001). Second, in the PICCOLO trial, irinotecan plus panitumumab was compared with irinotecan alone as a salvage treatment in patients with fluorouracil-resistant mCRC^[79]. Whereas no significant difference was observed in OS between the groups (10.4 mo vs 10.9 mo; HR = 1.01, *P* = 0.91), the irinotecan plus panitumumab group had a longer PFS (5.5 mo vs 4.7 mo; HR = 0.78, *P* = 0.015) and a higher RR (34% vs 12%; OR = 4.12, *P* < 0.0001) than the irinotecan monotherapy group.

The efficacy of panitumumab monotherapy for *KRAS* wild type mCRC was evaluated in the phase III 20020408 study^[17,80]. Patients with mCRC were randomly assigned to either panitumumab monotherapy or BSC alone. Patients treated with panitumumab had better RR and PFS compared with those with BSC (RR 17% vs 0%; PFS 12.3 wk vs 7.3 wk, HR = 0.45, *P* < 0.0001). Although no significant difference in OS was observed between the panitumumab arm and the BSC arm (8.1 mo vs 7.6 mo; HR = 0.99), this was because 76% (90/119) of patients with BSC received panitumumab treatment after progression under the cross-over protocol (Table 2).

The benefit of panitumumab treatment for mCRC patients with cetuximab-refractory disease was evaluated in several clinical trials. In the PANERB trial, 106 mCRC patients with *KRAS* wild type who experienced progression on cetuximab-based chemotherapy were enrolled^[81]. Of the 106 patients, 48 (45%) had an objective response with the cetuximab-containing treatment. Among these 48 patients, 15 (31%) had an objective response, and 23 (47%) in total had a clinical benefit with panitumumab therapy. On the other hand, 28 of 106 patients had disease progression on cetuximab-based treatment. Of these 28 patients, only 4 patients (14%) had clinical benefit with panitumumab therapy. Moreover, some clinical trials showed that panitumumab was not active (RR, 0%) as a salvage therapy in patients with cetuximab-resistant *KRAS* wild type mCRC^[82,83].

TREATMENT STRATEGY ACCORDING TO *KRAS* OR ALL *RAS* WILD TYPE mCRC

Anti-VEGFR and anti-EGFR treatments for patients with KRAS wild type mCRC

Recently, three large randomized clinical trials (PEAK, FIRE-3, and CALGB/SWOG 80405) were conducted to compare anti-EGFR agent-containing chemotherapy with bevacizumab-containing chemotherapy in *KRAS* wild type mCRC patients in the first-line setting (Table 3).

First, the phase II PEAK study was conducted in 285 mCRC patients with wild-type *KRAS* to compare FOLFOX plus panitumumab with FOLFOX plus bevacizumab as first-line treatment^[18]. Although median PFS was similar between the panitumumab arm and the bevacizumab arm (10.9 mo vs 10.1 mo; HR = 0.87, *P* = 0.353), median OS was significantly prolonged in the panitumumab arm compared with the bevacizumab arm (34.2 mo vs 24.3 mo; HR = 0.62, *P* = 0.009). The RR was 57.8% in the panitumumab arm and 53.5% in the bevacizumab arm. Second, the phase III FIRE-3 study was conducted to evaluate the superiority of FOLFIRI plus cetuximab to FOLFIRI plus bevacizumab in mCRC patients with *KRAS* wild type as first-line treatment^[19]. A total of 592 patients with *KRAS* wild type tumors were randomly assigned and received treatment, with 297 in the FOLFIRI plus cetuximab group and 295 in the FOLFIRI plus bevacizumab group. The FIRE-3 study did not show differences in terms of RR (62% in the cetuximab group vs 58% in the bevacizumab group; OR = 1.18, *P* = 0.18) and PFS (10.0 mo in the cetuximab group vs 10.3 mo in the bevacizumab group; HR = 1.06, *P* = 0.55), while OS was prolonged in the cetuximab-containing regimen (28.7 mo in the cetuximab group vs 25.0 mo in the bevacizumab group; HR = 0.77, *P* = 0.017). Finally, in the CALGB/SWOG 80405 study, 1137 patients with *KRAS* wild type were randomized to two arms: Cytotoxic chemotherapy (FOLFOX or FOLFIRI) plus cetuximab or bevacizumab^[84]. No benefit of the cetuximab-containing regimen was observed for PFS (10.4 mo vs 10.8 mo; HR = 1.04, *P* = 0.55) or OS (29.9 mo vs 29.0 mo; HR = 0.925, *P* = 0.34) compared with the bevacizumab-containing regimen. The RR was significantly higher in the cetuximab arm than in the bevacizumab arm (65.6% vs 57.2%, *P* = 0.02).

The efficacy of panitumumab combined with FOLFIRI in the second-line setting was evaluated in the phase II SPIRITT study^[85]. The SPIRITT study compared FOLFIRI in combination with panitumumab or bevacizumab for *KRAS* wild type mCRC patients with progression on a bevacizumab-containing oxaliplatin-based regimen. A total of 182 patients were randomly assigned to FOLFIRI combined with panitumumab or bevacizumab. Median PFS and OS were similar between the FOLFIRI with panitumumab arm and the FOLFIRI with bevacizumab arm (PFS 7.7 mo vs 9.4 mo, HR = 1.01, *P* = 0.97; OS 18.0 mo vs 21.4 mo; HR = 1.06, *P* = 0.75). The RR

Table 3 Clinical trials comparing anti-epidermal growth factor receptor therapy *vs* anti-vascular endothelial growth factor therapy in metastatic colorectal cancer with *KRAS* wild type

Trial name	Regimens	n	ORR	PFS (mo)	OS (mo)
First-line chemotherapy PEAK	FOLFOX + Panitumumab	142	57.8%	10.9	34.2
	FOLFOX + Bevacizumab	143	53.5%	10.1	24.3
	(HR, <i>P</i> -value)		-	HR = 0.87	HR = 0.62
				<i>P</i> = 0.353	<i>P</i> = 0.009
FIRE-3	FOLFIRI + Cetuximab	297	62%	10.0	28.7
	FOLFIRI + Bevacizumab	295	58%	10.3	25.0
	(HR, <i>P</i> -value)		OR = 1.18	HR = 1.06	HR = 0.77
			<i>P</i> = 0.18	<i>P</i> = 0.55	<i>P</i> = 0.017
CALGB/SWOG 80405	Chemotherapy ¹ + Cetuximab	578	65.6%	10.4	29.9
	Chemotherapy ¹ + Bevacizumab	559	57.2%	10.8	29.0
	(HR, <i>P</i> -value)		<i>P</i> = 0.02	HR = 1.04	HR = 0.925
				<i>P</i> = 0.55	<i>P</i> = 0.34
Second-line chemotherapy SPIRITT	FOLFIRI + Panitumumab	91	32%	7.7	18.0
	FOLFIRI + Bevacizumab	91	19%	9.2	21.4
	(HR, <i>P</i> -value)		-	HR = 1.01	HR = 1.06
				<i>P</i> = 0.97	<i>P</i> = 0.75

¹Chemotherapy regimens were FOLFOX or FOLFIRI. ORR: Objective response rate; PFS: Progression-free survival; OS: Overall survival; HR: Hazard ratio; OR: Odds ratio; FOLFIRI: Bolus and infusional 5-fluorouracil/leucovorin/irinotecan; FOLFOX: Bolus and infusional 5-fluorouracil/leucovorin/oxaliplatin.

was 32% in the panitumumab arm and 19% in the bevacizumab arm.

Treatment outcome of anti-EGFR therapy for patients with *RAS* wild type mCRC

An activating mutation of *KRAS* exon 2 has been found to be a negative predictive marker in mCRC, as described above. *KRAS* status was used for patient selection for anti-EGFR treatment. *NRAS* is one of the *RAS* oncogene family members, and somatic mutations like *KRAS* gene have been detected within the *NRAS* gene. A retrospective analysis of the PRIME study showed that 17% of patients with *KRAS* exon 2 wild type had mutations in *RAS* exons (*KRAS* exon 3, 4 and *NRAS* exon 2, 3). An activating mutation of *NRAS* exon 4 was not detected in this analysis^[19]. Patients with all-*RAS* wild type who received panitumumab plus FOLFOX had a prolonged OS compared with those with *KRAS* exon 2 wild type (25.8 mo in *RAS* wild type and 23.9 mo in *KRAS* wild type). A similar outcome was demonstrated in the CRYSTAL study^[86]. From these results, the negative predictive factors were any mutations in either *KRAS* or *NRAS* codons 12, 13, 59, 61, 117, and 146 hotspots. Now, all-*RAS* wild type patients can be defined as those without the above mutations.

All-*RAS* subset analyses were performed in three randomized clinical trials that compared anti-EGFR agent-containing chemotherapy with bevacizumab-containing chemotherapy: PEAK, FIRE-3, and CALGB/SWOG 80405. In PEAK and FIRE-3, a further survival benefit was observed in the anti-EGFR arm compared with the bevacizumab arm in mCRC patients with *RAS* wild type (OS in PEAK, 41.3 mo vs 28.9 mo, HR = 0.63, *P* = 0.058; OS in FIRE-3, 33.1 mo vs 25.6 mo, HR = 0.70, *P* = 0.011)^[18,19]. In contrast, CALGB/SWOG 80405

demonstrated no significant difference in OS between cetuximab plus chemotherapy and bevacizumab plus chemotherapy even in the *RAS* wild type population (32.0 mo vs 31.2 mo, HR = 0.90, *P* = 0.40)^[84]. The outcomes of these trials were discussed in several groups^[87-90]. Meta-analyses of the three studies were performed for the *RAS* wild type subset in order to compare anti-EGFR therapy with anti-VEGF therapy^[87]. Although no significant difference in PFS was observed between anti-EGFR and anti-VEGF agents combined with chemotherapy (HR = 0.92, 95%CI: 0.71-1.18, *P* = 0.50), the anti-EGFR arm had better OS (HR = 0.77, 95%CI: 0.63-0.95, *P* = 0.016) and RR (OR = 1.46, 95%CI: 1.13-1.90, *P* = 0.004) compared with the anti-VEGF arm. On the other hand, these three clinical trials aimed to reveal the superiority of anti-EGFR therapy compared with anti-VEGF therapy in *KRAS* wild type mCRC, but they did not meet the primary endpoints of their studies; the primary endpoint was PFS in PEAK, RR in FIRE-3, and OS in CALGB/SWOG 80405. Although anti-EGFR therapy in the first-line setting has a favorable trend compared with anti-VEGF therapy, the treatment strategy in *RAS* wild type mCRC has been controversial. In the future, one ongoing clinical trial may resolve this problem. The phase III STRATEGIC-1 by GERCORE is now ongoing to investigate the appropriate sequential strategy for *RAS* wild type mCRC^[91]. In the STRATEGIC-1 trial, patients are randomized to receive either FOLFIRI plus cetuximab as first-line followed by oxaliplatin-based regimen combined with bevacizumab as second-line, or an oxaliplatin-based regimen by OPTIMOX plus bevacizumab as first-line followed by an irinotecan-based regimen combined with bevacizumab as second-line and by anti-EGFR therapy with or without irinotecan as third-line. We eagerly await the results of this trial (Table 4).

Table 4 Treatment outcome by anti-epidermal growth factor receptor therapy as first-line treatment in metastatic colorectal cancer with *RAS* wild type

Trial name	Regimens	<i>n</i>	ORR	PFS (mo)	OS (mo)
CRYSTAL	FOLFIRI + Cetuximab	178	66.3%	11.4	28.4
	FOLFIRI	189	38.6%	8.4	20.2
	(HR, <i>P</i> -value)		OR = 3.31 <i>P</i> < 0.001	HR = 0.56 <i>P</i> = 0.0002	HR = 0.69 <i>P</i> = 0.0024
PRIME	FOLFOX + Panitumumab	259	-	10.1	25.8
	FOLFOX	253	-	7.9	20.2
	(HR, <i>P</i> -value)		-	HR = 0.72 <i>P</i> = 0.004	HR = 0.77 <i>P</i> = 0.009
PEAK	FOLFOX + Panitumumab	88	63.6%	13.0	41.3
	FOLFOX + Bevacizumab	82	60.5%	9.5	28.9
	(HR, <i>P</i> -value)		-	HR = 0.65 <i>P</i> = 0.029	HR = 0.63 <i>P</i> = 0.058
FIRE-3	FOLFIRI + Cetuximab	171	65%	10.4	33.1
	FOLFIRI + Bevacizumab	171	60%	10.2	25.6
	(HR, <i>P</i> -value)		OR = 1.28 <i>P</i> = 0.32	HR = 0.93 <i>P</i> = 0.54	HR = 0.70 <i>P</i> = 0.011
CALGB/SWOG 80405	Chemotherapy ¹ + Cetuximab	270	68.6%	11.4	32.0
	Chemotherapy ¹ + Bevacizumab	256	53.8%	11.3	31.2
	(HR, <i>P</i> -value)		<i>P</i> < 0.01	HR = 1.1 <i>P</i> = 0.31	HR = 0.9 <i>P</i> = 0.4

¹Chemotherapy regimens were FOLFOX or FOLFIRI. ORR: Objective response rate; PFS: Progression-free survival; OS: Overall survival; HR: Hazard ratio; OR: Odds ratio; FOLFIRI: Bolus and infusional 5-fluorouracil/leucovorin/irinotecan; FOLFOX: Bolus and infusional 5-fluorouracil/leucovorin/oxaliplatin.

OTHER TARGETED AGENTS

The efficacy of chemotherapy combined with tyrosine kinase inhibitor of the EGFR (gefitinib or erlotinib) was evaluated in several phase II studies. First, a total of 27 patients with pretreated mCRC received FOLFOX plus gefitinib in the single-arm phase II study^[92]. The RR was 33% and median PFS was 5.4 mo. Most common grade 3/4 toxicities were neutropenia (48%) and diarrhea (48%). Second, the phase II study was conducted in 100 mCRC patients to compare FOLFIRI plus gefitinib with FOLFIRI alone as first-line setting^[93]. The adding gefitinib to FOLFIRI demonstrate no improvement of RR (47.9% vs 45.1%) or PFS (8.3 mo vs 8.3 mo) compared with FOLFIRI alone, but had more toxicities with grade 3/4 (67.3% vs 52.1%). Finally, the efficacy of capecitabine plus erlotinib in chemo-naïve mCRC patients was evaluated in a small sample size phase II study^[94]. A total of thirteen patients with mCRC were enrolled in this phase II study. The RR was 20% (2/10), but 4 of 13 patients discontinued therapy because of adverse events. From these results, the adding the EGFR tyrosine kinase inhibitor with chemotherapy showed high toxicities and no improvement of ORR.

Two targeted agents, ganitumab and conatumumab, were evaluated in the randomized phase II study in mCRC patients with mutant *KRAS* as second-line setting^[95]. Ganitumab is a human IgG monoclonal antibody targeting the type I insulin-like growth factor receptor and conatumumab is a fully human monoclonal IgG1 antibody targeting the proapoptotic death receptors 5. A total of 155 patients were randomized 1:1:1 to receive FOLFIRI plus conatumumab, ganitumab, or placebo. The median PFS was 6.5 mo (HR = 0.69; *P* = 0.147), 4.5 mo

(HR = 1.01; *P* = 0.998), and 4.6 mo. The median OS was similar between three arms (12.3 mo vs 12.4 mo vs 12.0 mo).

Recently, the clinical benefit of dual-targeted therapy with trastuzumab and lapatinib in patients with *KRAS* wild type, HER2-positive mCRC in the phase II HERACLES study^[96]. In the HERACLES study, 914 patients with *KRAS* exon 2 wild type were screened, and 48 (5%) patients were identified as HER2-positive status. A total of 27 patients with HER2-positive received trastuzumab plus lapatinib treatment as salvage setting. The ORR was 30% and the toxicity was tolerable. The combination of trastuzumab plus lapatinib might be a novel therapeutic option for patients with HER2-positive mCRC.

CONCLUSION

The development of biological and cytotoxic agents has contributed to prolonged survival in mCRC patients, with a median OS of approximately two years and more. In the past two decades, many beneficial therapeutic options and regimens have appeared in daily practice for mCRC, based on the results from randomized clinical trials. Personalized therapy should be performed for mCRC patients according to their clinical and biological factors, such as performance status, organ function, metastasis sites, and tumor biology including *RAS* status. Especially in *RAS* wild type mCRC, anti-EGFR agents, such as cetuximab and panitumumab, have been shown to improve objective response and survival in several clinical trials. Anti-EGFR agents are absolutely key drugs for the *RAS* wild type population. Based on the evidence for anti-EGFR therapy as first-line, second-

line, and salvage therapy, we should plan personalized treatment strategies for patients with RAS wild type mCRC. On the other hand, bevacizumab in combination with chemotherapy has demonstrated clinical benefits in any treatment line. Bevacizumab has been shown to fit any cytotoxic regimens, such as FOLFOX, CapeOX, FOLFIRI, FOLFOXIRI, SOX, or TAS-102. Moreover, continuing bevacizumab beyond progression prolonged survival in mCRC patients who experienced clinical benefit in prior bevacizumab-containing chemotherapy. In addition, we have many biological agents for the second-line or salvage therapy, such as aflibercept, ramucirumab, and regorafenib. Based on the evidence and patients' characteristics, it will be necessary to construct personalized therapy for mCRC patients.

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Role of the preoperative usefulness of the pathological diagnosis of pancreatic diseases

Kazuya Matsumoto, Yohei Takeda, Takumi Onoyama, Soichiro Kawata, Hiroki Kurumi, Masaru Ueki, Norimasa Miura, Hajime Isomoto

Kazuya Matsumoto, Yohei Takeda, Takumi Onoyama, Soichiro Kawata, Hiroki Kurumi, Hajime Isomoto, Department of Gastroenterology, Tottori University Hospital, Yonago 683-8504, Japan

Masaru Ueki, Department of Next Generation Advanced Medical Promotion Center, Tottori University Hospital, Yonago 683-8504, Japan

Norimasa Miura, Division of Pharmacotherapeutics, Department of Pathophysiological and Therapeutic Science, Faculty of Medicine, Tottori University, Yonago 683-8504, Japan

Author contributions: Matsumoto K drafted the manuscript; Matsumoto K and Ueki M concept and designed the study; Takeda Y, Onoyama T, Kawata S and Kurumi H collected the data; Ueki M and Miura N analyzed and interpreted the data; Isomoto H revised the manuscript.

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Correspondence to: Kazuya Matsumoto, MD, PhD, Department of Gastroenterology, Tottori University Hospital, 86 Nishi-cho, Yonago 683-8504, Japan. matsumot@med.tottori-u.ac.jp
Telephone: +81-859-386527

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Abstract

Pancreatic cancer is the fifth leading cause of cancer death and has the lowest survival rate of any solid cancer. Endoscopic ultrasound-guided fine-needle aspiration biopsy (EUS-FNA) is currently capable of providing a cytopathological diagnosis of pancreatic malignancies with a higher diagnostic power, with a sensitivity and specificity of 85%-89% and 98%-99%, compared to pancreatic juice cytology (PJC), whose sensitivity and specificity are only 33.3%-93% and 83.3%-100%. However, EUS-FNA is not effective in the cases of carcinoma *in situ* and minimally invasive carcinoma because both are undetectable by endoscopic ultrasonography, although PJC is able to detect them. As for the frequency of complications such as post endoscopic retrograde cholangiopancreatography pancreatitis, EUS-FNA is safer than PJC. To diagnose pancreatic cancer appropriately, it is necessary for us to master both procedures so that we can select the best methods of sampling tissues while considering the patient's safety and condition.

Key words: Endoscopic ultrasound-guided fine-needle aspiration biopsy; Cytology; Pathology; Pancreatic juice cytology; Pancreatic cancer

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Core tip: In the era of cyto-pathological diagnosis of pancreatic cancer, endoscopic ultrasound-guided fine-needle aspiration biopsy (EUS-FNA) and pancreatic juice cytology (PJC) represent the most promising procedures for diagnosing pancreatic malignancies. However, there haven't been any reports that compared the utilities and faults of these procedures. In this review we have

highlighted the current role of EUS-FNA and PJC in the diagnosis process for pancreatic malignancies.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) currently ranks fifth when it comes to death involving cancer. It also, when it comes to solid cancers, has the lowest survival rate^[1,2]. The current survival rate for patients with PDAC after 5 years with the condition is less than 3.5%^[3,4]. An early diagnosis is crucial to improve the prognosis. However, for a number of reasons, including the inaccessibility of the pancreas and the highly malignant property of the disease, an early diagnosis is still difficult to obtain, despite the constant improvements in diagnostic imaging. Furthermore, it is especially difficult to distinguish between a PDAC and a pancreatic inflammatory lesion, which includes chronic pancreatitis (CP), or a benign stricture of the main pancreatic duct (MPD), and between intra-ductal papillary mucinous carcinoma IPMC and intra-ductal papillary mucinous neoplasm (IPMN). Being able to differentiate PDAC from other conditions is important, because not only are the treatments for each of these conditions different, but the prognosis for CP and other rare tumors is better than that for PDAC. A cyto-pathological diagnosis is desirable before beginning therapy in cases in which a qualitative diagnosis for the pancreatic mass by various imaging studies is not possible. In fact, 5%-10% underwent pancreatoduodenectomy based on a diagnosis that was made before surgery. However, after performing surgery of the primary pancreatic or periampullary malignancy, it is later proven histopathologically to be CP, a benign fibrous common bile duct stricture or so on^[5-7]. After performing endoscopic ultrasound-guided fine-needle aspiration biopsy (EUS-FNA) for a pancreatic mass, the frequency of a PDAC does not reach 80%^[8,9], which is very important because the treatment strategy for a resection case and an unresectable case are different between PDAC and pancreatic neuroendocrine tumor cases^[10-14].

There are many diagnostic procedures in cytopathological treatment including, abdominal ultrasound guided fine-needle aspiration biopsy, computed tomography guided fine-needle aspiration biopsy, EUS-FNA, pancreatic juice cytology (PJC), and Endoscopic pancreatography guided biopsy. I will give an outline mainly on EUS-FNA and PJC in this review.

PROCEDURE OF AN EUS-FNA

Vilmann *et al.*^[15] was the first person to describe the EUS-FNA of a pancreatic mass in 1992. These days, EUS-FNA is the preferred method to sample pancreatic mass lesions, replacing for the most part other methods because EUS-FNA is considered the best diagnostic modality for pancreatic masses with a higher accuracy than that of biopsies under CT or US guidance.

There is a door knocking method and a fanning method in EUS-FNA. The door knocking method is a nice procedure that is useful in obtaining a specimen from a mass, especially one with fibrotic tissue, and, as for the fanning method, the utility is proved by RCT^[16].

FNA needles, which are available in sizes from 19 to 25 gauge (G), are available commercially. A recent meta-analysis suggests that a 22-G and a 25-G needle have a similar specificity rate after being used with 1292 patients being diagnosed with pancreatic malignancies^[17]. The same study showed that the 25-G needle did appear to have a higher sensitivity when compared to the 22-G needle. Another study found that 25-G needles seemed to be more advantageous over the 22-G needles when it comes to the adequacy of passes. No difference in accuracy, number of passes or complications was found^[18]. However, 25-G needles should be considered first in cases in which one must sample from the pancreatic head or uncinate process lesions, as in some studies it has appeared that the 25-G needle has a reduced chance of experiencing technical failures over 22-G needles in such situations^[19,20]. 19-G needles, on the other hand, are not often used in the duodenum because of their natural rigidity. However, recently, a more flexible needle has been made of nitinol to improve its ability to function well (Flex 19, Boston Scientific, Natick, MA). An initial study using these new and improved needles included 38 patients. Thirty-two of the 38 patients had pancreatic head/uncinate lesions. The use of the needles provided adequate samples for cytological analysis in all 32 patients. There were no reported technical failures or procedure related complications^[21]. Ramesh *et al.*^[22] reported that there is no significant difference in the performance of flexible 19-G and 25-G needles although the procurement of histological core tissue with the flexible 19-G needles was significantly higher (88% vs 44%, $P < 0.001$).

As for aspiration, there is a report that compared non-aspiration, aspiration of 10 mL, the aspiration of the slow pull method, and 10-20 mL, but a constant opinion was not obtained from the sampling rate about accuracy^[23-27].

EUS-FNA accuracy is also impacted by the skill level and whether or not a cytopathologist is available^[28-30]. It has recently been shown, in a meta-analysis that covered 34 studies, that rapid on-site evaluation had a significant determinant on the accuracy of EUS-FNA when it comes to the diagnosis of pancreatic masses^[28]. Two studies have evaluated the optimal number of EUS-

FNA passes^[29,31] to be 5-7 passes for pancreatic masses in order to get the best diagnostic yield. For situations in which rapid pathology interpretation is not possible, this information may prove to be useful.

It is considered that the white specimens in EUS-FNA samples include histological evidence, and, as for the red specimen, it is thought to be the blood component. When inspected by a 19-G needle, a histologic core was found to be present in white specimens 78.9% of the time, and in red specimens 9.3% of the time^[32]. It is reported in multiple meta-analysis that ROSE is useful in solving the problem mentioned above^[28,33].

Whereas, a meta-analysis suggests 25-G needles have a higher sensitivity rate than 22-G needles when it comes to diagnosing pancreatic malignancy^[17], it is expected in the future that EUS-FNA by using a 25-G needle will become more mainstream because of the ease of its puncture. At that time, reexamination re-examination may be required if it is necessary to perform immunohistochemical staining after performing ROSE, due to the smaller sample size meaning a decreased chance of there being a histologic core in the sample. Furthermore, there is a fundamental problem in that globally, there are not enough pathologists capable of performing ROSE.

We developed the target sample check illuminator (TSCI) to be a device that would solve the above problem^[34]. The mean number of needle punctures was 2.4 (range, 1-5), and the agreement rate between TSCI and histopathology in 142 samples was 93.7% (133/142). No differences in detection capacity were observed in cancerous or non-cancerous lesions. When presence of the target specimen was confirmed by TSCI, 91.4% (53/58) of the patients were able to finish the tests, and the mean number of needle punctures was 1.2 (67/58).

DIAGNOSTIC POWER OF EUS-FNA

Two recent studies reported a sensitivity of 85% and 89% based on cytology for the diagnosis of malignancy. The specificity for the same was found to be 98% and 99% respectively^[28,35].

It is useful in the improvement of the diagnostic ability of EUS-FNA to use a genetic analysis from EUS-FNA samples. Recent meta-analysis reported that combining K-ras mutation analysis with routine cytology moderately improves the ability of EUS-FNA to differentially diagnose between PDAC and pancreatic inflammatory masses. In a total of eight studies, with 696 cases of PDAC and 138 cases of pancreatic inflammatory masses, the pooled sensitivity, specificity, positive likely ratio and negative likely ratio of K-ras mutation analysis combined with cytopathology for diagnosis of PDAC vs pancreatic inflammatory masses were 90%, 95%, 13.45, and 0.13, respectively. Especially, among total 123 patients whose EUS-FNA results were inconclusive or negative, fifty-nine

had K-ras mutations and were finally diagnosed with PDAC (48%, 59/123)^[36]. In addition, there are several possible means of processing aspirated samples obtained by EUS-FNA for molecular and other ancillary tests^[37].

COMPLICATION WITH EUS-FNA

Complications from EUS-FNA include pain, bleeding, fever, and infection. Rare complications such as, acute portal vein thrombosis^[38], peritoneal seeding of tumor cells^[39], and ruptured pseudoaneurysm of the splenic artery^[40] have also been reported. A recent systematic review by Wang *et al.*^[41], who identified 51 articles with a total of 10941 patients, reported that the mortality rate attributable to EUS-FNA-specific morbidity was 0.02% (2/10941) and that out of 8246 patients with pancreatic lesions only 60 (0.82%) patients reported any complications. About 36/8246 patients had pancreatitis. Of those patients, 75% of the cases were mild. Out of the total number of patients, one of them with severe pancreatitis died. The total rates of pain, bleeding, fever and infection were 0.38%, 0.10%, 0.08% and 0.02% respectively. Two point two percent of patients were reported to have peritoneal seeding of tumor cells after receiving EUS-FNA. However, it seems to be lower than that caused by CT-guided FNA (16.3%)^[42]. There was no increase in the risk of peritoneal carcinomatosis in pancreatic masses to be found^[43]. Beane *et al.*^[44] found there to be no difference in the survival rate of patients with PDAC who underwent EUS-FNA than with those who did not. Not only was there no difference, but a recent study that looked at the risk of gastric/peritoneal recurrence in cases where EUS-FNA was performed found EUS-FNA was not associated with increased needle track seeding^[45]. Furthermore, preoperative EUS-FNA was evaluated in 498 patients, and it was found that it had no negative effect on the survival rate of patients with resected pancreatic cancer^[46].

LIMITATION FOR EUS-FNA

Even though EUS-FNA has an excellent accuracy and a low incidence of major complications, it does have several limitations. We cannot perform EUS-FNA when we cannot detect a tumor in EUS. Actually, we cannot identify the carcinoma *in situ* (CIS) in EUS^[47]. Secondly, even though EUS-FNA has a very high sensitivity rate, when it comes to pancreatic tumors, its negative predictive value is only 55%-65%^[35,48]. As such, EUS-FNA does not allow us to rule out the possibility of a malignancy. Third, if the patient has CP the diagnostic accuracy of EUS-FNA decreases^[49,50]. It might also hinder cytological interpretation of pancreatic FNA, thus giving EUS-FNA a decreased sensitivity^[51]. Fourth, EUS-FNA for pancreatic cancer has a false-positive rate of 1.1%, usually in patients with CP^[52]. Fifth, we may not be able to perform EUS-FNA when we cannot

discontinue the use of an antithrombotic drug.

PROCEDURE, DIAGNOSTIC POWER, AND COMPLICATION OF PJC

McCune *et al.*^[53] developed the ERCP process in 1968. As for the sampling of the pancreas lesion, Endo was the first person to perform a collection of pancreatic juice under the ERP^[54]. The process of PJC is used in all of the following procedures: Brushing cytology, cytodiagnosis with pancreatic duct lavage fluid (PDLF), cytodiagnosis by using endoscopic nasopancreatic drainage (ENPD), and cytodiagnosis by using secretin. Now I will present the methods, diagnosis results, and complications of each procedure.

Brushing cytology

A cytopathological diagnosis by using brushing cytology is easier than conventional aspiration cytology because it can collect fresh cells.

However, the sensitivity (33.3%-65.8%) and the accuracy (46.7%-76.4%) are not so good because it is difficult to perform and collect enough cells^[55,56]. Recently, scraping cytology with a guide-wire yielded 71.4%-93% sensitivity, 100% specificity, 100% positive predictive value, 75%-84.4% negative predictive value, and 88.8%-94% accuracy^[8,57].

However, this diagnosis rate is shown to improve by mastering the procedure^[56].

When we diagnose a CIS by PJC for a pancreatic duct stenosis case, when we are unable to see the pancreatic mass in imaging studies, and resected it, it is usually the case that there is no cancer at the site of stenosis in the MPD. The stenosis is caused by inflammation due to a CIS, which was derived from a branch duct. For this reason, the diagnostic power of brushing cytology is uncertain. As for the complications rate of brush cytology, it has been reported that acute pancreatitis is a possible complication with a rate of 4.2%-33.3%^[8,55-57].

ENPD method

The ENPD method places 5 or 6-French ENPD tubes in the patient for up to 2-3 d^[58,59]. Iiboshi *et al.*^[58] diagnosed 15 CIS using this method. Sensitivity, specificity, positive predictive value, negative predictive value, and overall accuracy of the ENPD method for pancreatic cancer were 80%-100%, 83.3%-100%, 93.3%-100%, 71%-100%, and 87%-95%, respectively, revealing significantly higher sensitivity than the conventional method ($P = 0.0001$)^[58]. As for the complications of the ENPD method, post endoscopic pancreatitis (PEP) has a rate of 7.5%. In particular, the incidence rate of PEP of ENPD method for BD-IPMN is higher than the conventional method^[60].

Cytodiagnosis by using secretin

Due to the fact that secretin stimulates pancreatic exocrine function, we are able to obtain more pancreatic

juice when secretin is present than without it. Finally, we can obtain pancreatic epithelial cells by using secretin. Administration of secretin was performed conventionally before collecting pancreatic juice for cytodiagnosis^[54,60]. Secretin may be required in cases in which a sufficient amount of material was not able to be obtained by conventional methods or it may be needed to aspirate mucous fluid in intraductal papillary mucinous neoplasm^[57]. Nakaizumi reported that the sensitivity for PDAC was 76% in PJC by using secretin^[61]. As for the complications of secretin, at the top of the attached document, it shows a rate of 1.9% for nausea, 0.7% for flushing, and 0.5% for stomachache and vomiting^[62]. We have not experienced any adverse events with the secretin administration. Also, we confirmed that the quantity of pancreatic juice significantly increases even though the secretin load in diluted form is 1/32.

Cytodiagnosis with pancreatic duct lavage fluid

Imamura's process requires us to inject a saline from injection lumen, before aspirating it by the negative pressure from a guide-wire lumen with a different syringe at the same time by using double or triple-lumen cannule after brushing cytology in ERP. The sensitivity of pancreatic cancer diagnosis by this procedure is 83%, and pancreatitis was not a side-effect due to PDLF^[63]. We choose to do PJC by using secretin if a catheter is able to pass through the narrow segment of the MPD, and PDLF if the catheter cannot pass.

If secretin is used in cases where a catheter is unable to pass the stenosis of the MPD, the pancreatic ductal pressure in the caudad area past the stenosis increases, and this causes pancreatitis.

GENETIC ANALYSIS WITH PANCREATIC JUICE

It is useful in the improvement of the diagnostic ability of PJC to use a genetic and molecular analysis from PJC samples in cases in which a small quantity of specimen was obtained from PJC and the adjuvant diagnosis of the cytodiagnosis is negative. In a diagnosis for pancreatic cancer, sensitivity improves by adding the *K-ras* mutation analysis with routine cytology^[64]. There are some reports about the utilities of telomerase activity^[65], DNA methylation^[66], Smad4^[67], and KL-6^[68,69] measurement in pancreatic juice.

LIMITATION OF PJC

First, the accuracy of PJC is generally only around 40%-70%^[55,56] except in some institutions^[8,57,58]. Second, we cannot diagnose pancreatic neuroendocrine tumor, solid pseudopapillary neoplasm, or pancreatic acinar cell carcinoma, because they are not connected to the MPD. Third, it is hard to perform immunostaining because it is difficult to obtain a specimen as compared with EUS-FNA. Fourth, around 4.2%-33.3% of complications such as PEP

can occur after PJC^[8,55-57,60], but it is reported that the risk decreases for PEP with diclofenac administration. Elmunzer *et al.*^[70] reported that post-ERCP pancreatitis developed in 27 of 295 patients (9.2%) in the indomethacin group and in 52 of 307 patients (16.9%) in the placebo group ($P = 0.005$). Moderate-to-severe pancreatitis developed in 13 patients (4.4%) in the indomethacin group and in 27 patients (8.8%) in the placebo group ($P = 0.03$).

USE OF EUS-FNA AND PJC

Generally, EUS-FNA is better in diagnostic ability and adverse events than PJC. Therefore, if we can perform EUS-FNA, we should choose EUS-FNA, and it is desirable to only choose PJC in the following cases: (1) we can not detect a mass in EUS; (2) it is difficult to perform EUS-FNA when avoiding blood vessels and the MPD; (3) it is difficult to stop use of antithrombotic medicine; and (4) there aren't any institutions capable of performing EUS-FNA in the neighborhood.

Furthermore, there are some reports that the diagnostic accuracy of EUS-FNA and/or PJC was significantly higher than that of EUS-FNA or PJC alone^[8,71].

In conclusion, although there are some complications such as acute pancreatitis and dissemination, if the frequency of complications and the physical burden of surgery for patients are taken into consideration, it is perhaps better to obtain tissue before treatment begins. Since there are various methods of sampling tissue, it is important to choose the procedure while considering the patient's condition and safety.

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Pathogenesis and risk factors for gastric cancer after *Helicobacter pylori* eradication

Reina Ohba, Katsunori Iijima

Reina Ohba, Katsunori Iijima, Department of Gastroenterology, Akita University Graduate School of Medicine, Akita 010-8543, Japan

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Correspondence to: Reina Ohba, MD, PhD, Department of Gastroenterology, Akita University Graduate School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan. reina@doc.med.akita-u.ac.jp
Telephone: +81-18-8846573
Fax: +81-18-8017501

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Abstract

Helicobacter pylori (*H. pylori*) infection was thought to be the main cause of gastric cancer, and its eradication showed improvement in gastric inflammation and dec-

reased the risk of gastric cancer. Recently, a number of studies reported the occurrence of gastric cancer after successful eradication. Patients infected with *H. pylori*, even after eradication, have a higher risk for the occurrence of gastric cancer when compared with uninfected patients. Metachronous gastric cancer occurs frequently following the endoscopic removal of early gastric cancer. These data indicate that metachronous cancer leads to the occurrence of gastric cancer even after successful eradication of *H. pylori*. The pathogenesis of this metachronous cancer remains unclear. Further research is needed to identify biomarkers to predict the development of metachronous gastric cancer and methods for gastric cancer screening. In this article, we review the role of the *H. pylori* in carcinogenesis and the histological and endoscopic characteristics and risk factors for metachronous gastric cancer after eradication. Additionally, we discuss recent risk predictions and possible approaches for reducing the risk of metachronous gastric cancer after eradication.

Key words: *Helicobacter pylori*; Eradication; Atrophic gastritis; Intestinal metaplasia; Metachronous gastric cancer

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Core tip: *Helicobacter pylori* (*H. pylori*) eradication and endoscopic resection appeared to reduce the risk of gastric cancer. However, recent studies show that the risk of metachronous gastric cancer increases in the background of gastric mucosal atrophy even after successful eradication. Thus, curing *H. pylori* infections may not prevent metachronous gastric cancer in background mucosa with intestinal metaplasia. We review the risk factors and possible approaches for reducing the risk of metachronous gastric cancer.

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INTRODUCTION

Gastric cancer is the fourth most common cancer in the world and the second leading cause of cancer deaths worldwide with more than 700000 deaths annually^[1]. After the discovery of *Helicobacter pylori* (*H. pylori*) in 1983, the casual relationship between this bacterium and gastritis or gastric cancer has been steadily elucidated. In 1994, *H. pylori* was classified as a carcinogen by the International Agency for Research on Cancer of the World Health Organization (WHO). A 2009 meta-analysis showed that *H. pylori* eradication appeared to reduce the risk of gastric cancer^[2]. In Japan, approximately 99% of gastric cancers are caused by *H. pylori*; thus, *H. pylori*-negative gastric cancer constitutes less than 1% of all cases^[3].

H. pylori eradication has been one of the major therapeutic strategies to reduce gastric cancer incidence in healthy individuals and gastric cancer patients who have undergone endoscopic mucosal resection. The number of gastric cancers diagnosed and treated at an early stage increased after the development of endoscopic treatments. Ten years ago, more than 50% of early-stage gastric cancers were endoscopically treated, and the 5-year survival rate of early-stage gastric cancer patients after endoscopic treatment was 90%^[4]. Large lesions can be resected *en block*, including both the mucosa and submucosa, by endoscopic submucosal resection (ESD), which has improved histopathological diagnoses and decreased tumor recurrence. The endoscopic removal of early-stage gastric tumors does not affect the overall cancer risk. Research on gastric cancers after *H. pylori* eradication has been conducted for more than a decade. In 2008, an open-label, randomized controlled trial indicated that the occurrence of metachronous gastric cancer is reduced by approximately 1/3 after eradication^[5]. This study led to the recommendation of *H. pylori* eradication in patients with endoscopically treated gastric cancer. In Japan in 2013, health insurance covered *H. pylori* eradication as a treatment for gastritis, and this treatment was expected to reduce the incidence of gastric cancer^[6]. However, a subsequent Japanese study indicated that even after *H. pylori* infection is cured and gastric inflammation is eliminated, the risk for developing gastric cancer remains; furthermore, this risk was dependent on the level of gastric mucosal atrophy present before eradication therapy^[7]. Thus, the gastric mucosa endures continuous *H. pylori*-induced inflammation that increases the risk of metachronous gastric cancer even after treatment. Previous studies revealed that *H. pylori* eradication does not reduce the incidence of metachronous gastric cancer in patients who underwent endoscopic resection and recommended that

eradication should be performed before the progression of gastric mucosal atrophy. Extensive atrophy in the stomach and intestinal metaplasia of multiple areas causes gastric cancer and may increase the risk for metachronous gastric cancer when compared with cases of chronic gastritis mucosa^[8-11].

Currently, the success of a gastric cancer prevention strategy depends on timing because the treatment must be introduced before the progression of gastric carcinogenesis. However, recent studies on gastric cancer suppression suggested that critical features of gastric carcinogenesis can be reversed *via* molecular mechanisms.

Thus, monitoring patients for signs of gastric cancer after eradication is important. Here, we review the observed macroscopic and histological gastric mucosal changes, risks for metachronous gastric cancer, and possible approaches for reducing gastric cancer. We also discuss some of the potential molecular mechanisms for gastric cancer development after eradication.

H. PYLORI-INDUCED GASTRIC CANCER

A mechanism for carcinogenesis from *H. pylori*-triggered inflammation was first proposed by Correa^[12,13]. Correa proposed that chronic inflammation causes superficial gastritis that progresses to multifocal atrophic gastritis, followed by intestinal metaplasia, wherein gastric epithelium undergoes an "epithelial-mesenchymal transition" and begins to exhibit an intestinal phenotype. The subsequent stage consists of dysplasia culminating in invasive carcinoma, thus completing the "pre-cancerous cascade". This mechanism can be influenced by interactions between host and pathogen genotypes and environmental factors, such as socioeconomic indicators, a high-salt diet, low fruit/vegetable intake and smoking^[14]. In particular, *H. pylori* is the sole bacterium to be classified by the WHO as a class I carcinogen^[15].

Gastric cancer is an inflammation-associated cancer that occurs as a result of the infection which causes chronic, life-long, gastric mucosal inflammation. The pathogenesis of gastric cancer depends on the presence of genetic instability, that is, a consequence of *H. pylori*-induced acute and chronic inflammation, direct bacterial host interactions, and interactions with exogenous factors to produce carcinogens locally in the stomach^[16]. However it should be added that *H. pylori* is recognized as the primary cause of gastric cancer, it is a necessary but insufficient cause of gastric cancer which is typical of infectious cause of cancer such as relation between hepatitis C virus and liver cancer or human papilloma virus and cervical cancer.

Recent studies have shown that *H. pylori* infection causes gastric cancer by inducing gene mutations, aberrant DNA methylation, and disturbance of intracellular signaling pathways. Point mutations and aberrant DNA methylation accumulated even in normal mucosa, leading to field cancerization^[17,18]. We describe below a

molecular mechanism of the gastric cancer.

Field cancerization

Field cancerization was first proposed by Slaughter *et al.*^[19] based on a study of oral stratified squamous epithelium in 1953. This phenomenon, also known as widespread carcinogenesis, can be caused by long-term exposure to common cancer inducers in several body areas. During field cancerization, abnormalities caused by exposure to cancer inducers can accumulate while the organism continues to appear normal. *H. pylori* infections contribute to carcinogenesis and field cancerization by causing point mutations and DNA methylation abnormalities in the gastric mucosa.

Gene mutations caused by activation-induced deaminase

Activation-induced deaminase (AID) is a member of the cytidine deaminase family, which includes DNA- and RNA-editing enzymes. AID expression is highly regulated, restricted to germinal center B cells and essential for somatic hypermutation and class-switch recombination in B cells^[20]. Infection with cag pathogenicity island (cag PAI)-positive *H. pylori* ectopically induced the high expression of AID in human gastric epithelial cells, leading to multiple mutations in the *TP53* tumor suppressor gene.

AID expression is induced by the activation of NF- κ B caused by the *H. pylori*-induced inflammation in gastric epithelium cells. Additionally, *TP53* mutations are induced by AID-producing gastric epithelium cells, which play an important role in stomach carcinogenesis.

AID upregulation in *H. pylori*-infected stomachs occurs *via* the introduction of bacterial macromolecules through the type IV secretion system encoded by cag PAI and inflammatory cytokines, such as tumor necrosis factor (TNF), that are produced by *H. pylori*-related gastric inflammation. Infection with cag PAI-positive *H. pylori* resulted in aberrant AID expression in gastric epithelial cells, leading to the generation of somatic mutations in the host genome, such as the *TP53* gene. Cag PAI-positive *H. pylori* is more commonly associated with AID upregulation than cag PAI-negative *H. pylori*, and NF- κ B activation provides evidence linking the pathogenic strain of *H. pylori* to the accumulation of nucleotide alterations and the subsequent development of gastric cancer^[21].

DNA methylation abnormalities caused by chronic inflammation

H. pylori infection potently and temporarily induces methylation of multiple CpG islands (CGIs) in specific promoter regions, including tumor suppressor genes. Aberrant DNA methylation in gastric mucosa is associated with an increased risk of gastric cancer. Methylation levels in *H. pylori*-positive individuals were higher when compared with cases of *H. pylori*-negative gastric cancer^[22]. The persistence of DNA methylation in the

gastric mucosa decreases after *H. pylori* eradication^[17]. Chronic inflammation causes *H. pylori*-induced DNA methylation and thus may be more important than the infection itself. The expression of certain inflammatory genes, such as *TNF*, *IL-1 β* , and *Nos2*, increases DNA methylation^[23]. These data suggest a relationship between inflammatory signals and DNA methylation.

Disruption of intracellular signaling by the direct action of the virulence factor CagA

CagA is a protein produced by *H. pylori* that is directly injected into gastric epithelium cells through a type IV secretion system. Next, CagA activates Ras-Erk signaling by binding to Src homology 2-containing protein tyrosine phosphatase^[24,25]. Cell death in the gastric mucosa is inhibited by activating extracellular signal-regulated kinase and promoting myeloid cell leukemia-1 protein expression, which subsequently inhibits apoptotic cell death and delays the turnover of epithelial cells^[26].

Influence of *H. pylori* on carcinogenesis

Accumulation of point mutations: Frequent *TP53* mutations were discovered in the *H. pylori*-infected gastric mucosa of non-cancer patients using new sequencing technologies. Increased cytidine deaminase activity in these tissues appeared to increase these mutations and thus may promote gastric carcinogenesis in patients with *H. pylori* infection because most of the mutations were C: G to T: A^[27].

Accumulation of DNA methylation: The DNA methylation induced by helicobacter infection remains at the stem cell level in non-infected mucosa after eradication, and the residual methylation level correlates with carcinogenic risk^[17].

PREVENTION OF GASTRIC CANCER BY *H. PYLORI* ERADICATION

For one approach to inhibit gastric cancer, early prevention is important. *H. pylori* eradication stops the progression cancer risk and reverse some of mucosal damage. Multicenter clinical study results that the incidence of new gastric cancers who have a history of such disease and are thus at high risk for developing further gastric cancers was reduced by one-third among those with *H. pylori* eradication compared to no eradication therapy^[5]. This study also added that eradication did not prevent development on gastric cancer completely, the risk for gastric cancer is directly related to the degree of atrophy. A large-scale cohort study from Taiwan followed 80000 patients with peptic ulcer for 10 years after *H. pylori* eradication therapy^[28]. They reached the conclusions that the earlier eradication obviously reduce the incidence of gastric cancer. A meta-analysis of randomized controlled trials in 2014 showed that *H. pylori* eradication decreased the risk for gastric cancer in healthy asymptomatic infected individuals by

34%^[29]. However it remains unclear whether *H. pylori* eradication among those at lower risk. On the other hand, when there is not *H. pylori* infection, and there is not inflammation of the stomach, it may be said that you do not need to worry about gastric cancer immediately.

GASTRIC CANCER DEVELOPMENT AFTER ERADICATION

Endoscopic resection is widely applied as a curative treatment for gastric cancer. However, metachronous gastric cancer following endoscopic resection is becoming a major problem due to gastric mucosa atrophy and chronic inflammation of the intestinal epithelium caused by *H. pylori*.

Metachronous gastric cancer means here that a new cancer is found separately from an initial cancer with or without *H. pylori*. The metachronous gastric cancer contains gastric cancer after *H. pylori* eradication.

Uemura *et al*^[30] were the first to report that *H. pylori* eradication after endoscopic resection decreased the occurrence of metachronous cancer. A prospective randomized trial in Japan showed that *H. pylori* eradication reduced the risk of metachronous gastric cancer during a 3-year follow-up period. In contrast, a prospective randomized trial in South Korea showed that eradication after endoscopic resection did not reduce the incidence of metachronous gastric cancer^[31]. The percentage of metachronous gastric cancers after endoscopic treatment was 8.5% during a follow-up period of up to 11.1 years, which did not significantly differ from the 14.3% cancer rate in the eradicated group^[8]. And other studies conducted in Japan did not support eradication after endoscopic resection^[6,9].

The efficacy of *H. pylori* eradication at preventing metachronous gastric cancer after endoscopic resection remains controversial. One of the causes which has no significant difference between eradicated group and not eradicated group, there exists nearly 30% overlooked cancers and those are often found by whole follow-up for 1 or 2 years. There is the report that cumulative incidence rate of metachronous gastric cancer was lower in the eradication group and the high risk of metachronous gastric cancer probably does not continue after 10 years^[32]. Yoon *et al*^[33] reviewed 13 studies and 6237 patients in a meta-analysis of the beneficial effects of *H. pylori* eradication and recommended that patients who received endoscopic treatment should also receive eradication therapy. Similarly, Yuhara *et al*^[34] assessed 2 randomized control trials and 5 retrospective cohorts. There was variability in the observation period, atrophic degree of the background gastric mucosa, sanitization period and definition of the eradication group; however, the risk of metachronous gastric cancer in the non-eradication group was higher when compared with eradication group. Thus, eradication after endoscopic treatment may reduce the risk of metachronous gastric cancer.

There is one more thing to be added. That is, re-current infection after successful *H. pylori* eradication. The causes to become positive again after *H. pylori* eradication was classified in "relapse" and "re-infection" by various kinds of judging methods. "Relapse" is the phenomenon that increases again after quantity of bacteria decreased in sensitivity or less of the testing concerned temporarily, and becomes positive by reexamination. "Re-infection" means *H. pylori* completely disappeared after eradication, they were infected with different *helicobacter* newly. The reinfection rate of *H. pylori* is reported 0.22%-11.5% and the variations date may reflect differences in the prevalence of *H. pylori*, hygienic environment, and false-negative eradication judgment^[35,36]. Generally, it is not necessary to mind gastric cancer risk of re-infection because there are few re-infection.

Gastric mucosal changes after successful *H. pylori* eradication

Atrophic mucosa with intestinal metaplasia in differentiated gastric cancer and undifferentiated cancer of the gastric fundic gland mucosa are well-known examples of the relationship between gastric cancer and the background mucosa^[37].

The continued study of *H. pylori* and gastric cancer has revealed a link between nodular gastritis in young women and undifferentiated gastric cancer^[38]. Yagi *et al*^[39] reported the regular arrangement of collecting venules (RAC) using a magnifying endoscope, and Nakajima^[40,41] reported the RAC based on radiography. Both studies noted the importance of assessing background gastric mucosa with diagnostic imaging. *H. pylori* infected gastric mucosa presents with strong active gastritis that is immediately improved after eradication.

Kato *et al*^[42] reported that spotty redness is significantly improved and related to eradication success in a multicenter study comparing unsuccessful and successful groups.

Using NBI magnifying observation, Okubo *et al*^[43] showed that enlarged or elongated pits improved to small oval or pinhole-like round pits and the density of fine irregular vessels decreased after successful eradication without severe gastric atrophy or intestinal metaplasia. Kong *et al*^[44] reported histological changes after successful eradication using a meta-analysis. This study illustrates a very strong correlation between the eradication of *H. pylori* infection and improvement in intestinal metaplasia in the gastric antrum but not in the corpus and between gastric atrophy in both the antrum and the corpus. After eradication, the neutrophilic infiltration of the lamina propria of the gastric mucosa and the lymphocytic infiltration of plasma cells were immediately improved^[45,46].

Endoscopic detection of gastric cancer after successful eradication

Gastric cancer after successful *H. pylori* eradication is often difficult to diagnose by endoscope because

of the indistinct borderline or disappearance of the characteristic surface structures of tumors.

In 2005, Ito *et al.*^[47] discovered that metachronous gastric cancers were difficult to identify endoscopically due to flattened and obscured tumor cells with an outer layer that lacked atypical columnar epithelium. Saka *et al.*^[48] described many characteristic changes in gastric cancer detection after successful *H. pylori* eradication: (1) a gastritis-like mucosal pattern that is often ill-delineated; (2) a portion of the gastritis-like gastric mucosa that contains a pattern distinct from the background mucosa; and (3) a mucosal pattern of a white zone that exhibits “morphological heterogeneity” and “direction diversity” using NBI magnifying endoscopy. These changes can impair the detection of gastric cancer after eradication; however, an area exhibiting “morphological heterogeneity” and “direction diversity” when compared with the background mucosa is relatively easy to diagnose as cancer^[49].

With regard to the gastritis-like gastric mucosa, Kitamura *et al.*^[50] reported that “epithelium with low-grade atypia (ELA)” is frequently observed on the surface of gastric tumors after successful eradication therapy. Caudal-related homeobox 2 (CDX2) was not expressed, and neither p53- nor Ki67-positive cells were found in ELA, regardless of their expression in tumors. The presence of ELA positively correlated with the clinical interval between eradication and gastric cancer detection. Moreover, Yamamoto *et al.*^[51] reported that the average diameter of gastric tumors was smaller and the Ki-67 index was lower in patients who underwent *H. pylori* eradication. An analysis of macroscopic morphology revealed mainly depressed-type tumors and a high ratio of gastric differentiated-predominant mixed type lesions after a long eradication interval. *H. pylori* eradication may suppress growth, intestinalization, and acid hyposecretion during the development of gastric cancer.

These studies show a long-term risk for gastric cancer after successful eradication. Thus, endoscopic follow-ups must consider the distinct characteristics of gastric cancer after eradication.

Development and risk of metachronous gastric cancer

Various risks have been associated with the development of atrophic mucosa. Patients with precancerous changes in the gastric mucosa show an increased risk of gastric cancer. *H. pylori* eradication does not prevent the development of gastric cancer in all patients; furthermore the risk of cancer is higher in patients with precancerous changes prior to eradication^[52-55].

There is continued speculation on gastric cancer diagnoses after eradication. Some researchers question the discovery of gastric cancer after eradication. Asaka *et al.*^[56] suggested a cancer diagnosis could be due to a preexisting tumor that was not detected endoscopically prior to eradication and the potential inhibitory effect of *H. pylori* eradication on tumor growth. That is, there are two kinds of the gastric cancer that discovery after

eradication even if it occurs before eradication and the new cancer occurred after eradication truly. Take *et al.*^[55] found that the incidence of developing gastric cancer after amelioration of an *H. pylori* infection was 0.30% per year; furthermore, the cancer could develop as long as 10 years after *H. pylori* eradication even without gastric inflammation. It was also reported that doubling time of the intramucosal carcinoma is approximately 16.6 mo^[57] and it takes more than 10 years from occurrence of gastric cancer cell until we can recognize visually endoscopically^[48]. Thus, gastric cancer cannot be completely prevented by eradication of *H. pylori*.

Well-known risk factors of gastric cancer after successful eradication include another active *H. pylori* infection, increased age, and atrophic gastritis severity at the time of eradication therapy^[11,58,59]. The independent risk factors of metachronous gastric cancer are male sex, severe gastric mucosal atrophy, and multiple gastric cancers prior to a successful *H. pylori* eradication^[8,58,60,61].

Shiotani *et al.*^[62] showed that atrophy in biopsy specimens from the lesser curvature of the corpus was strongly associated with gastric cancer risk. A serum pepsinogen I level less than 25 ng/mL prior to eradication was significantly associated with subsequent tumor development.

Other reported risk factors include aberrant DNA methylation, microsatellite instability, aberrant expression of miRNAs, CD44v9 expression by tumor cells, and microscopic foci of intramucosal neoplasia elsewhere in the stomach.

The next chapter describes the prevention of metachronous gastric cancer through the use of predictive markers.

POSSIBLE APPROACHES FOR REDUCING CANCER RISK

Arginine adenosine-5'-diphosphate ribosylation inhibitors and prostaglandin E2

Patients presenting with atrophic gastritis, metaplasia, or dysplasia are routinely subjected to eradication therapy targeting the underlying infection; however, eradication is only partly effective at reversing atrophy and often fails to treat metaplasia and dysplasia^[63].

Patients with any of these conditions have at least a 10-fold increased risk of developing gastric cancer; thus, a “watch-and-wait” strategy is not appropriate. For high-risk patients, improved treatments for metaplasia have been reported.

Recently, patients and animals taking tamoxifen have been shown to have regression in intestinal metaplasia^[64]. Olaparib (Lynparza™), an Arginine adenosine-5'-diphosphate ribosylation (ADP-ribosylation) inhibitor used for ovarian cancer, has been shown to reverse intestinal metaplasia. ADP-ribosylation inhibitors may successfully prevent and cure helicobacter-induced gastric preneoplasia^[65]. Prostaglandin E2 showed efficacy as a

treatment during the early stages of *Helicobacter*-induced gastric carcinogenesis^[66].

Interactions between *H. pylori* and CD44v9-positive cancer stem cells

Researchers have sought to understand why all *H. pylori* infected people do not develop gastric cancer.

CD44v9, one of the main surface marker proteins of cancer stem cells, prevents the accumulation of reactive oxygen species and contributes to the increased resistance of tumors to anticancer drugs^[67]. CD44 overexpression, especially variant 9 (CD44v9), has been implicated in the local inflammatory response and metaplasia-carcinoma sequence in the human stomach^[68].

As a long-term survival strategy for the stomach, the CagA released by *H. pylori* is degraded via VacA-mediated autophagy^[69]. In contrast, CD44v9-expressing cancer stem cells accumulate intracellular CagA by suppressing autophagy. Therefore, the presence of CD44v9-expressing cancer stem cells is strongly associated with an increased risk of gastric carcinogenesis in the presence of *H. pylori*. CD44v9-positive cancer stem cells can appear after long-term inflammation. Chemopreventive treatments targeting this cancer protein may restore autophagy^[69,70]. It would be important index to examine CD44v9-expressing when we evaluate a recurrence risk of the gastric cancer that occur after eradication of *H. pylori*.

Treating DNA methylation abnormalities with demethylating agents

Reversal of DNA methylation abnormalities may be effective for gastric cancer prevention. *H. pylori* infection induces DNA methylation abnormalities, which create the groundwork for cancerogenesis in the gastric mucosa. The carcinogenic risk can be assessed by measuring the extent of DNA methylation abnormalities that result in a "point of no return". Nanjo *et al.*^[71] identified seven specific CGIs that show increased methylation levels after *H. pylori* infection. EMX1, NKX6-1, and NEFM were particularly influential, and the carcinogenic gastric cancer risk was 23.8 times higher in cases of increased EMX1 methylation. These cancer risks also apply to individuals with past infections.

A multicenter prospective cohort study by Asada *et al.*^[72] showed that the methylation level in the non-cancerous gastric mucosa of patients with gastric cancer was significantly ($P = 0.042$) associated with an increased risk of developing metachronous gastric cancers. Specifically, the methylation level of miR-124a-3 results in an elevated risk of metachronous gastric cancer; furthermore, similar trends were observed for EMX1 and NKX6-1. In conclusion, miR-124a-3 is an informative biomarker for predicting the risk of metachronous gastric cancer^[73].

CDX2 is a transcriptional control factor that is indispensable for intestinal epithelium differentiation, and it functions as a tumor suppressing factor for tumors derived from intestinal epithelium. DNA methylation is frequently found in the *CDX2* gene promoter region

due to the development of intestinal gastric tumors that inactivate the gene. In addition, CDX2 is not expressed in stomachs uninfected by *H. pylori*. Early eradication prevents gastric cancer by inhibiting aberrant CDX2 expression. *H. pylori* eradication can reverse the gastric phenotype and diminish aberrant CDX2 expression in the early stages of intestinalization^[11,74].

The technique used to determine the presence or absence of gastric cancer consists of harvesting DNA with an endoscope. The genetic DNA methylation rate of SRY-box containing gene 17 (*SOX17*) was assessed before and after endoscopic submucosal dissection. The pathological examination revealed that the DNA methylation rate of *SOX17* was significantly decreased in all of the patients after endoscopic submucosal dissection. A decreased DNA methylation rate of *SOX17* can be used to infer the extent of the resection. A decreased DNA methylation ratio after ESD should be observed during routine follow-ups. In contrast, an increased ratio indicates an incomplete resection and may suggest the need for additional surgery^[75].

DNA demethylating agents are used for myelodysplastic syndrome patients. Demethylation of the gastric mucosa has been considered as a potential treatment. Additional research on treatment adaptation and side effects are needed to determine the applicability of preventing gastric cancer by inhibiting aberrant DNA methylation.

Chromosomal aberration of carcinoma tissue is not found in precancerous lesions; however, DNA methylation abnormalities can be used to identify both carcinomas and precancerous lesions. Examination of aberrant DNA methylation after eradication can be used to differentiate a high risk group from the sample population (Figure 1). Additional studies are needed to determine the relationships between aberrant DNA methylation and the invasion degree and cancer prognosis.

Utilization of pepsinogen serum levels and an *H. pylori* antibody titer

A novel and rapid diagnostic method has been introduced in Japan. This simple method, which consists of a pepsinogen serum level assay and helicobacter antibody titer, can be easily applied to large populations.

Yoshida *et al.*^[76] reported that altered DNA methylation levels in the stomach mucosa closely correlated with *H. pylori*-associated gastritis as assessed by serum pepsinogen II levels and a helicobacter antibody titer. Moreover, 4655 healthy asymptomatic subjects with no eradication treatment and who were followed-up for 16 years were divided into four groups based on the pepsinogen and antibody levels. This study showed a graded and significant rise in the hazard ratio for gastric cancer as chronic gastritis worsened. The mild atrophic gastritis group showed high gastric mucosa inflammation, which is a risk factor for diffuse-type cancer^[77]. These results indicate that gastric cancer mainly develops from the gastritis-atrophy-metaplasia-cancer sequence and

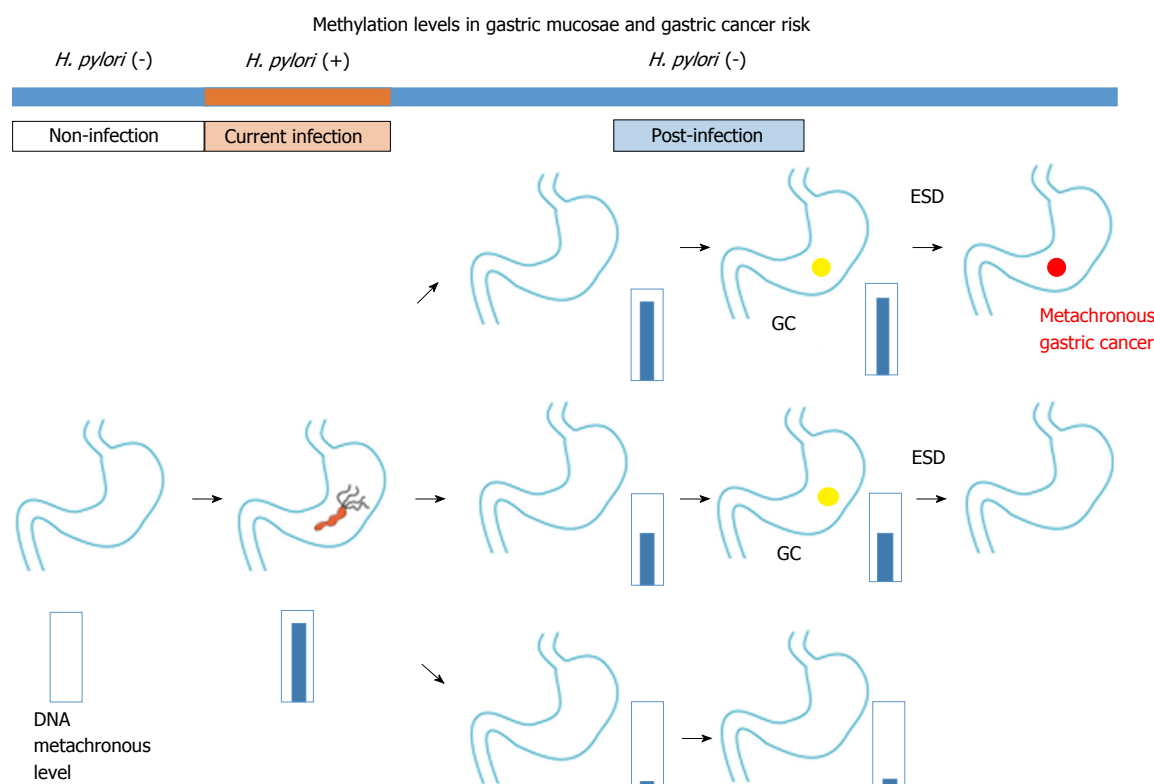


Figure 1 Relationship between gastric mucosae methylation levels and *Helicobacter pylori* infection/gastric cancer (modified from Maekita *et al*^[22]). Residual aberrant methylation even after eradication is thought to reflect methylation in gastric gland stem cells. From endoscopically biopsied tissue, predicting GC risk based on the accumulation of aberrant DNA methylation in the gastric mucosae. ESD: Endoscopic submucosal resection; GC: Gastric cancer; *H. pylori*: *Helicobacter pylori*.

partly from active inflammation-based carcinogenesis. Notably, at-risk individuals require follow-up because this serum method should be used as a risk examination and not as a cancer screening. We previously reported that Congo-red chromoendoscopy methods can be used to identify high-risk areas after eradication. Biopsies of high-risk areas (non-acid-secreting area) revealed sustained hyperproliferation, accumulation of p53 protein and immunoreactivity for Ki-67^[78]. Moreover, we demonstrated that a slow-releasing L-cysteine capsule effectively eliminated acetaldehyde from the gastric juice of PPI-treated aldehyde dehydrogenase 2 (ALDH2)-active and ALDH2-deficient subjects. These novel methods may aid in the prevention of gastric cancer, especially in established high-risk groups^[79].

Eventually, we have got one method to make *H. pylori* may become extinct before the future children infect. However, we should take measures to cope with the gastric cancer which may occur after *H. pylori* eradication. Surveillance and follow-up based on the feature or the gastric cancer including remnant stomach after *H. pylori* eradication is important, and it can be said there is little invasive method more than a stomach is removed. Additional studies are needed to clarify these surveillance methods under multiple conditions and determine their reliability as biomarkers for metachronous gastric cancers.

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Molecular mechanisms of chemoresistance in gastric cancer

Wen-Jia Shi, Jin-Bo Gao

Wen-Jia Shi, Department of Pediatric Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, Hubei Province, China

Jin-Bo Gao, Department of Gastrointestinal Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, Hubei Province, China

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Correspondence to: Dr. Jin-Bo Gao, Associate Professor, Department of Gastrointestinal Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1277 Jiefang Avenue, Wuhan 430022, Hubei Province, China. gaojinbo@163.com
Telephone: +86-27-85351619
Fax: +86-27-85351619

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Abstract

Gastric cancer is the fourth most common cancer and

the second leading cause of cancer deaths worldwide. Chemotherapy is one of the major treatments for gastric cancer, but drug resistance limits the effectiveness of chemotherapy, which results in treatment failure. Resistance to chemotherapy can be present intrinsically before the administration of chemotherapy or it can develop during chemotherapy. The mechanisms of chemotherapy resistance in gastric cancer are complex and multifactorial. A variety of factors have been demonstrated to be involved in chemoresistance, including the reduced intracellular concentrations of drugs, alterations in drug targets, the dysregulation of cell survival and death signaling pathways, and interactions between cancer cells and the tumor microenvironment. This review focuses on the molecular mechanisms of chemoresistance in gastric cancer and on recent studies that have sought to overcome the underlying mechanisms of chemoresistance.

Key words: Drug resistance; Gastric cancer; Chemotherapy

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Core tip: Although chemotherapy remains one of the primary therapeutic modalities used in the treatment of gastric cancer, chemoresistance limits the effectiveness of chemotherapy and results in treatment failure. The elucidation of the mechanisms of drug resistance will be very helpful for the prediction of sensitivity to chemotherapy and the reversal of drug resistance to improve therapeutic efficacy. The mechanisms of drug resistance have been broadly investigated in recent years. In this review, we summarize the molecular mechanisms of chemoresistance in gastric cancer and discuss the progress in the reversal of drug resistance.

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INTRODUCTION

Gastric cancer is one of most common malignant tumors. It currently ranks as the fourth most common cancer and is the second leading cause of cancer deaths worldwide. The incidence of gastric cancer varies greatly in different regions, and over 70% of new cases and deaths occur in developing countries. The highest incidence rates are observed in Eastern Asia, Eastern Europe, and South America, whereas the lowest rates are observed in North America and most parts of Africa^[1].

Although the incidence of gastric cancer has declined due to improved living standards, a reduction in chronic *H. pylori* infection and increased screening activities, the overall outcome has not significantly improved over the last few decades. The treatment outcomes for gastric cancer are determined by the stage of the tumor at presentation and the condition of the patients. Surgery is the only potentially curative treatment for gastric cancer. The five-year overall survival rate after surgery varies from 70%-95% in early stage patients to 20%-30% in advanced-stage patients. Moreover, more than two-thirds of patients have unresectable disease when they are diagnosed^[2]. Therefore, chemotherapy is used to relieve symptoms in patients with unresectable tumors and to reduce the risk of recurrence and metastasis in patients with localized disease after surgery. Perioperative chemotherapy can improve the 5-year survival rate from 23% to 36.3% among patients with resectable adenocarcinoma of the stomach compared with surgery alone^[3]. In addition, chemotherapy has shown only a modest benefit in patients with metastatic disease with an average survival of approximately ten months^[4,5].

Although chemotherapy plays an important role in the treatment of both local and metastatic gastric cancer, the efficacy of chemotherapy is limited by chemoresistance. Chemotherapeutic resistance, whether intrinsic or acquired, is a complex and multifactorial phenomenon that is associated with tumor cells as well as with the tumor microenvironment^[6]. With the development of modern biological techniques, the mechanisms of chemoresistance have been broadly investigated in recent years. This review focuses on the molecular mechanisms of chemoresistance in gastric cancer and on recent studies that have sought to overcome the underlying mechanisms of chemoresistance.

REDUCED INTRACELLULAR CONCENTRATION OF DRUGS

Drug efflux

The ATP-binding cassette (ABC) transporter family has been shown to be associated with chemoresistance. These transmembrane proteins can reduce the intracellular concentrations of drugs *via* an increase in the efflux of drugs and the redistribution of drugs away from the site of action. This family of proteins is composed of 49 members that are divided into 7 subclasses (ABCA-

ABCG). ABCB1, also known as P-glycoprotein and MDR1, was the first ABC transporter to be identified and has been studied extensively. The overexpression of ABCB1 has been found in human gastric cancer cell lines and in clinical gastric cancer tissues^[7-9]. The association between ABCB1 expression and the clinicopathological characteristics of patients with gastric cancer is not fully understood. According to one study, ABCB1 expression was less frequent in locally advanced tumors and was absent in primary tumors where distant metastases were also present^[8]. In another study, ABCB1 expression was also associated with well and moderately differentiated tumors and intestinal-type tumors, but it did not indicate poor prognosis of gastric cancer patients treated with 5-fluorouracil (5-FU) and doxorubicin-based adjuvant chemotherapy^[10]. Recent reports have suggested that the expression of ABCB1 is related to poor prognosis in gastric cancer patients^[9,11]. Further studies have indicated that the expression of ABCB1 is associated with chemoresistance in patients with gastric cancer, as its presence in tumor cells may be an indicator of a lack of sensitivity to chemotherapy^[12-15]. The expression of ABCB1, which results in acquired chemoresistance, can be induced by chemotherapy. The expression rate of ABCB1 increased from 27.8% to 37.5% after the administration of adriamycin-based chemotherapy. ABCB1 expression after chemotherapy has been correlated with a higher rate of systemic recurrence^[16]. ABCB1 has been demonstrated to affect intrinsic and acquired resistance of gastric cancer cells to chemotherapeutic agents. Blocking the expression of ABCB1 can reverse multidrug resistance in human gastric carcinoma cells^[17,18]. Other ABC transmembrane proteins, such as ABCC1, which is also known as multidrug resistance-associated protein, are also associated with multidrug resistance in gastric cancer^[9,19,20].

The expression of ABCB1 is regulated by a variety of factors. NF-kappa B is a transcriptional factor that can bind to gene promoters or enhancer sites to promote the transcription of those genes. Bentires-Alj *et al.*^[21] identified a consensus NF-kappa B binding site in the first intron of the human *ABCB1* gene and demonstrated that NF-kappa B can bind to this intronic site and activate reporter gene transcription. Gu *et al.*^[22] demonstrated that upon paclitaxel stimulation, cyclooxygenase-2 induced the expression of ABCB1 in gastric cancer cells *via* the NF-kappa B pathway. Another study found a positive association between phosphorylated AKT (p-AKT) and ABCB1 expression in both gastric cancer tissues and gastric cancer cell lines. Moreover, it was shown that the expression of ABCB1 was reduced by the inhibition of the phosphatidylinositol-3 kinase (PI3K)/AKT pathway in SGC7901/ADR cells. Ubiquitin ligase Cbl-b can also down-regulate the expression of ABCB1 through the suppression of the PI3K/AKT signaling pathway. These findings indicated that the PI3K/AKT pathway might regulate the expression of ABCB1 and may be correlated with chemoresistance^[23]. Recently, some studies have demonstrated that microRNAs play an important role

in chemoresistance *via* the regulation of the expression of ABCB1. miR-508-5p can repress the expression of ABCB1 by targeting the 3'UTR of ABCB1^[24]. miR-106a and miR-27a, through the up-regulation of ABCB1 expression, are also involved in chemoresistance in gastric cancer^[25,26]. In addition, the long non-coding RNA PVT1 has been shown to increase the expression of multidrug resistance-related genes [*ABCB1*, *ABCC1*, *mTOR* and Hypoxia-inducible factor-1 α (HIF-1 α)], which in turn results in the development of chemoresistance in gastric cancer^[27].

Drug inactivation

Glutathione *S*-transferases (GSTs) are a family of phase II detoxification enzymes that catalyze the conjugation of glutathione (GSH) to a broad variety of hydrophobic and electrophilic compounds. GSTs are involved in chemoresistance because they inactivate drugs. The expression of glutathione *S*-transferase-pi (GST-pi) has been found in both gastric cancers and in normal gastric mucosa, but the total GST enzyme activity and the absolute amounts of GST-pi protein were significantly higher in tumors compared with those of matched normal mucosa^[28]. Differences were found in the GSH and GST parameters between responsive and progressive patients with gastric cancer who were treated with chemotherapy, which suggests a role for the GSH/GST system in the susceptibility of gastric tumor cells to chemotherapy^[29]. The overexpression of GST-pi has been found to be significantly related to the sensitivity of gastric cancer to cisplatin^[30]. It was reported that GST- α is correlated with cisplatin resistance in gastric cancer, and the quantification of GST- α can be used to predict the clinical effects of cisplatin in patients with gastric cancer^[31]. Lastly, 3 β -acetyl tormentic acid has been shown to sensitize multidrug-resistant cells to antineoplastic drugs through the modulation of intracellular levels of GSH and GST activity^[32].

Reduced prodrug activation

The reduced activation of prodrugs may decrease the intracellular concentrations of the corresponding active drugs, which results in the reduction of chemotherapeutic efficacy. 5-FU is a common chemotherapy drug whose activation involves thymidine phosphorylase, uridine phosphorylase and orotate phosphoribosyl transferase. Lower expression or impaired activity of these enzymes has been associated with chemoresistance to 5-FU in gastric cancer^[33-35].

ALTERATIONS IN DRUG TARGETS

DNA topoisomerases are a class of nuclear enzymes that modulate DNA topology during chromosomal transactions, such as gene transcription and DNA replication, recombination and repair. Topoisomerases are targets of various chemotherapeutic agents such as doxorubicin, etoposide, mitoxantrone and irinotecan. Alterations

in topoisomerases could affect a patient's response to chemotherapy as well as resistance. A series of studies revealed higher Topo-II expression in gastric carcinomas compared with normal gastric mucosa, and this increased expression was correlated with clinicopathological parameters such as tumor location, histological type, infiltration depth, distant metastases and tumor stage^[36-39]. A reduction in Topo-II expression was also found to contribute to the resistance of human gastric cancer cells to adriamycin and other topo II-targeted drugs *in vitro*^[40]. Furthermore, Topo-II expression has been negatively correlated with hydroxycamptothecin, adriamycin and mitomycin C resistance in gastric cancer tissues^[41].

Paclitaxel is an anti-microtubule drug that interferes with tubulin and that stabilizes microtubule composition, normal spindle assembly and cell division, which all result in cancer cell death. The clinical effectiveness of paclitaxel and the expression of the microtubule-associated protein tau have therefore been investigated^[42]. Among 20 cases of inoperable or noncurative, resected gastric cancer, 14 demonstrated positive tau expression while 6 were negative for tau expression. All six tau-negative cases showed a favorable response to paclitaxel, whereas 12 of the 14 tau-positive cases showed progressive disease or no change after paclitaxel administration. These results indicated that tau-negativity may be used to select gastric cancer patients who will respond favorably to paclitaxel treatment. Another study demonstrated that the sensitivity of gastric cancer patients to paclitaxel treatment was inversely correlated with the expression of class III β -tubulin and the microtubule-associated protein tau^[43]. Additionally, low miR-34c-5p expression and high microtubule-associated protein tau protein expression were found in paclitaxel-resistant gastric cancer samples. The overexpression of miR-34c-5p causes a significant down-regulation of tau protein expression, which leads to an increase in the chemosensitivity of paclitaxel-resistant gastric cancer cells. Therefore, the modulation of microtubule-associated proteins might play an important role in the chemoresistance of gastric cancer cells to paclitaxel^[44].

DYSREGULATION OF CELL SURVIVAL AND DEATH

Chemotherapeutic drugs cause DNA damage and induce cell death, and escape from cell death is one of the mechanisms of chemoresistance. The promotion of cell survival and resistance to apoptosis are both hallmarks of cancer cells. Accumulating evidence has shown that the dysregulation of cell survival and death is involved in the resistance of cancer cells to chemotherapeutic drugs.

BCL-2 family members

The BCL-2 protein family comprises a group of apoptosis regulators. These proteins can be divided into the following three subfamilies: The anti-apoptotic subfamily,

which contains the BCL-2, BCL-xL, BCL-w, MCL-1, BFL1/A-1, and BCL-B proteins; the pro-apoptotic subfamily, which contains the BAK, BAX, and BOK proteins; and the BH3-only protein subfamily, which contains the pro-apoptotic BIM, BID, BIK, BAD, BMF, HRK, PUMA, and NOXA proteins^[45]. Interactions among the BCL-2 protein family members within the mitochondrial outer membrane control cellular commitment to apoptosis^[46]. The role of the BCL-2 family of proteins in chemoresistance has been studied extensively.

Studies have demonstrated that the overexpression of BCL-2 is associated with chemoresistance to cytotoxic chemotherapeutic agents in patients with gastric cancer^[47,48]. The silencing of BCL-2 increased cell apoptosis and decreased resistance to 5-FU in gastric adenocarcinoma cells^[49]. This suggested that the modulation of BCL-2 expression could affect chemosensitivity in gastric cancer. A recent study showed that Rho GDP dissociation inhibitor 2 rendered gastric cancer cells resistant to cisplatin *via* the up-regulation of BCL-2 expression^[50]. In addition, microRNAs are small, endogenous noncoding RNAs that negatively regulate gene expression at the posttranscriptional level. Several microRNAs, such as miR-204, miR-181b, miR-15b and miR-16, were found to up-regulate the expression of BCL-2, which resulted in multidrug resistance in human gastric cancer cells^[51-53].

The pro-apoptotic protein BAX has been demonstrated to predict clinical responsiveness to chemotherapy in patients with gastric cancer^[54]. Increased BAX expression has also been shown to sensitize KATO III cells to chemotherapeutic agent-induced apoptosis through the enhancement of the release of cytochrome c from mitochondria^[55]. Our studies showed that interferon regulatory factor 1 enhanced the chemosensitivity of gastric cancer cells to 5-FU through the induction of PUMA-mediated apoptosis^[56,57]. Other BCL-2 family members (BCL-xL, BAK, MCL-1) have also been demonstrated to function in the regulation of chemotherapy-induced apoptosis^[58,59]. This indicated that proteins in the BCL-2 family, through interactions among its members, play a pivotal role in the determination of cell fate following chemotherapy.

p53

The p53 tumor suppressor gene plays an important role in various processes, including cell cycle regulation, DNA repair and apoptosis. In one study, mutations in the p53 gene were found in 0%–77% of gastric carcinomas^[60]. Moreover, p53 alterations including a high frequency of p53 mutations, loss of heterozygosity, overexpression of the p53 protein, and consequently, the loss of p53 function, are early events in gastric cancers; they are also important biomarkers that are used to determine prognosis and treatment response^[61]. Although the relationship between p53 and chemoresistance in gastric cancer has been studied for many years, the results are not consistent. Recently, a meta-analysis was performed

to expound the relationship between p53 status and the response to chemotherapy^[62]. Thirteen published studies were eligible, including 564 cases, which were identified and analyzed. The results showed that p53 positive status (*i.e.*, high expression of p53 protein and/or a mutant p53 gene) was associated with an improved response in patients with gastric cancer who received chemotherapy. This indicated that p53 status might be a useful predictive biomarker for response to chemotherapy in gastric cancer. A later study showed that rAd-p53 enhanced the sensitivity of gastric cancer cells to chemotherapy *via* the promotion of apoptosis^[63]. The restoration of p53 was able to overcome cisplatin resistance in gastric cancer through the inhibition of AKT as well as through the induction of BAX^[64].

PI3K/AKT pathway

The PI3K/AKT pathway is a vital regulator of cell growth, proliferation and survival. The stimulation of receptor tyrosine kinases or G-coupled proteins activates PI3K, which in turn activates AKT; the phosphorylation of AKT is required for the complete activation of AKT. Activated AKT then phosphorylates various substrates so that it can exert its functions in cell proliferation, growth, anti-apoptosis and cell cycle progression. Aberrant activation of the PI3K/AKT pathway, which is believed to play an important role in resistance to chemotherapy, has been reported in human malignancies including gastric cancer.

Mutations in PIK3CA, which lead to increased PI3K activity, have been reported in gastric cancer^[65,66]. The expression of AKT and p-AKT was found in 74% and 78% of gastric carcinomas, respectively^[67]. It has been reported that the expression of p-AKT is correlated with depth of infiltration of the tumor, number of infiltrated lymph nodes, and overall survival^[68]. Moreover, several studies have shown that activated AKT is associated with increased resistance to multiple chemotherapeutic agents including 5-FU, adriamycin, mitomycin C and cis-platinum^[69,70]. Further studies demonstrated that chemotherapeutic reagents can induce activation of the PI3K/AKT signaling pathway, which results in acquired chemoresistance in gastric cancer cells^[71,72]. In addition, in one study, the overexpression of AKT decreased the chemosensitivity of gastric cancer cells to cisplatin, whereas the down-regulation of AKT reversed the resistant phenotype of gastric cancer cells *in vitro* and *in vivo*^[73,74].

Studies have reported that the aberrant activation of the PI3K/AKT pathway can be induced by various factors, including mutations in PIK3CA^[65], loss of PTEN function^[69], mutations in AKT isoforms^[75], and upstream activation of other growth pathways (*e.g.*, EGFR signaling pathway)^[76]. Although the PI3K/AKT pathway plays an important role in chemoresistance, the mechanism of PI3K/AKT activation that results in chemoresistance is not fully understood. It has been reported that NF-kappa B is a downstream target of AKT and that chemothera-

peutics induce AKT activation, I κ B α phosphorylation and degradation, and finally, NF-kappa B activation. Inducible AKT and NF-kappa B activities are involved in the chemoresistance of gastric cancer cells. The activation of NF-kappa B is one part of the mechanism of chemoresistance induced by AKT^[77]. Survivin is another downstream target of AKT. In cisplatin-resistant gastric cancer cells, higher levels of survivin and p-AKT have been observed. According to one study, specific inhibition of AKT reduced the expression of survivin and enhanced the sensitivity of cisplatin-resistant cells to cisplatin^[78]. Another study showed that the up-regulation of p-AKT expression could confer multidrug resistance in gastric cancer cells through the up-regulation of BCL-2 expression and the down-regulation of BAX expression^[79].

Because the PI3K/AKT pathway plays a vital role in chemoresistance in gastric cancer, the targeting of PI3K/AKT has emerged as a promising approach to reverse chemotherapy resistance. A recent study reported that LY294002, a selective inhibitor of PI3K, might overcome intrinsic and acquired resistance to 5-FU *via* the down-regulation of activated p-AKT and mitochondria-dependent apoptosis in gastric cancer cells^[80]. An AKT inhibitor (MK-2206) has also been demonstrated to augment the efficacy of chemotherapeutics in gastric cancer, but the magnitude of synergy depends on the treatment sequence. Furthermore, in one study, MK-2206 administered before chemotherapy resulted in the highest synergistic effect compared to the effects when it was administered after or concurrently with chemotherapy^[81].

Mitogen-activated protein kinase pathway

The mitogen-activated protein kinase (MAPK) signaling pathway is widely expressed in multicellular organisms, where it plays a critical role in multiple biological processes, such as cell proliferation, differentiation, and cell death. Dysregulation of the MAPK signaling pathway is associated with the occurrence and progression of various cancers including gastric carcinoma^[82]. Moreover, numerous studies have demonstrated that the MAPK pathway is also involved in chemotherapy resistance in gastric cancer. According to one study, phosphorylated mitogen-activated protein kinase (p-MAPK) was positive in 59.6% of patients with metastatic gastric cancer. Moreover, the expression of p-MAPK in primary tumors and metastatic lesions was similar. The overall survival was found to be significantly shorter in p-MAPK-positive patients. This indicated that p-MAPK expression might be a potential negative prognostic parameter in patients with metastatic gastric cancer who are treated with chemotherapy^[83]. The activation of the p38-MAPK pathway was found in vincristine-resistant gastric cancer SGC7901/VCR cells and was determined to be responsible for the modulation of multidrug resistance^[84]. In addition, the inhibition of p38 MAPK significantly increased gastric cancer cell sensitivity to doxorubicin through the induced expression of the pro-apoptotic protein BAX and a concomitant decrease in BCL-2 expression^[85].

TUMOR MICROENVIRONMENT

The tumor microenvironment consists of the extracellular matrix (ECM), various cells including cancer-associated fibroblasts, immune and inflammatory cells, and blood or lymph vessels. Increasing evidence has shown that the tumor microenvironment has multiple functions in tumorigenesis, invasion, and metastasis, as well as in drug resistance.

Hypoxia

Hypoxia, which is a common feature of solid tumors, results in tumor progression and treatment resistance. HIF-1 α is one of the most important regulators of the cellular response to hypoxia. HIF-1 α expression was found to be positive in 65.6% of gastric cancers. The overexpression of HIF-1 α was found to be an indicator of poor prognosis for patients with gastric cancer and was significantly correlated with histology, depth of invasion, VEGF expression, and MVD^[86]. It has also been reported that HIF-1 α expression could predict the response of patients with advanced gastric cancer to 5-FU-based adjuvant chemotherapy^[87]. Another study showed that HIF-1 α determines gastric cancer chemosensitivity through the modulation of p53 and NF-kappa B^[88]. Additional studies demonstrated that HIF-1 α overexpression increases the expression of BCL-2, decreases the expression of BAX, and also significantly induces the expression of ABCB1 and ABCC1. This indicates that HIF-1 α may confer hypoxia-induced drug resistance *via* the inhibition of drug-induced apoptosis and decreases in intracellular drug accumulation^[89].

Alterations of the extracellular matrix

The ECM is a complicated network of multifunctional molecules that influence major malignant phenotypes of cancer cells, including oncogenesis, progression and drug resistance. Laminin and collagen IV are natural basement membrane components that constitute a specific ECM that maintains malignant phenotypes in gastric adenocarcinoma cells^[90]. Recent findings showed that the adhesive ability of multidrug-resistant gastric cancer cells was significantly increased compared with parental cells, which were sensitive to chemotherapeutic drugs. The ECM component laminin increased the resistance of gastric cancer cells to vincristine and adriamycin by binding to the receptor MGr1-Ag/37LRP. This suggested that the chemoresistant phenotype of gastric cancer cells is associated with a state of increased cell adhesion. Laminin can modify the response to chemotherapeutic agents by various mechanisms, including regulation of MDR-related proteins (ABCB1 and ABCC1), apoptosis-related genes (BCL-2 and BAX), and signaling pathways (PI3K/AKT and MAPK/ERK)^[91,92]. It has been demonstrated that extracellular high mobility group box chromosomal protein 1 might promote drug resistance to adriamycin and vincristine *via* the up-regulation of ABCB1 in human gastric adenocarcinoma cells^[93].

Cytokines and growth factors

Soluble factors in the tumor microenvironment such as cytokines and growth factors exhibit key functions in chemotherapeutic resistance, as they maintain the activation of various survival-related signaling pathways. In a recent study, the serum levels of 52 types of cytokines and angiogenic factors were measured in 68 patients with gastric cancer who were treated with fluoropyrimidine and platinum combination chemotherapy. The following eleven cytokines and angiogenic factors were found to be independently correlated with poor overall survival: Interleukin-2 receptor-alpha, growth-regulated alpha protein, hepatocyte growth factor, macrophage colony-stimulating factor, stromal cell-derived factor, IL-6, IL-8, IL-10, interferon-gamma, vascular endothelial growth factor, and osteopontin^[94]. IL-33 has been reported to confer resistance to chemotherapy in gastric cancer cells through activation of the JNK signaling pathway^[95]. IL-6 can trigger the activation of STAT3 and has been found to be associated with acquisition of resistance of gastric cancer cells to trastuzumab^[96]. Tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) is a member of the TNF superfamily of structurally related cytokines. It was revealed that TWEAK promotes resistance to 5-FU in gastric cancer cells through NF-kappa B activation^[97].

CONCLUSION

Resistance to chemotherapy is a major challenge for patients who currently undergo therapy for gastric cancer. A wide range of molecular mechanisms of chemoresistance has been implicated in gastric cancer, including reduced intracellular concentrations of drugs and alterations of drug targets. The dysregulation of cell survival and death signaling pathways can also lead to resistance to chemotherapeutic drugs. In addition, the interactions between cancer cells and the tumor microenvironment also plays an important role in chemoresistance in gastric cancer. These emerging findings are very helpful for the development of personalized therapies based on the prediction of the chemosensitivity of cancer cells as well as for the establishment of novel therapeutic strategies to reverse the chemoresistance of tumors. However, the mechanisms of chemoresistance are complex and multifactorial. The chemotherapeutic resistance of tumors may be caused by different molecular mechanisms in different patients due to tumor heterogeneity and drug variety. Therefore, more extensive studies are needed for a more comprehensive elucidation of the mechanisms of chemotherapy resistance in gastric cancer.

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Pancreatic cancer: New hopes after first line treatment

Francesca Aroldi, Paola Bertocchi, Giordano Savelli, Edoardo Rosso, Alberto Zaniboni

Francesca Aroldi, Paola Bertocchi, Alberto Zaniboni,
Department of Medical Oncology, Poliambulanza Foundation,
25124 Brescia, Italy

Giordano Savelli, Department of Nuclear Medicine, Polia-
mbulanza Foundation, 25124 Brescia, Italy

Edoardo Rosso, Department of General Surgery, Poliambulanza
Foundation, 25124 Brescia, Italy

Author contributions: Aroldi F, Bertocchi P, Savelli G and Rosso E contributed equally to this work and wrote the paper; while Zaniboni A made critical revisions related to important intellectual content of the manuscript and approved the final version of the article.

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Correspondence to: Alberto Zaniboni, MD, Department of Medical Oncology, Poliambulanza Foundation, via Bissolati 57, 25124 Brescia, Italy. zanib@numerica.it
Telephone: +39-30-3515553
Fax: +39-30-3518270

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Abstract

Pancreatic cancer is the fourth leading cause of cancer-related death worldwide. Extensive research has yielded advances in first-line treatment strategies, but there is no standardized second-line therapy. In this review, we examine the literature trying to establish a possible therapeutic algorithm.

Key words: Nab-paclitaxel; Nal-iri; Pancreatic cancer; Second line; Algorithm

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Core tip: Pancreatic cancer is an emerging disease. In this review a possible therapeutic algorithm for second line treatment is hypothesized.

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INTRODUCTION

Pancreatic cancer (PDA) is an emerging disease and is the fourth leading cause of cancer death worldwide^[1]. The prognosis is very poor; the 5-year survival rate is 5%, and the life expectancy of patients with metastatic PDA is approximately 2.8-5.7 mo^[2]. These poor outcomes are likely due to resistance to chemotherapy and PDA biology. PDA often presents with micro-metastatic lesions at diagnosis, which are subsequently confirmed by radiological evidence. The mechanisms underlying PDA development and progression have not been fully elucidated, hindering the development of

therapeutic strategies for disease management. National Comprehensive Cancer Network guidelines recommend 5-fluorouracil-oxaliplatin-irinotecan (FOLFIRINOX) or nab-paclitaxel plus gemcitabine as first-line therapy. Gemcitabine (alone or in combination) is recommended as second-line therapy, with capecitabine or fluoropyrimidine plus oxaliplatin as the final option^[3]. In fact gemcitabine seems to be more effective than fluoropyrimidine^[4]. Second-line therapy is not standardized and depends on patient performance status (PS) (which frequently worsens after first-line therapy), comorbidities, previous treatments, and residual toxicities. Notably, only 40% of patients can receive an additional line of therapy after the first treatment^[5].

However, active treatment has been linked to improved outcomes in PDA compared to best supportive care (BSC)^[6].

In this review, we focus on significant second-line studies and subsequently propose a therapeutic algorithm for PDA.

SECOND-LINE THERAPY

Although studies of efficient target therapies are promising, the backbone of second-line strategies for PDA involves a combination of chemotherapeutic agents. Currently, gemcitabine-based chemotherapy or fluoropyrimidine-based therapy, depending on previous drug regimens, remains the standard of care in the second-line setting.

Target therapy

PDA is a heterogeneous cancer and identifying plausible therapeutic targets is consequently difficult. The efficacy of a variety of drugs targeting different pathways has been evaluated, including targets in angiogenesis, farnesyl-transferases, cancer stem cells, hyaluronic acid, EGFR-MEK, m-TOR, and JAK-STAT pathways. Bevacizumab has been investigated in PDA therapy due to the favourable results achieved in other gastrointestinal cancers. Although the addition of bevacizumab to gemcitabine in first-line therapy failed to improve overall survival (OS), it has been evaluated in second-line settings. A small randomized trial comparing bevacizumab monotherapy to the combination of bevacizumab (10 mg/kg q14) plus docetaxel (35 mg-m² day 1.8.15 q 28) reported a detrimental effect of combination therapy^[7]. The only targeted therapy approved by the Food and Drug Administration for PDA treatment is erlotinib. This drug produced a small advantage in first-line treatment and also demonstrated activity (4.1 mo in OS) in second-line treatment^[8]. However, the combination of erlotinib with bevacizumab failed to improve patient outcomes^[9].

The EGFR and VEGFR pathways play major roles in PDA carcinogenesis and have been investigated as potential targets for second-line treatment. Inhibition of MEK1/2 by selumetinib failed to improve OS in gemcitabine-refractory patients compared to capecitabine^[10],

most likely due to activation of the EGFR pathway. On the contrary, a phase II trial with a small sample size, demonstrated that the combination of erlotinib and selumetinib achieved better outcomes (7.3 mo OS)^[11]. The EGFR inhibitor gefitinib failed to produce satisfactory results when used in combination with docetaxel using different schedules^[12,13]. The JAK-STAT inhibitor ruxolitinib achieved promising outcomes in preclinical and phase II trials^[14], but the subsequent phase III trial was terminated prematurely due to inefficacy. Instead encouraging results (median OS 9.8 mo) were reported with the use of olaparib, a poly (ADP-ribose) polymerase inhibitor, in BRCA1 and BRCA2 (BRCA1/2) mutated patients prior exposed to at least one line of therapy^[15]. Reni *et al.*^[16] investigated the maintenance with sunitinib in advanced PDA not progressed after six months of first line therapy, reporting an interesting result: An high 2-year OS, 22.9% vs 7.1% with sunitinib and placebo respectively.

Immunotherapy

Recent studies have focused on the new and attractive field of immunotherapy, which has achieved excellent results in the treatment of other malignancies. A variety of strategies had been tested in the following therapeutic settings: Vaccines, immune checkpoint blockade, anti-cytotoxic T-lymphocytes-associate protein 4 inhibitors, anti-PD-1 and anti-PDL1 inhibitors, and oncolytic virotherapy^[17]. Good outcomes have not been obtained, most likely due to the immune suppressive nature of the PDA microenvironment, which is characterized by desmoplastic reactions. Predictive biomarkers are not available but are actively being sought^[17].

Chemotherapy

Extensive data on potential treatments for gemcitabine-refractory patients are available because gemcitabine was previously the most frequently used agent for PDA. The roles of oxaliplatin and irinotecan in PDA have been evaluated due to the effectiveness of these drugs for other gastrointestinal cancers. Based on the promising results of a phase II trial^[18], three phase III randomized studies have investigated the efficacy of oxaliplatin-based regimens in gemcitabine-refractory patients. The CONKO-01 trial compared the efficacy of oxaliplatin, folinic acid (LLV), and 5-fluorouracil (5-FU) 24 h (OFF) with BSC. Although the patient cohort was small due to premature accrual closure, the study represents the first second-line trial demonstrating an advantage of chemotherapy over BSC (median OS 4.8 mo vs 2.3 mo). Patient randomization was terminated early because of the hesitation of the clinicians to administer BSC when a potentially active therapy was available^[19].

Afterwards in the CONKO-003 trial the OFF arm was compared to an active control arm (5-FU). The results of this study confirmed OFF schedule efficacy. OS increased significantly in the experimental arm (5.9 mo vs 3.3 mo), and there was a low rate of adverse events^[6]. These

data are more reliable than those of the CONKO-01 trial because of the high number of randomized patients (168). Smaller, non-randomized studies reported similar results (approximately 5 mo in OS) for combination oxaliplatin-capecitabine (xelox)^[20].

However, PANCREOX, which randomized 108 patients to oxaliplatin and 5-FU, the FOLFOX6m schedule, or 5-FU plus leucovorin, demonstrated a detrimental effect of FOLFOX6m on both OS and quality of life (median OS 6.1 mo vs 9.9 mo, $P = 0.02$), although the high OS in the 5-FU arm appears disputable^[21].

Other schedules after progression to gemcitabine-based therapy have been investigated in non-randomized studies. The combination of oxaliplatin plus raltitrexed^[22] or gemcitabine^[23] led to OS of 5.2 and 6 mo, respectively. A recent small phase II study reported outstanding OS (10.4 mo) for the combination of oxaliplatin and docetaxel^[24]. The use of docetaxel was suggested by the result of a previous retrospective trial that reported OS of 4.0 mo with docetaxel monotherapy^[25]. By contrast, the combination of docetaxel with irinotecan yielded disappointing outcomes, with OS of 4.1 mo, comparable to the median OS for other schedules in this setting^[26].

Limited experience with docetaxel^[27], paclitaxel^[28], and eribulin^[29] monotherapies has not yielded satisfactory results.

Similar to oxaliplatin, irinotecan has been evaluated in association with other agents such as raltitrexed, resulting in OS of 6.5 mo^[30]. Patients pre-treated with platinoid therapy frequently experienced peripheral neuropathy, limiting therapy options. Irinotecan monotherapy exhibited moderate activity and an acceptable safety profile as second-line treatment^[31]. A subsequent multicentre phase II study was conducted to investigate the combination of irinotecan with fluoropyrimidine using the FOLFIRI schedule (irinotecan 180 mg/m², leucovorin 200 mg/m², 5-FU 400 mg/m² bolus and 5-FU 600 mg/m² for 22 h). This schedule was well tolerated and led to acceptable outcomes (OS 5 mo)^[32].

Recent interesting data emerging from two relevant studies (PRODIGE 4, ACCORD 11) of FOLFIRINOX first-line therapy suggest an important impact of this schedule on OS; approximately 45% of patients underwent second-line therapy, despite the high rate of adverse events in the experimental arm compared to the gemcitabine control arm, including febrile neutropenia (5.4% vs 1.2%), neutropenia (45.7% vs 21%) and diarrhoea grade 3-4 (12.7% vs 1.8%)^[33].

The optimal treatment choice after FOLFIRINOX progression has not been established. The PRODIGE intergroup reported the most common second-line therapy administered to patients progressing in the FOLFIRINOX arm was gemcitabine (82.5%) or a gemcitabine-based combination (12.5%). Conversely, in the gemcitabine-resistant group, the second-line treatment was FOLFOX (49.4%), gemcitabine plus oxaliplatin (17.6%), fluorouracil and leucovorin plus cisplatin (16.5%) or FOLFIRINOX (4.7%)^[34]. Studies have also investigated nab-paclitaxel plus gemcitabine^[35,36]

and gemcitabine alone^[37] after first-line FOLFIRINOX. In gemcitabine-refractory patients, second-line therapy with FOLFIRINOX is marginally effective and less manageable in terms of toxicities^[38].

The most accepted hypothesis explaining rapid PDA progression during chemotherapy is the presence of a dense stroma surrounding the tumour cells that prevents drug activity. To overcome this obstacle, a new formulation of paclitaxel (nab-paclitaxel) was used in combination with gemcitabine as a first-line regimen^[39]. This combination exhibited activity in the second-line setting both in gemcitabine-refractory patients^[40,41] and after progression on FOLFIRINOX^[42]. Nab-paclitaxel exhibited a clinical benefit and is also feasible in elderly patients^[43]. Based on these results, irinotecan encapsulated in liposome nanoparticles was evaluated. A phase II trial of nanoliposomal irinotecan (nal-iri) monotherapy reported OS of 5.2 mo^[44]. The phase III study (NAPOLI-1) randomized (1:1:1) 417 patients worldwide to nanoliposomal irinotecan monotherapy, 5-FU and leucovorin, or nanoliposomal irinotecan plus 5-FU and leucovorin. The reported median OS was 4.9, 4.2 and 6.1 mo, respectively^[45]. This multicentre study had a large accrual, and nanoliposomal irinotecan plus 5-FU and leucovorin achieved the highest OS reported (Tables 1 and 2). Nanoliposomal irinotecan monotherapy was comparable to the combination of other chemotherapy agents.

All available agents have been tested in PDA therapy in different combinations and schedules. However, as in other cancers, the sequence of treatments and the optimization of the few efficient agents are critical. Therefore, it is very important to define an algorithm based on PS, patient age, and comorbidities for the first line decision (Figure 1).

To determine the first-line therapy, patients can be classified into three categories depending on age, PS, and comorbidities: Patients with PS 0-1 and < 65 years can be treated with FOLFIRINOX; patients with PS 0-1 and > 65 years can receive nab-paclitaxel; and patients with PS > 1 elderly patients, or patients with serious comorbidities receive gemcitabine monotherapy. The selection of subsequent therapies is affected by previous treatment and depends on residual toxicities, PS after first-line therapy, and patient preference.

Based on the literature, we hypothesize that patients treated with FOLFIRINOX can receive nab-paclitaxel plus gemcitabine or gemcitabine monotherapy. However, patients treated with first-line gemcitabine have additional options, including fluoropyrimidine and irinotecan.

There are currently no studies assessing treatment progression following nab-paclitaxel. However, if the patient recovers from peripheral neurotoxicity, we suggest platinoid-based chemotherapy or irinotecan-based therapy. A recent post hoc analysis of the MPACT trial showed that second-line therapy is feasible and more beneficial in patients previously treated with an efficient first-line treatment, particularly nab-paclitaxel plus gemcitabine, than in patients treated with gemcitabine

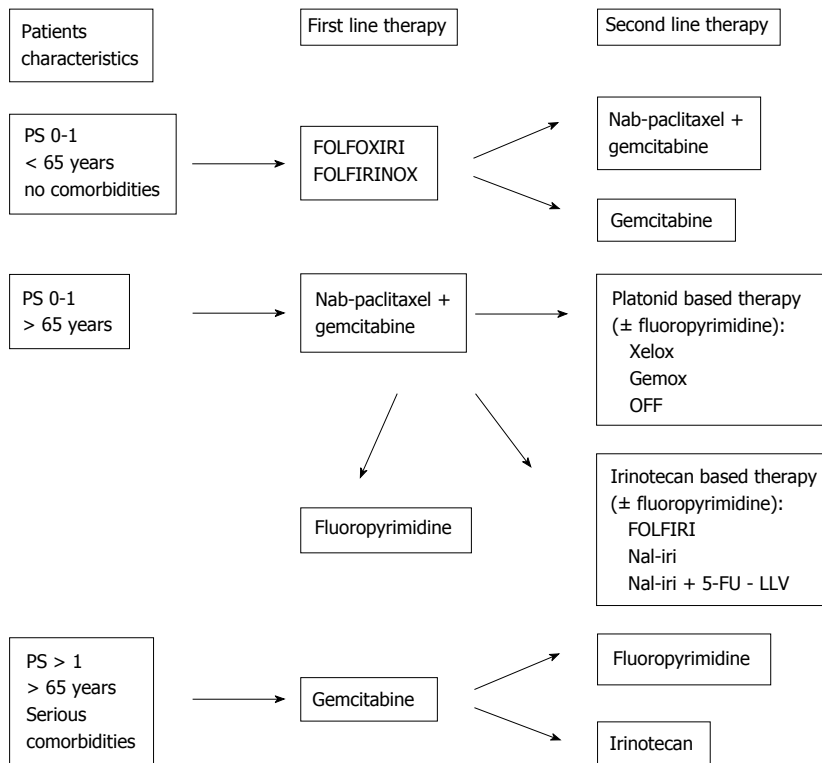


Figure 1 Proposed algorithm of pancreatic cancer therapy. 5-FU: 5-fluorouracil; LLV: L-leucovorin.

Table 1 Randomized second line studies

Ref.	Year	Study	Regimens	Patients	OS (mo)
Pelzer <i>et al</i> ^[19]	2011	CONKO-01	OFF <i>vs</i> BSC	23	4.8 <i>vs</i> 2.3
Oettle <i>et al</i> ^[6]	2014	CONKO-003	OFF <i>vs</i> 5-FU	76	5.9 <i>vs</i> 3.3
Wang-Gillam <i>et al</i> ^[45]	2015	NAPOLI 1	Nal-iri + 5-FU + LLV <i>vs</i> Nal-iri <i>vs</i> 5-FU + LLV	417	6.1 <i>vs</i> 4.9 <i>vs</i> 4.2
Ulrich-Pur <i>et al</i> ^[30]	2003		Raltitrexed + irinotecan <i>vs</i> raltitrexed	38	6.5 <i>vs</i> 4.3

BSC: Best supportive care; 5-FU: 5-fluorouracil; OS: Overall survival; LLV: L-leucovorin.

Table 2 Most significant not randomized second line studies

Ref.	Year study	Regimens	Patients	OS (mo)
Reni <i>et al</i> ^[22]	2006	Oxaliplatin + Raltitrexed	41	5.2
Demols <i>et al</i> ^[23]	2006	Oxaliplatin + Gemcitabine	33	6
Saif <i>et al</i> ^[25]	2010	Docetaxel	17	4
Yi <i>et al</i> ^[31]	2009	Irinotecan	33	6.6
Zaniboni <i>et al</i> ^[32]	2012	Folfiri	50	5
Bertocchi <i>et al</i> ^[40]	2015	Abiraxane + gemcitabine	23	5

OS: Overall survival.

alone^[46].

Improved outcomes may be obtained by treatment with all active agents (nab-paclitaxel, gemcitabine, irinotecan, oxaliplatin, fluoropyrimidine) during the entire patient history.

CONCLUSION

Many drugs have been investigated for PDA treatment, but outstanding OS results have only recently been obtained in the second-line setting. The median OS remains approximately 5 mo that is a good results in comparison with that achieved by BSC. Current targeted therapies have not demonstrated efficacy in phase III trial, and future studies must strengthen the efficacy of current chemotherapy agents. Drugs such as nal-iri and nab-paclitaxel represent the first real change in this landscape and may provide new hope for PDA treatment.

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Retrospective Cohort Study

Association between serum vitamin D levels and gastric cancer: A retrospective chart analysis

Neil Vyas, Rafael Ching Companioni, Melik Tiba, Hassan Alkhawam, Carmine Catalano, Robert Sogomonian, Joel Baum, Aaron Walfish

Neil Vyas, Hassan Alkhawam, Carmine Catalano, Robert Sogomonian, Department of Internal Medicine, Elmhurst Hospital Center, Elmhurst, NY 11373, United States

Rafael Ching Companioni, Melik Tiba, Joel Baum, Aaron Walfish, Department of Gastroenterology, Elmhurst Hospital Center, Elmhurst, NY 11373, United States

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Correspondence to: Neil Vyas, MD, Department of Internal Medicine, Elmhurst Hospital Center, 79-01 Broadway, Elmhurst, NY 11373, United States. neil.vyas@mssm.edu
Telephone: +1-347-5638628

Fax: +1-718-3344000

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Abstract

AIM

To determine whether there is an increased risk of gastric adenocarcinoma associated with vitamin D deficiency (VDd).

METHODS

A retrospective case control study was performed of all patients diagnosed with gastric adenocarcinoma between 2005 and 2015. After we excluded the patients without a documented vitamin D level, 49 patients were included in our study.

RESULTS

The average age of patients with gastric adenocarcinoma and documented vitamin D level was 64 years old (95%CI: 27-86) and average vitamin D level was 20.8 mg/dL (95%CI: 4-44). Compared to a matched control group, the prevalence of VDd/insufficiency in patients with gastric adenocarcinoma was significantly higher than normal vitamin D levels (83.7% vs 16.3%). Forty-one patients (83.7%) with adenocarcinoma showed VDd/insufficiency compared to 18 (37%) patients with normal vitamin D level without gastric cancer (OR: 8.8, 95%CI: 5-22, P value < 0.0001). The average age of males with gastric adenocarcinoma diagnosis was 60 years old vs 68 years old for females (P = 0.01). Stage

II gastric adenocarcinoma was the most prevalent in our study (37%).

CONCLUSION

We reported a positive relationship between VDd and gastric adenocarcinoma, that is to say, patients with decreased VDd levels have an increased propensity for gastric adenocarcinoma.

Key words: Gastric cancer; Adenocarcinoma; Vitamin D

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Core tip: In recent years, vitamin D deficiency (VDd) has been associated with several gastrointestinal malignancies largely mediated by vitamin D receptors. It affects multiple cellular processes such as inhibiting differentiation, metastasis, proliferation and inducing apoptosis and cell cycle arrest. All these mechanisms support vitamin D's anti-cancer role. VDd removes the tumorigenic activity that it elicits from regulating cell cycle, inhibiting cellular proliferation, angiogenesis and molecular signaling. Several studies have revealed that vitamin D₃ substantially promotes apoptosis in undifferentiated gastric malignant cells, specifically HCG-27. A retrospective research was conducted to find an association between vitamin D serum levels and gastric cancer.

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INTRODUCTION

Vitamin D is a fat soluble secosteroid responsible for enhancing intestinal absorption of several key nutrients such as calcium, magnesium, iron and phosphate. It is known to affect intestinal, skeletal and biologic pathways such as immune cells and tumor microenvironment. Vitamin D exists in two forms - vitamin D₂ (ergocalciferol) and vitamin D₃ (cholecalciferol), which is obtained from foods such as fortified dairy products, eggs, fish and liver^[1]. The development of vitamin D₃ also stems from the conversion process of 7-hydrocholesterol in the dermis after exposure to sunlight (ultraviolet-B radiation)^[2]. Vitamin D₂ and D₃, whether from diet or dermal synthesis, is considered biologically inactive. The activation process begins in the liver where hydroxylation occurs by enzyme 25-hydroxylase, which results in 25-hydroxy vitamin D [25(OH)D]^[1-3]. Next, 25(OH)D travels to the kidneys to become the active form of vitamin D; 1,25(OH)₂D₃ by 1 alpha hydroxylase^[2,3]. Subsequently, vitamin D₃ is released to serum where it

acts on receptors of the intestine, bone and kidney to regulate calcium metabolism^[2,4,5].

Measurement of 25(OH)D level is the highly indicative for overall vitamin D status. 25(OH)D accounts for sunlight exposure, dietary intake and conversion from adipose tissue^[3,6,7]. Vitamin D deficiency (VDd) is defined as 25(OH)D < 15-20 mg/dL and sufficient range > 30 mg/dL.

In recent years, VDd has been associated with a multitude of gastrointestinal malignancies largely mediated by vitamin D receptors (VDRs). It affects multiple cellular processes such as inhibiting differentiation, metastasis, proliferation and inducing apoptosis and cell cycle arrest. All these mechanisms support vitamin D's anti-cancer role. Numerous studies have associated colon cancer with low vitamin D, however limited data suggests an association with gastric cancer^[8-11].

Gastric adenocarcinoma is considered the fourth most common cancer worldwide. A major risk factor for development of gastric adenocarcinoma is *Helicobacter pylori* (*H. pylori*) infection^[2]. Other risk factors include smoking and alcohol use. However, recent studies show that VDd is associated with poor prognosis in gastric adenocarcinoma^[3]. Recent cohort studies revealed that increased serum vitamin D levels are linked to a decreased risk of gastric cancer.

VDd removes the tumorigenic activity that it elicits from regulating cell cycle, inhibiting cellular proliferation, angiogenesis and molecular signaling^[12]. Current research illustrates that vitamin D₃ increases apoptosis in undifferentiated gastric malignant cells, specifically HCG-27 cell lines^[3]. In order to find a correlation between serum levels of vitamin D and gastric adenocarcinoma a retrospective research was conducted.

MATERIALS AND METHODS

Study design

A retrospective, single-centered study was performed of all subjects diagnosed with gastric adenocarcinoma between 2005 and 2015. This study was conducted at a tertiary medical center in one of the most diverse communities in the United States, which provided a significant cultural and epidemiological advantage. This study was approved by Icahn School of Medicine at Mount Sinai, Institutional Review Board.

Selection criteria

Among 3200 patients with gastrointestinal malignancies diagnosed between 2005 and 2015, 304 patients had gastric adenocarcinoma. Patients who did not have a vitamin D level were excluded. Of the 304 patients with gastric adenocarcinoma, 49 patients had documented vitamin D levels, documented gastric pathology by pathologist report and were over the age of 18. Vitamin D levels were defined as following: 25(OH)D deficiency (< 20 ng/mL), insufficiency (20-29 ng/mL) and normal 25(OH)D level (≥ 30 ng/mL).

Demographic data such as age, gender and ethnicity

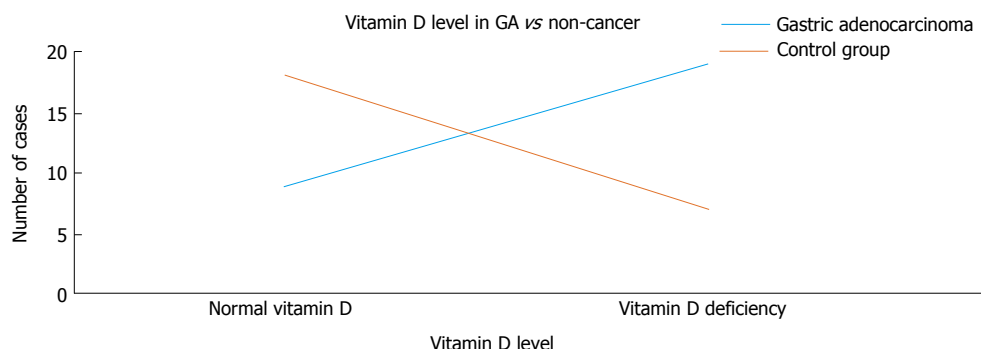


Figure 1 Average vitamin D level in gastric adenocarcinoma vs control group. GA: Gastric adenocarcinoma.

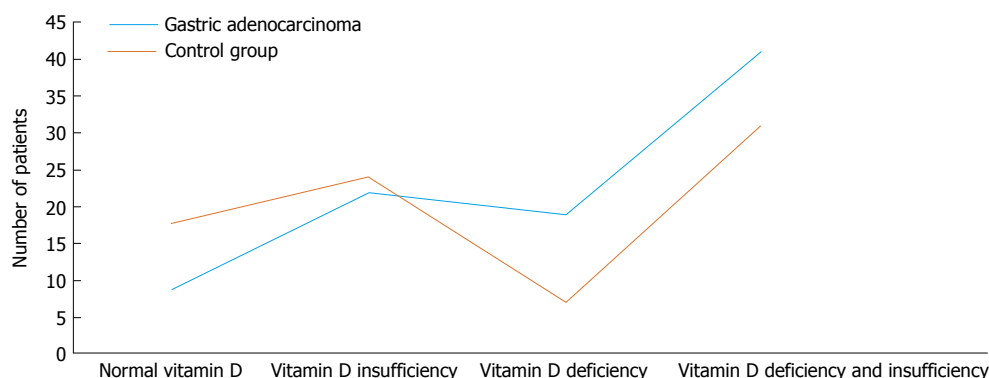


Figure 2 Vitamin D level distribution among gastric cancer adenocarcinoma and control group.

Table 1 Baseline characteristics of gastric adenocarcinoma and control group

Characteristics	Case (gastric adenocarcinoma) (n = 49)	Control (n = 49)
Age, yr	63.9%	60.43%
Male	24 (49%)	24 (49%)
Female	25 (51%)	25 (51%)
Hispanic	30 (61.2%)	30 (61.2%)
Body mass index	24 kg/m ²	25 kg/m ²
	SD (22-27)	SD (23-28)
Vitamin D deficiency	19 (38.8%)	7 (14.3%)
Vitamin insufficiency	22 (44%)	24 (49.0%)
Combined vitamin D deficiency and insufficiency	41 (83.68%)	31 (63.27%)

as well as smoking, body mass index, *H. pylori* infection and stage of adenocarcinoma were assessed. Our cohort was compared to a matched control group of subjects who had no known history of cancers and with documented level of vitamin D (Table 1).

For all statistical analyses, the results were considered significant when two-tailed *P*-values were < 0.05. The distributions of demographic information and baseline comorbidity were compared between both groups.

RESULTS

The average age of patients with gastric adenocarcinoma and documented vitamin D level was 64 years old

(95%CI: 27-86) and average vitamin D level was 20.8 (95%CI: 4-44). Compared to the matched controls, the prevalence of VDd and vitamin D insufficiency in patients who had gastric adenocarcinoma was significantly higher than in patients with normal vitamin D levels (83.7% vs 63.27%). There were 41 gastric adenocarcinoma patients with VDd/insufficiency (83.7%) compared to 18 (37%) patients with normal vitamin D level without gastric adenocarcinoma (OR: 8.8, 95%CI: 5-22, *P* value < 0.0001) (Figure 1).

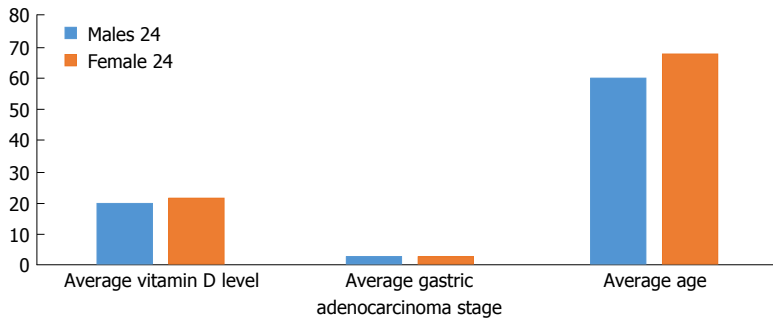
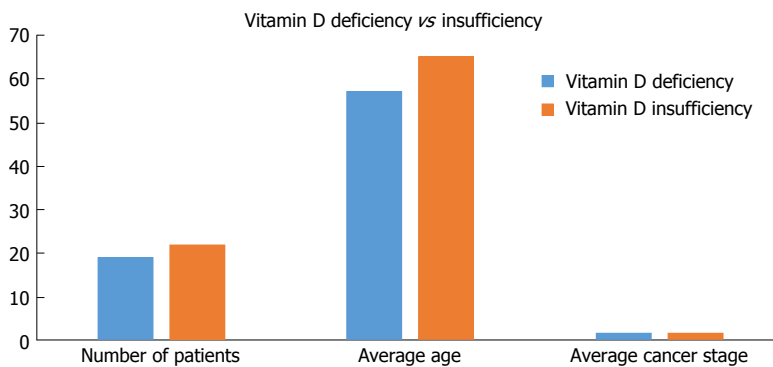
Furthermore, patients with VDd had an OR that was 2.7 times higher for gastric adenocarcinoma compared to the normal vitamin D population (95%CI: 1.4-5, *P* value 0.002), while patients with VDd had a 1.4 OR. However, endpoints did not achieve statistical significance (*P* value 0.1) (Figure 2).

Among gastric adenocarcinoma groups, stage II gastric adenocarcinoma was most common (37%) followed by stages III (25%), I (20%) and IV (18%). The vitamin D level did not differ between different cancer stages. There were higher rates of Hispanics (61%) followed by the East Asian population (13%) (Table 2). The average age in males with a gastric adenocarcinoma diagnosis was 60 years old vs 68 years old for females (*P* = 0.01). There was no significant difference in average vitamin D level (20 ng/mL in males vs 22 ng/mL in females) or stage of cancer (Figure 3).

We sub grouped the patients with an abnormal vitamin D values into VDd and insufficiency; the average age of gastric adenocarcinoma in the deficiency group

Table 2 Average vitamin D level, age and gastric adenocarcinoma cancer stage based on ethnicity

	Number of patients	Average vitamin D level	Average age	Average adenocarcinoma stage
Hispanic	30 (61%)	20	63	3
East Asian	13 (27%)	20	60	2
Middle Eastern	4 (8%)	21	70	3
Indian	2 (4%)	30	75	3

**Figure 3** The average vitamin D level, age and stage of gastric adenocarcinoma between males and females.**Figure 4** Vitamin D deficiency vs insufficiency among gastric adenocarcinoma group.

was 57.7 years old vs 65.5 years old in the insufficiency group ($P = 0.07$). There were no differences in gastric adenocarcinoma stage between the two groups (Figure 4).

DISCUSSION

In our study, our inclusion criteria were patients diagnosed with gastric adenocarcinoma with a vitamin D level. We had 49 patients as part of our cohorts. These cases were then matched to a cohort of similar age, gender and ethnicity with measured serum vitamin D level and no history of cancer. The mean age of the cases were 64 as opposed to the control group at 60. Gender ratio was similar - 49% male and 51% female. The predominant ethnicity was Hispanic, accounting for 61.2%. This can be attributed to the increased Hispanic patient population at Elmhurst Hospital Center. The diagnoses of gastric adenocarcinoma were by biopsy: 18% with stage IV, 25% with stage III, 37% with stage II and finally 20% with stage I. For our case group, the vitamin D value recorded was the one measured at the time of diagnoses and prior to repletion. We defined

vitamin D levels in association with widely accepted thresholds. VDd is defined as < 20 mg/dL, insufficiency 20-29 mg/dL and sufficiency was ≥ 30 mg/dL.

The prevalence of VDd was compared between the gastric adenocarcinoma group and matched control group, using ORs and 95%CI. Our finding showed an increased prevalence of VDd in the case group as compared to the matched group - 39% vs 14% respectively. The primary end point suggests an association between VDd and gastric adenocarcinoma; however, the study also found no correlation between the degree of deficiency and the stage of gastric adenocarcinoma.

In general, there is now accumulating evidence of VDd associated with multiple malignancies due to its anticancer effects. There has been increasing data for the association of VDd with colon cancer^[2,5,10]. More recently, there is some new research suggesting an association with esophageal, pancreatic and liver cancer as well^[2]. However, there is limited data showing gastric adenocarcinoma. The anticancer biochemical role of vitamin D is largely mediated by the VDR. VDR is a steroid in the thyroid hormone receptor family. Free

1,25 D₃ binds to the VDR causing phosphorylation of the receptor then the ligand activated VDR interacts with the retinoid X receptor (RXR) to form a heterodimer^[8,9,11-14]. Subsequently, the heterodimer 1,25-VDR-RXR complex translocate to the nucleus and thus binds to vitamin D response elements (VDREs) in multiple regulatory regions located in the promoters of target genes^[5,8,9,11-14]. The receptor signaling between RXR and VDR is a vital step for VDR transcriptional activity. VDREs are also utilized to initiate gene transcription. The RXR-VDR complex recruit specific coactivator molecules such as steroid receptor coactivators, histone acetyltransferases and its mediators. The significance of the RXR-VDR complex translocating to the nuclei and binding to VDREs is to allow for promotion or suppression of precise cellular events of tumorigenesis^[2,4,5,15].

The most common malignancy of the stomach is adenocarcinomas arising from gastric epithelium. Gastric adenocarcinoma is the fourth most common malignancy worldwide. In the United States, gastric adenocarcinoma accounts for 1.5% of new cancer cases and 1.8% of cancer deaths. The 5-year relative survival rate for gastric cancer increased from 14.3% in 1975 to 29.3% from 2006-2011^[16]. The 5-year observed survival rate for gastric adenocarcinoma patients after surgery ranges from 71% for stage IA to as low as 4% for stage IV^[17].

Most gastric cancer patients are elderly when diagnosed with a median age of 69 in the United States. Less than 2% of gastric cancer cases arise in patients under 35 years of age. Asia and South America have higher rates of gastric cancer than in the United States. Gastric adenocarcinoma is marginally more prominent in males in the United States, whereas in Japan, it is the most common cancer diagnosed in men. However, worldwide, gastric cancer rates are twice as high in men compared to women. American Cancer Society's estimates for stomach cancer in the United States for 2015 revealed that approximately 24590 cases will be diagnosed (15540 in men and 9050 in women) and that 10720 people will die from this type of cancer (6500 men and 4220 women).

Gastric adenocarcinoma is often asymptomatic, but some non-specific symptoms reported include indigestion, abdominal discomfort and appetite loss. Later in the disease phase there is bleeding which leads to anemia. Although causes of gastric cancer are multifactorial, *H. pylori* infections are an essential risk factor^[17]. However, only 2% of people with *H. pylori* infections develop stomach cancer^[18]. Duodenal ulcers are most likely due to inflammation of the pyloric antrum, whereas inflammation of the corpus causes gastric ulcers and gastric carcinoma^[19]. There is a counter correlation between low socioeconomic status and this disease due to factors such as poor nutrition, poor sanitation and scarce treatment and conservation of food and water. Individuals who maintain diets high in fruits, green vegetables, beta-carotene and ascorbic acid are less at risk. There is also research that indicates decreased use of nitrites in prepared foods reduces the

risk as well. It has also been well studied that dietary factors such as smoked foods and red meat^[20] also increase the risk. With respect to vitamin D, there is now supporting evidence for the association of deficient levels and an increase risk for gastric adenocarcinoma. Several *in vitro* studies proved that 1,25-dihydroxyvitamin D₃ has the anticarcinoma effects of anti-proliferation, promoting apoptosis^[9,20-22]. More specifically, it has been demonstrated that vitamin D₃ substantially stimulates apoptosis in the undifferentiated gastric cancer cell line HCG-27^[3,23]. Moreover, specific to gastric adenocarcinoma, vitamin D₃ inhibits gastric cancer cell growth and induces cell cycle arrest^[9,20,23]. Bao *et al.*^[12] found that direct usage of 1,25 dihydroxy vitamin D₃ induces cellular apoptosis in gastric cancer cells and also increases the expression of VDR, further supporting the antitumor role that vitamin D may activate in gastric adenocarcinoma^[2]. One of the limitations of our study is the small population number as not every patient with gastric adenocarcinoma had documented vitamin D level. Another limitation that must be addressed is the role malnutrition in cancer patients. There are several pathogenesis of cancer associated malnutrition. The primary reason is decreased food intake due to systemic effects of the disease, local tumor effects, psychological effects or adverse effects of treatment. Other contributing factor is the malignancy releasing inflammatory markers systemically such as procytokines have been linked to malnutrition and cachexia^[24]. However, this study is unable to assess the nutrition status of the patients included in this study but one must be aware malnutrition which includes low vitamin D could be a result of the malignancy itself.

The question becomes, can vitamin D supplementation decrease incidence of gastric adenocarcinoma? It is reasonable to suggest the role of supplementation of vitamin D in deficient patients because of indirect etiologies related to gastric adenocarcinoma. Patients who underwent gastrectomy subsequently altered vitamin D metabolism, especially amongst those who received total gastrectomy^[3,25]. Supplementation is also recommended for patients who have malnutrition and malabsorption disorders, which is a consequence of malignancy or diseases such as celiac sprue. Lastly, there is new evidence that high doses of vitamin D plus calcium show significant reduction in cancer incidence in women^[26]. With expanding evidence of vitamin D supplementation and its benefits as mentioned above, avoiding deficiency or adding vitamin D supplements might be an economical and safe way to reduce cancer incidence, or act as a supplement to chemotherapeutic agents^[9,11,24,27]. Overall, VDD can be easily corrected by taking supplements, changing dietary habits or increasing sunlight exposure, which is why it is a practical reason for clinicians to assess vitamin D status. To further assess this association, additional prospective research should be done to clarify the benefit of vitamin D supplementation and possible prevention of gastric adenocarcinoma.

In summary, there is increasing research on the role of vitamin D and gastrointestinal cancers, but there is still limited data on the association of vitamin D and gastric adenocarcinoma. Our study showed VDd has an increased predisposition for gastric adenocarcinoma. There is however no correlation between the severity of VDd and stage of gastric adenocarcinoma.

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COMMENTS

Background

In recent years, vitamin D deficiency (VDd) has been associated with a multitude of gastrointestinal malignancies. It affects multiple cellular processes such as inhibiting differentiation, metastasis, proliferation and inducing apoptosis and cell cycle arrest. All these mechanisms support vitamin D's anticancer role. Numerous studies have associated colon cancer with low vitamin D, however limited data suggests an association with gastric cancer.

Research frontiers

Vitamin D is a fat soluble secosteroid that is known to affect intestinal, skeletal and biologic pathways such as immune cells and tumor microenvironment. Vitamin D is related to many cancers and the current research hotspot is the potential use of vitamin D screening and supplementation to prevent or slow the progression of malignancy.

Innovations and breakthroughs

Understand the pathogenesis of vitamin D anticancer role. Several studies have found a link between low vitamin D levels and gastrointestinal malignancy. However, there is limited data suggesting a relationship between VDd/insufficiency in gastric adenocarcinoma.

Applications

This study showed VDd has an increased predisposition for gastric adenocarcinoma. Further studies are warranted to assess this association and the possible risk stratification of vitamin D supplementation in preventing or slowing the progression of gastric adenocarcinoma.

Terminology

The anticancer biochemical role of vitamin D is largely mediated by the vitamin D receptor (VDR). VDR is a steroid in the thyroid hormone receptor family. Free 1,25 D₃ binds to the VDR causing phosphorylation of the receptor then the ligand activated VDR interacts with the retinoid X receptor to form a heterodimer. This allows for the suppression or promotion of specific cellular events of tumorigenesis.

Peer-review

It is interesting that the VDd is associated with gastric adenocarcinoma. The article fits in perfectly with the global trend.

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Retrospective Study

Delaying surgery after neoadjuvant chemoradiotherapy improves prognosis of rectal cancer

Mehmet Mihmanlı, Esin Kabul Gürbulak, İsmail Ethem Akgün, Mustafa Fevzi Celayir, Pinar Yazıcı, Deniz Tunçel, Tuba Tülin Bek, Ayhan Öz, Sinan Ömeroğlu

Mehmet Mihmanlı, Esin Kabul Gürbulak, İsmail Ethem Akgün, Mustafa Fevzi Celayir, Pinar Yazıcı, Ayhan Öz, Sinan Ömeroğlu, Department of General Surgery, Şişli Etfal Training and Research Hospital, Şişli 34371, Istanbul, Turkey

Deniz Tunçel, Department of Pathology, Şişli Etfal Training and Research Hospital, Şişli 34371, Istanbul, Turkey

Tuba Tülin Bek, Department of Radiation Oncology, Şişli Etfal Training and Research Hospital, Şişli 34371, Istanbul, Turkey

Author contributions: Mihmanlı M, Kabul Gürbulak E and Akgün İE contributed equally to this work; Mihmanlı M and Kabul Gürbulak E collected and analyzed the data, and drafted the manuscript; Akgün İE provided analytical oversight; Kabul Gürbulak E designed and supervised the study; Celayir MF, Yazıcı P and Tunçel D revised the manuscript for important intellectual content; Bek TT, Öz A and Ömeroğlu S offered technical or material support; Mihmanlı M and Kabul Gürbulak E provided administrative support; all authors have read and approved the final version to be published.

Institutional review board statement: The clinical study entitled "Delaying surgery after neoadjuvant chemoradiotherapy improves prognosis of rectal cancer" is acceptable for clinical research and approved by the Ethical Committee of Sisli Hamidiye Etfal Training and Research Hospital.

Informed consent statement: Informed written consent was obtained from all study participants, or their legal guardian for being included in the study.

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Data sharing statement: Technical appendix, statistical code and dataset available from the corresponding author at (ekabul@gmail.com). Participants gave informed consent for data sharing. No additional data are available.

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Correspondence to: Esin Kabul Gürbulak, MD, Department of General Surgery, Şişli Etfal Training and Research Hospital, Halaskargazi Cad, Etfal Sk, Şişli 34371, Istanbul, Turkey. ekabul@gmail.com
Telephone: +90-212-3735000
Fax: +90-212-2240772

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Abstract

AIM

To investigate the prognostic effect of a delayed interval between neoadjuvant chemoradiotherapy (CRT) and surgery in locally advanced rectal cancer.

METHODS

We evaluated 87 patients with locally advanced mid- or distal rectal cancer undergoing total mesorectal excision following an interval period after neoadjuvant CRT at Şişli Hamidiye Etfal Training and Research Hospital, Istanbul between January 2009 and January 2014. Patients were divided into two groups according to the interval

before surgery: < 8 wk (group I) and \geq 8 wk (group II). Data related to patients, cancer characteristics and pathological examination were collected and analyzed.

RESULTS

When the distribution of timing between group I ($n = 45$) and group II ($n = 42$) was viewed, comparison of interval periods (median \pm SD) of groups showed a significant difference of as 5 ± 1.28 wk in group I and 10.1 ± 2.2 wk in group II ($P < 0.001$). The median follow-up period for all patients was 34.5 (9.9-81) mo. group II had significantly higher rates of pathological complete response (pCR) than group I had (19% *vs* 8.9%, $P = 0.002$). Rate of tumor regression grade (TRG) poor response was 44.4% in group I and 9.5% in group II ($P < 0.002$). A poor pathological response was associated with worse disease-free survival ($P = 0.009$). The interval time did not show any association with local recurrence ($P = 0.79$).

CONCLUSION

Delaying the neoadjuvant CRT-surgery interval may provide nodal down-staging, improve pCR rate, and decrease the rate of TRG poor response.

Key words: Rectal carcinoma; Pathological tumor response; Neoadjuvant chemoradiotherapy; Interval timing; Tumor down-staging

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Core tip: Delaying the neoadjuvant chemoradiotherapy (CRT)-surgery interval for treatment of locally advanced rectal carcinoma may improve pathological complete response rates by providing nodal down-staging, as well as decreasing the rate of tumor regression grade (TRG) poor response. TRG may be an important predictive factor for disease-free survival. Extending the interval between CRT and surgery may improve the survival through tumor down-staging without increasing the rate of surgical complications.

Mihmanlı M, Kabul Gürbulak E, Akgün İE, Celayir MF, Yazıcı P, Tunçel D, Bek TT, Öz A, Ömeroğlu S. Delaying surgery after neoadjuvant chemoradiotherapy improves prognosis of rectal cancer. *World J Gastrointest Oncol* 2016; 8(9): 695-706 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v8/i9/695.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v8.i9.695>

INTRODUCTION

Locally advanced distal and mid-rectal tumors are commonly treated with preoperative combined chemoradiotherapy (CRT) followed by total mesorectal excision (TME)^[1-3]. Previously conducted studies have recommended a treatment interval time between preoperative neoadjuvant CRT and surgery for the

treatment of locally advanced rectal cancer^[4,5]. The first prospective trial (The Lyon Trial R90-01), which assigned patients randomly to have surgery at two different time intervals following CRT, was conducted in 1999. That trial showed that a 6-8-wk treatment interval between radiotherapy and surgery improved tumor down-staging and yielded a higher pathological response rate compared with a 2-wk interval. Since then, a 6-8-wk interval has been accepted as the appropriate treatment interval between neoadjuvant therapy and surgery^[6]. However, a definite definition for an optimum interval period is still lacking in the medical literature. In addition, even though the known effect of an extended interval on pathological complete response (pCR), the impact of pCR on disease-free survival (DFS) and overall survival (OS) has not been clearly described^[7].

The aim of this study was to determine whether the interval time between preoperative neoadjuvant CRT and surgery affected the rates of pCR, perioperative surgical complications, sphincter-saving surgery, DFS and OS in locally advanced mid-or distal rectal cancer.

MATERIALS AND METHODS

Patients

This was a retrospective review of a series of 113 consecutive patients who underwent preoperative neoadjuvant CRT followed by radical resection with TME for curative intent of locally advanced mid- or distal rectal cancer between January 2009 and January 2014 at Şişli Hamidiye Etfal Training and Research Hospital, General Surgery and Oncology Departments. The study was approved by the local ethics committee. Written informed consent was obtained from all study participants, or their legal guardian for being included in the study. All patients included in the study (1) were aged \geq 18 years; (2) had pathological diagnosis of adenocarcinoma of mid-rectal (located between 5 and 10 cm from the anal verge) and distal rectal (situated in the first 5 cm from the anal verge, excluding the anal canal) tumors by endoscopic biopsy; (3) had tumors with T3/T4 stage or N (+) as demonstrated on pelvic phased-array magnetic resonance imaging; and (4) underwent TME after neoadjuvant CRT. Study parameters including interval period between neoadjuvant CRT and surgery, operation time and type, intraoperative and early postoperative morbidity and mortality, and hospital stay were recorded. Reports from pathological examinations were interrogated to extract data on total and metastatic lymph node numbers and surgical margins. Data on local recurrence, organ metastases that occurred during postoperative follow-up period, DFS and OS rates were also recorded. Postoperative anastomotic complications were defined according to severity grading of anastomotic leakage of the International Study Group of Rectal Cancer^[8]. Twenty-six patients, including those who had widespread metastasis at the time of diagnosis ($n = 3$), patients who underwent short-term radiotherapy (5×5 Gy)

($n = 7$), individuals with synchronized tumors or had treatment due to other malignancies with rectal cancer as secondary ($n = 5$), patients who could not tolerate neoadjuvant chemotherapy ($n = 3$), and patients who were lost to follow-up ($n = 8$) were excluded. The remaining 87 patients constituted the study population. The study patients were divided into two groups according to the interval time between neoadjuvant CRT and surgery: < 8 wk (group I) and ≥ 8 wk (group II).

In all patients, tumor localization and pathological diagnosis were made by using procto-sigmoidoscopy and endoscopic biopsy, respectively. Systemic staging was performed using thoracic and abdominal computed tomography (CT), while local staging was performed using phased-array magnetic resonance imaging. Preoperative neoadjuvant CRT was given for a total of 5 wk and it included 45-50.4 Gy radiotherapy (5×1.8 -2.0 Gy/wk) and concomitant 5-fluorouracil (180 mg/m² per day) for 5 d/wk. Surgery was performed in all patients at the earliest interval of 4 wk after neoadjuvant chemotherapy. The interval between neoadjuvant therapy and surgery varied according to logistics and scheduling preferences of the attending surgeon.

Postoperative follow-up protocol was performed every 3-4 mo during the first year, once every 6 mo during the second year, and once every year after the second year. During follow-up, local recurrences were determined using thoracoabdominal CT, positron emission tomography-CT or colonoscopy.

Pathological examination

Histopathological examination of the resected specimens including the mesorectum was performed to identify as many lymph nodes as possible. Evaluation of tumor regression grade (TRG) after CRT in the primary tumor of the rectal wall was performed by experienced pathologists according to the Ryan scheme for tumor regression score^[9], which was suggested by the College of American Pathologists protocol. The absence of viable cancer cells or acellular pools of mucin in resected specimens were considered as complete response (TRG 1). Single cells or microscopic foci of cancer cells in samples were assessed as near complete response (TRG 2). Residual cancer outgrown by fibrosis was considered as the minimal response (TRG 3). Minimal or no tumor kill, or extensive residual cancer in specimens were found as a poor response (TRG 4). T stage 0 was considered as complete tumor response in the rectal wall, while near complete, minimal or inadequate responses were examined at any T stage. In the present study, tumoral down-staging or pathological tumor response was expressed by TNM classification, according to American Joint Committee on Cancer, and pCR was defined as T stage 0 in the rectal wall without metastatic lymph node in the mesorectum.

Statistical analysis

Data were analyzed using SPSS for Windows, version

17.0 (SPSS, Chicago IL, United States) by the official biostatistician of the hospital. Continuous variables are represented as mean \pm SD and categorical variables as numbers and percentages. Intergroup analyses were performed using Students' *t* test and Mann-Whitney *U* test for continuous variables and χ^2 test and Fisher's exact test for categorical variables. The Spearman correlation test was used to investigate the relationship between continuous variables and intervals between CRT and surgery. Paired *t* and Wilcoxon tests were used to compare dependent groups. Oncological outcomes of patients were classified as 2-year and 5-year DFS and OS. The Kaplan-Meier test, log-rank test, and Cox regression analyses were used to determine the relationship between potential risk factors and DFS and OS. OS was defined as the period between diagnosis of the disease until death that occurred as a result of the disease. DFS was defined as the time between diagnosis of the disease until local recurrence or far-organ metastasis. Patients who died from other causes or died within the early postoperative period were censored. Results were evaluated between 95%CIs, and the level of statistical significance was set at $P < 0.05$.

RESULTS

A total of 87 patients who had locally advanced mid- or distal rectal cancer underwent surgical resection with TME after neoadjuvant CRT. Of these 45 (group I) had a treatment interval < 8 wk and 42 (group II) had an interval ≥ 8 wk. Patient demographics and clinical characteristics were comparable in both groups (Table 1). When the distribution of timing in both groups was viewed, comparison of interval periods (median \pm SD) between the groups showed a significant difference; as 5 ± 1.28 (2-7.8) wk in group I and 10.1 ± 2.2 (8.2-20.2) wk in group II ($P < 0.001$) (Figure 1).

Effect of interval period on preoperative variables

Statistical analysis did not show any correlation between treatment interval and respective preoperative and postoperative variables (operation time and type, diverting ileostomy dehiscence rate, intraoperative and postoperative complication rate, hospital stay and early postoperative mortality rate) (Table 2). Despite all patients being informed about surgical approach and necessity of diverting stoma, six had not given consent to have a diverting loop stoma. Therefore, 42 patients (93.3%) in group I and 39 patients (92.9%) in group II were diverted at the time of TME.

For patients who had distally located tumors, sphincter-saving surgery was performed in 17 (64.7%) in group I and 11 (63.6%) in group II ($P = 0.86$). Intraoperative complications occurred in four patients from group I, left ureter injuries in two patients (4.4%), and presacral significant bleeding in the other two (4.4%). In group II, there was right ureter injuries in two patients (4.7%) and bladder injury in one patient (2.3%) ($P = 0.48$). During

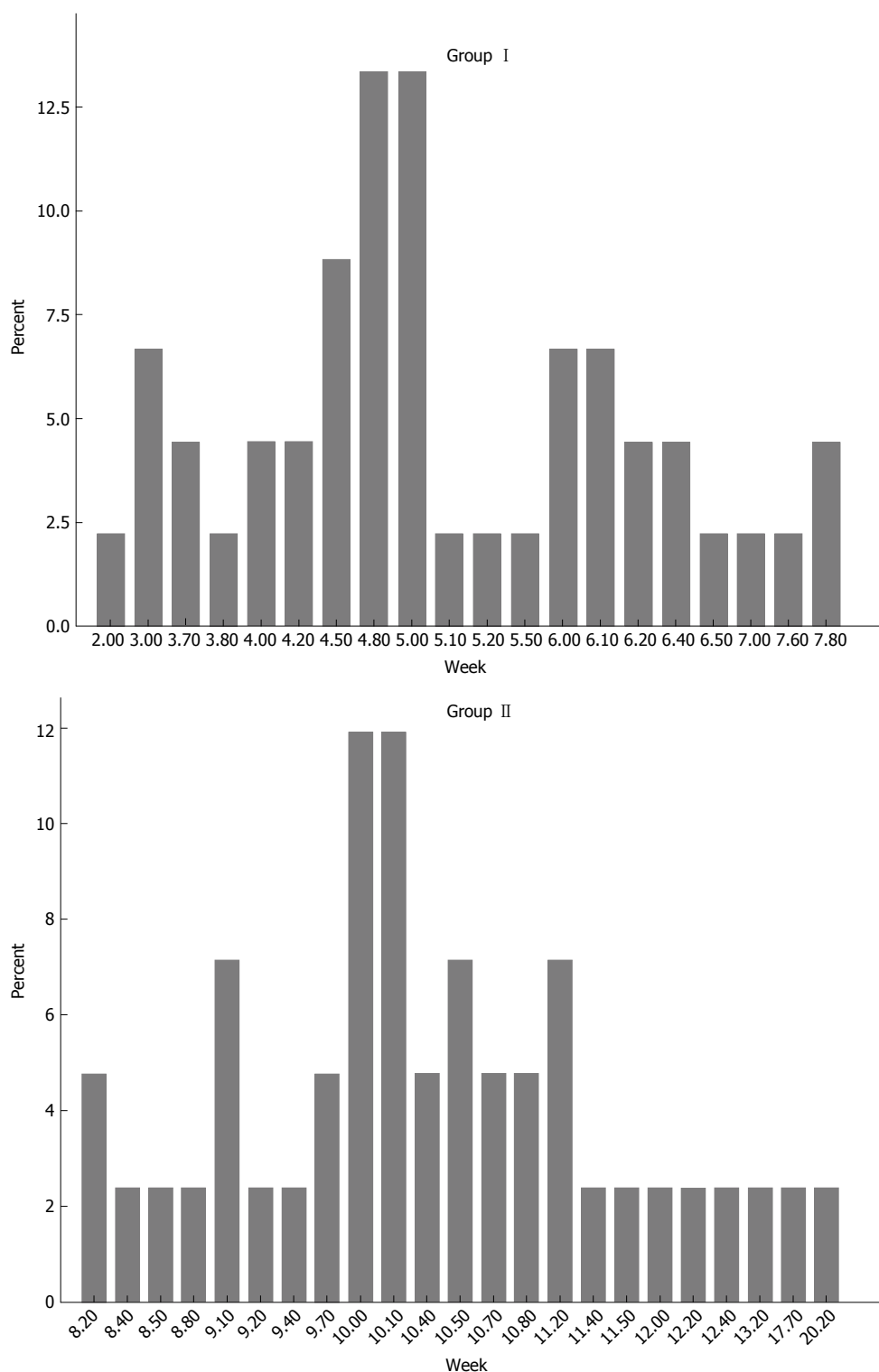


Figure 1 Distributions of groups with regard to interval time between neoadjuvant therapy and surgery. Median interval periods \pm SD were 5 ± 1.28 (2-7.8) wk in group I and 10.1 ± 2.2 (8.2-20.2) wk in group II ($P < 0.001$).

the postoperative period, complications occurred in 13 patients (28.9%) in group I and 11 patients (26.1%) in group II. Complications that occurred in group I were anastomotic leakage in five patients (11.2%) and wound infection in eight (17.8%). Surgical complications in both groups were comparable ($P = 0.42$).

Anastomotic complications were classified based

on the severity grading of anastomotic complications of the International Study Group of Rectal Cancer. In group I, three cases with diverting stoma developed perianastomotic abscess in the pelvis. These were classified as Grade B anastomotic complications, and managed successfully with percutaneous abscess drainage and antibiotics. Two cases that were not

Table 1 Demographic and clinical characteristics of patients

	Group I (<i>n</i> = 45)	Group II (<i>n</i> = 42)	<i>P</i> value
Age (mean ± SD)	53.7 ± 13.4	58 ± 13.2	0.82
Sex (male/female)	32/13	31/11	0.62
Localization of tumor from the anal verge (cm) (mean ± SD)	5.6 ± 3	6.1 ± 2.8	0.39
T stage (2-4)	8/28/9	6/32/4	0.56
Stage (II / III)	4/41	5/37	0.53
Preoperative radiation dose (Gy) (mean)	49.5 ± 1.99	49.5 ± 2	0.78
Follow-up time (mo) (mean ± SD)	37.2 ± 19.6	31.1 ± 20.7	0.51

Table 2 Effect of interval time on the perioperative variables *n* (%)

	Group I (<i>n</i> = 45)	Group II (<i>n</i> = 42)	<i>P</i> value
Procedure type			
LAR	28 (62.2)	31 (73.8)	0.06
ULAR	11 (24.4)	7 (16.7)	0.09
APR	6 (13.3)	4 (9.5)	0.50
Diverting ileostomy	42 (93.3)	39 (92.9)	0.90
Operative time (min) (mean ± SD)	134.2 ± 19.9	133.4 ± 23.5	0.62
Intraoperative complications	8 (8.9)	3 (7.1)	0.48
Postoperative complications	13 (28.9)	11 (26.1)	0.42
Early postoperative mortality	1 (2.3)	2 (4.7)	0.37
Hospital stay (d) (mean ± SD)	11 ± 10.5	10 ± 9.3	0.32

APR: Abdominoperineal resection; LAR: Low anterior resection; ULAR: Ultralow anterior resection.

diverted at the time of TME required reoperation for Grade C anastomotic leakages. Mortality occurred in 1 (2.3%) of the patients who had Grade C anastomotic leakage on postoperative day 18.

In group II, anastomotic leakage was observed in 8 patients (19%), wound infection in 3 (7.1%) and mortality in 2 (4.7%). Mortality was due to postoperative pulmonary emboli and myocardial infarction. Grade A anastomotic complications appeared in four patients with diverting ileostomy who were treated with antibiotics, without the need for invasive interventions or surgical procedures. Grade B anastomotic complications occurred in three cases, and one of them did not have a diverting stoma. These patients underwent percutaneous abscess drainage and were treated with antibiotics. One patient who was not diverted with a stoma at the time of TME developed Grade C anastomotic leakage, and surgery was performed in this case. There was no mortality associated with anastomotic leakage in group II patients. There were no significant differences between the groups in terms of anastomotic complications or early postoperative mortality ($P = 0.07$ and $P = 0.37$, respectively).

Effect of interval period on stage and pathological response

In both groups, there were no significant differences in terms of postoperative T stage ($P = 0.17$). However, histopathological examination of TME specimens showed different nodal complete response in both groups (46.7%

vs 81%, $P = 0.001$) (Table 3). The pathological tumor down-staging was found to be related to a decreased number of metastatic lymph nodes in the mesorectum. Tumor down-staging rate was 57.4%, as the pCR [Stage 0 (TON0)] rate was 13.8%. In group I, tumor down-staging occurred in 22 patients (48.9%), while no down-staging was obtained in 23 patients (51.1%); of whom 22 patients had Stage 3 disease and the remaining one had Stage 2 disease. The pCR rate in group I was 8.9%. In group II, 33 patients (78.5%) developed tumor down-staging and nine (21.4%) showed no down-staging. Eight of the patients who showed no down-staging had Stage 3 disease and the other one had Stage 2 disease. The pCR rate in group II was 19%. A significant decrease in postoperative stage was seen in patients who had a longer interval period. Patients in group II had significantly higher rates of pCR than their counterparts in group I (19% vs 8.9%, $P = 0.002$).

Significant predictors of pathological response such as age, gender, tumor localization, preoperative stage, preoperative T stage, and interval time were investigated by univariate and multivariate analyses. Except for pathological TRG and interval time, no positive correlation was noted between other predictive factors and pathological response. However, while rate of poor response TRG (TRG-4) was found to be 44.4% in group I, it was 9.5% in group II ($P < 0.002$). Although, an extended interval between CRT and surgery was found to increase rates of complete or near-complete TRG response, this rate was not significant when both groups were compared (Table 4).

A total of 60 patients who were diagnosed with Stage 2 or 3 disease after histopathological examination of TME specimens, including 30 patients in each group, were recommended for postoperative adjuvant therapy. However, 57 patients eventually received adjuvant therapy after surgery due to early postoperative mortality in three patients.

Factors predicting local recurrence, DFS and OS

The median follow-up period for all patients was 34.5 (9.9-81) mo. Median follow-up time for group I was 37.5 (9.9-74.5) mo and group II was 31.2 (10.7-81) mo. Median follow-up was comparable in both groups ($P = 0.59$).

Analysis of OS in group I showed median survival duration of 62.8 (95%CI: 55.8-69.7) mo, and a 24-mo

Table 3 Comparison of pre- and post-treatment stages in both groups *n* (%)

	Group I (<i>n</i> = 45)		Group II (<i>n</i> = 42)		Comparison of groups I and II <i>P</i> value
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	
T stage					0.17
T0	-	9 (18.9)	-	8 (19)	
T1	-	2 (4.4)	-	2 (4.8)	
T2	8 (17.8)	14 (31.7)	6 (14.3)	14 (33.4)	
T3	28 (62.2)	19 (42.8)	32 (76.2)	16 (38.1)	
T4	9 (20)	1 (2.2)	4 (9.5)	2 (4.8)	
Stage					0.002
Stage 0	-	4 (8.9)	-	8 (19)	
Stage 1	-	11 (24.4)	-	15 (35.7)	
Stage 2	4 (8.9)	8 (17.8)	2 (11.9)	11 (26.2)	
Stage 3	41 (91.1)	22 (48.9)	37 (88.1)	8 (19)	
Postop LN without metastasis		21 (46.7)		34 (81)	0.001

Table 4 Analysis of the effect of factors on pathological tumor regression grade

TRG	Relationship between demographics and TRG (<i>p</i>) and OR with 95%CI					Distribution and comparison of TRG rates in both groups		
	Age	Sex	Tumor localization	Preop T stage	Preop stage	Group I (<i>n</i> = 45) %	Group II (<i>n</i> = 42) %	<i>P</i> value
Complete response	(0.46) 2.19, 95%CI: 0.55-8.72	(0.84) 0.54, 95%CI: 0.12-2.29	(0.17) 0.66, 95%CI: 0.15-2.92	(0.24) 1.00, 95%CI: 0.90-1.50	(0.48) 1.21, 95%CI: 0.12-11.8	(<i>n</i> = 4) 8.9	(<i>n</i> = 8) 19	0.36
Near complete response	(0.91) 1.02, 95%CI: 0.37-2.79	(0.79) 1.11, 95%CI: 0.36-3.38	(0.38) 1.93, 95%CI: 0.64-5.8	(0.75) 0.50, 95%CI: 0.12-2.57	(0.80) 0.36, 95%CI: 0.10-1.51	(<i>n</i> = 9) 20	(<i>n</i> = 14) 33.3	0.35
Minimal response	(0.79) 0.67, 95%CI: 0.25-1.76	(0.59) 1.25, 95%CI: 0.43-3.63	(0.12) 0.45, 95%CI: 0.16-1.20	(0.66) 2.25, 95%CI: 0.36-13.8	(0.38) 2.02, 95%CI: 0.37-10.9	(<i>n</i> = 12) 26.7	(<i>n</i> = 16) 38.1	0.15
Poor response	(0.48) 0.98, 95%CI: 0.35-2.76	(0.95) 0.94, 95%CI: 0.31-2.82	(0.11) 1.65, 95%CI: 0.56-4.84	(0.19) 3.22, 95%CI: 0.92-11.2	(0.70) 1.34, 95%CI: 0.23-7.62	(<i>n</i> = 20) 44.4	(<i>n</i> = 4) 9.5	0.002

P < 0.05 is statistical significance. OR: Odds ratio; TRG: Tumor regression grade.

survival rate of 91.5%, and 60-mo survival rate of 79.1%. For group II, the median OS was 77.9 (95%CI: 72-81) mo. Twenty-four-month survival rate was 100%, and 60-mo survival rate was 94.4%. OS showed a significant difference when both groups were compared (*P* = 0.02). The median DFS duration in group I was 50.8 (95%CI: 43.4-58.2) mo. DFS rates were 76.4% at 24 mo and 55.3% at 60 mo. For group II, median DFS was 71.2 (95%CI: 63.1-79.2) mo. DFS rate was 85.1% at 24 mo and remained unchanged at 85.1% until 60 mo in group II. DFS rates differed significantly when both groups were compared (*P* = 0.01) (Figure 2).

When potential factors affecting OS and DFS were analyzed, nodal down-staging was found to have a positive correlation with OS and DFS (Table 5). OS and DFS were better in patients who achieved nodal down-staging (OS: 78% vs 52.1%, *P* = 0.001; DFS: 72.3% vs 43.1%, *P* = 0.001) (Figure 3).

After investigating the correlation between survival rates and pathological TRG, lower and moderate pathological regression grades (TRG 1-3) provided similar survival benefit, but only a poor pathological response (TRG 4) was associated with worse DFS

(*P* = 0.009). However, TRG scores did not show any association with OS and local recurrence (*P* = 0.06 and *P* = 0.39, respectively) (Figure 4).

Local recurrence was found to be 8.9% after a mean duration of 71.1 ± 2.3 mo in group I. Group II had a local recurrence rate of 7.1% after a mean duration of 72.4 ± 4.6 mo. The interval time did not show any association with local recurrence (*P* = 0.79) (Figure 5).

DISCUSSION

Since the initial description of the TME technique for rectal cancer by Heald, TME has become the standard surgical treatment for mid- and distal rectal cancer^[10-12]. Previous prospective studies have shown that surgery alone is not sufficient for local disease control, but its combination with preoperative CRT reduces local recurrence and increases DFS^[3,13-19]. The Lyon R90-01 Trial was the first randomized prospective study to compare the effects of short and long intervals after neoadjuvant therapy on pathological tumor down-staging, and found that a 6-8-wk treatment interval between radiotherapy and surgery improved tumor

Table 5 Effect of factors on overall survival and disease-free survival

	OS		DFS	
	P value	HR with 95%CI	P value	HR with 95%CI
Sex	0.61	0.97, 95%CI: 0.19-4.99	0.69	0.50, 95%CI: 0.46-4.46
Age	0.57	1.01, 95%CI: 0.95-1.08	0.60	1.00, 95%CI: 0.94-1.06
Tumor localization	0.53	0.97, 95%CI: 0.72-1.30	0.88	1.17, 95%CI: 0.80-1.70
Pre-treatment stage	0.94	0.77, 95%CI: 0.80-7.50	0.45	0.90, 95%CI: 0.80-1.50
Pre-treatment T stage	0.59	1.08, 95%CI: 0.21-5.48	0.39	0.39, 95%CI: 0.15-3.02
Post-treatment stage	0.01	18.07, 95%CI: 0.60-53.9	0.007	0.82, 95%CI: 0.10-6.23
Post-treatment T stage	0.13	0.62, 95%CI: 0.34-11.3	0.07	0.25, 95%CI: 0.19-8.54
Postoperative metastatic lymph node (+)	0.001	0.91, 95%CI: 0.69-1.20	0.001	1.25, 95%CI: 0.93-1.67
Pathologic TRG	0.11	0.90, 95%CI: 1.28-6.35	0.04	1.19, 95%CI: 0.17-8.41

DFS: Disease-free survival; OS: Overall survival; TRG: Tumor regression grade.

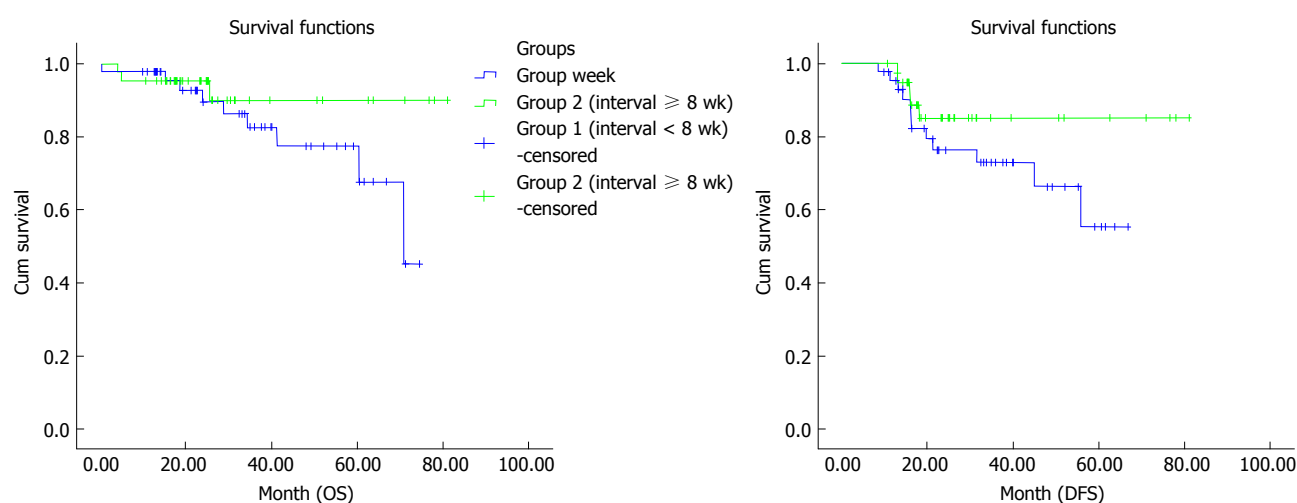


Figure 2 Comparison of overall survival and disease-free survival between the groups by Kaplan–Meier curves. The median DFS duration in group II was better than in group I ($P = 0.01$). DFS: Disease-free survival; OS: Overall survival.

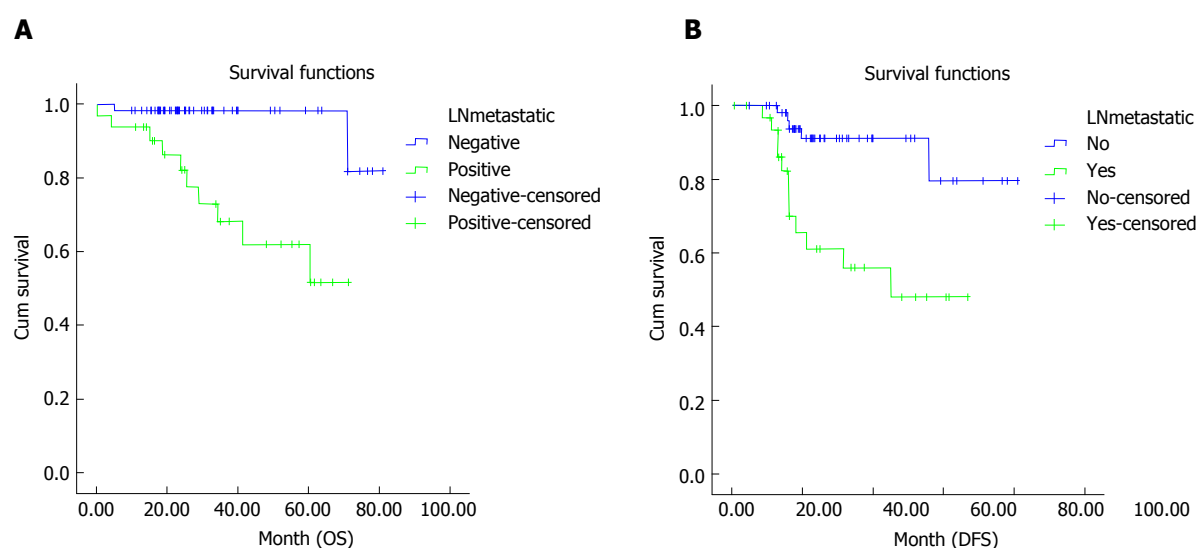


Figure 3 Effect of presence of tumor in lymph nodes and its correlation with overall survival (A) and disease-free survival (B). Survival rates were better in patients who achieved nodal down-staging ($P = 0.001$). OS: Overall survival; DFS: Disease-free survival.

down-staging and pCR. Since then, a 6-8-wk interval has been accepted as an appropriate interval between preoperative neoadjuvant therapy and surgery^[6].

In contrast, there have been rising concerns among surgeons regarding radiation-induced pelvic fibrosis that may occur as a result of a longer waiting period.

Table 6 Studies comparing the effects of the interval periods between neoadjuvant therapy and surgery on oncological outcome in locally advanced rectal cancer

Ref.	Total no. of patients	Design	Interval time (wk)	pCR	Local recurrence	OS
Francois <i>et al</i> ^[6]	201	Prospective, randomized	2/6-8	7%/14% ²	13%/10% ²	69%/66% ²
Wolthuis <i>et al</i> ^[20]	356	Retrospective	≤ 7/> 7	16%/28% ²	6%/3% ²	NA
Kalady <i>et al</i> ^[21]	306	Prospective	< 8/≥ 8	16.3%/28% ²	NA	NA
Garcia-Aguilar <i>et al</i> ^[22]	136	Prospective, nonrandomized	6/11	18%/25% ²	NA	NA
de Campos-Lobato <i>et al</i> ^[23]	177	Retrospective	< 8/≥ 8	16.5%/30.8% ²	1.2%/10.5% ²	NA
Tulchinsky <i>et al</i> ^[24]	132	Retrospective	≤ 7/> 7	17%/35% ²	6%/4% ¹	81%/93% ²
Sloothaak <i>et al</i> ^[25]	1593	Prospective	< 13/13-14/15-16	10%/13%/18% ¹	NA	NA
Saglam <i>et al</i> ^[31]	153	Prospective, randomized	4/8	19.7%/14.3% ²	11.8%/10.3% ²	76.5%/74.2% ²
Rödel <i>et al</i> ^[36]	385	Prospective	> 6	10.4%	3%	85%
Kerr <i>et al</i> ^[42]	189	Retrospective	Median 76 d (6-215 d)	15.9%	21%	NA

¹Significant difference statistically; ²Not significant difference statistically. pCR: Pathological complete response; NA: Not available; OS: Overall survival.

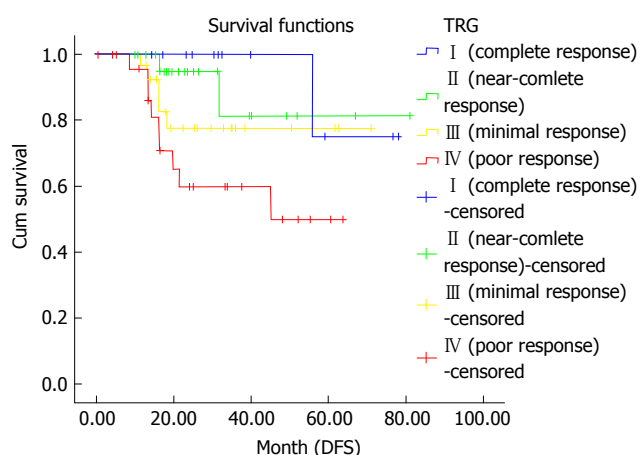


Figure 4 Correlation between level of the pathological tumor responses and disease-free survival. Only a poor pathologic response (TRG 4) was associated with worse DFS ($P = 0.009$). TRG: Tumor regression grade; DFS: Disease-free survival.

This is because fibrosis may cause operative difficulty and lead to an increased rate of surgical complications. Therefore, the issue of surgical timing has encouraged further studies to investigate the optimal interval time for surgical treatment in terms of oncological outcomes.

Several studies that have examined the effect of different intervals after neoadjuvant CRT on tumor response, pCR, local tumor control and survival have reported conflicting results (Table 6). Our study showed a higher rate of pCR among patients who had an interval > 8 wk. pCR was associated with a decreased number of metastatic lymph nodes in the mesorectum, which led to tumor down-staging. The only factor providing nodal down-staging was found to be interval period. In contrast, a shorter or longer interval time did not show any effect on T stage of the rectal wall. Specifically, a longer interval time was associated with a significant reduction in TRG 4 (poor response) rates. High TRG 4 rate (44%) in the shorter interval group in our study may be explained by performing surgery at 5 wk after neoadjuvant CRT in most patients.

It is questionable whether the poor tumor response

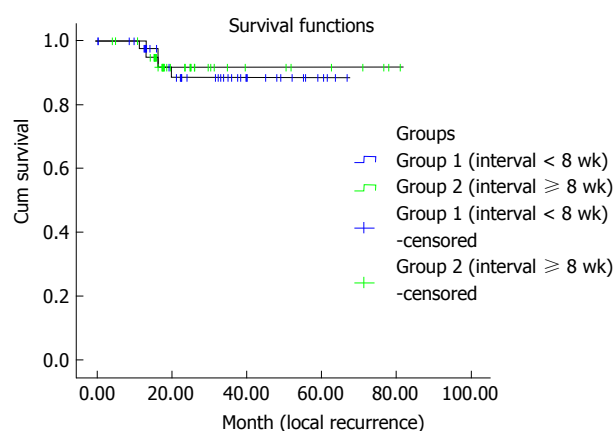


Figure 5 Local recurrences were similar in both interval groups. The interval duration did not show any association with local recurrence ($P = 0.79$).

also reduces DFS, while pCR is assumed as an indicator of improved DFS. Our findings established a negative correlation between TRG 4 (poor response) and DFS; an outcome that has not been mentioned in previous studies. Its clinical significance represents poor prognosis in terms of disease recurrence. Thus, the possibility of early recurrence of the disease should be considered in follow-up of patients who had TRG 4.

In a retrospective study by Wolthuis *et al*^[20], patients who underwent surgery after a treatment interval > 7 wk showed better pathological tumor response. However, pathological response in this study was evaluated based on the final pathological T staging of the rectal wall. In the Lyon R90-01 Trial by Francois *et al*^[6], despite the exclusion of preoperative chemotherapy, a longer interval time was again associated with better clinical tumor response. In another study, Kalady *et al*^[21] reported a pCR of 31.8% among patients who waited for > 8 wk before surgery and 16% in patients who had an interval < 8 wk. The authors further documented that an extended interval between completion of neoadjuvant therapy and surgery was the single most important determinant of better tumor response regarding final T stage in rectal wall as well as lymph nodes. Garcia-

Aguilar *et al.*^[22] in their prospective multicenter study investigated the effect of an extended interval between CRT and surgery on tumor response, CRT-related toxicity, and surgical complications. The authors also examined the impact of intense chemotherapy that was given during this interval period on pCR rates. They found that intense CRT in addition to increasing the time interval between neoadjuvant therapy and surgery may increase the pCR rate without significantly increasing CRT-related complications, operative difficulty or postoperative complications. Similarly, de Campos-Lobato *et al.*^[23] found an interval > 8 wk to be associated with a higher rate of pCR. In a recent study of predictive factors affecting pCR by Tulchinsky *et al.*^[24], neoadjuvant-surgery interval time was an independent predictive factor of tumor down-staging. In the largest and one of the most recent studies by the Dutch Surgical Colorectal Audit, a longer CRT-surgery interval of approximately 11 wk was related to the highest chance of pCR^[25]. Also, meta-analyses of several studies confirmed that an interval > 8 wk before surgery resulted in more tumor regression^[26,27].

In contrast, there have been studies reporting no correlation between duration of treatment interval and pCR. Findings from these studies have associated pCR with the longer period needed for a CRT response^[5,19,28-32]. In a study by Pucciarelli *et al.*^[33], tumor response was associated with preoperative chemotherapy regimen. A surgical interval > 6 wk was identified as a favorable prognostic factor for OS, although no differences were observed in pCR or DFS.

Our rates of tumor responses were lower than in the other studies. This was because of failure of tumor down-staging in half of the patients in group I. However, the longer interval group still had a better pCR than the shorter interval group had in the present study, and pCR rate was 19%, which was comparable with other studies. In the present study, tumor down-staging was related to a decrease in metastatic lymph nodes in the mesorectum, and a longer interval was needed for nodal down-staging.

A pCR is deemed to increase the chances of sphincter-saving surgery, decreases local recurrence, and has a positive prognostic impact on survival. Our study showed a positive correlation between pathological tumor response and OS and DFS. Patients who had an extended interval time had higher OS and DFS rates. These findings are in agreement with the study of Yeo *et al.*^[34]. In their multicenter retrospective study, which examined mesorectal nodal status in patients with T0 stage in the rectal wall after neoadjuvant therapy, nodal status was the most efficient independent prognostic factor for DFS and OS. Similarly, Abdul-Jalil *et al.*^[35] reported that pCR and nodal status after neoadjuvant therapy were important predictive factors for survival. Pucciarelli *et al.*^[33] showed that preoperative T staging was the only independent prognostic factor of DFS and OS; however, they reported that pathological tumor response did not affect survival. In the study by Francois *et al.*^[6] an extended interval time did not have any effect

on local recurrence and short-term survival. Also, in our study, rate of poor response TRG (TRG 4) was found to have negative effect on DFS, and was significantly lower in patients who had an extended interval before surgery. This shows the contribution of the prolonged interval to longer DFS. However, these factors were not associated with local recurrence. Similarly, Rödel *et al.*^[36] found that poor TRG was associated with worse DFS rate (63%), but not with local recurrence. In contrast, they found an association between TRG and presence of residual tumors in lymph nodes, but not between TRG and interval before surgery^[36]. In another study by Abdul-Jalil *et al.*^[35], TRG had no prognostic effect on survival.

Although it is suggested that neoadjuvant CRT increases the chances of sphincter-saving surgery, the benefit of extending the interval time from neoadjuvant therapy to surgery in reducing rates of abdominoperineal resection is controversial. Most studies investigating sphincter preservation rates by neoadjuvant therapy-surgery interval have reported no correlation between them^[5,6,22,24-26,29-31,37,38]. Our sphincter-saving surgery rates were similar in both groups, indicating no influence of neoadjuvant therapy-surgery interval. Also, its rate of 64% was comparable with the results of the other studies^[2,24,39].

One of the major concerns regarding extending the interval after neoadjuvant CRT is radiotherapy-induced fibrosis, which can lead to operative difficulty and an increased risk of intraoperative and postoperative morbidity. Current data on the effect of interval time and perioperative morbidity have varied. Buie *et al.*^[40] in their retrospective study of 246 patients, who underwent TME after neoadjuvant CRT, noted a significant increase in the rate of pelvic sepsis. In contrast, Martel *et al.*^[41] reported factors such as smoking, difficult anastomosis, and anastomosis located in the first 4 cm from the anal verge as significant predictors of pelvic sepsis, and not neoadjuvant CRT. In a recent prospective randomized study by Saglam *et al.*^[31], although overall surgical complication rate was higher in the short interval group, similar individual postoperative complications in both groups were observed. Similar findings were reported by Kerr *et al.*^[42] in another retrospective study that included 189 patients. Postoperative complication rates in that study were higher in patients operated on after < 8 wk delay, while the interval was not related to mortality. In contrast, several studies have shown no significant increase in postoperative complications by delaying surgery after neoadjuvant CRT^[6,20,22-24,30,32,43-45]. The findings of the present study have also shown similar postoperative complication rates in both short and long neoadjuvant therapy-surgery interval. Besides, we report a surgical complication rate of 26%-28%, which is considerably lower than the 40%-43% that previously reported^[24,39]. Our short-interval group had an anastomotic leakage rate of 11.1% compared with 19% in the long-interval group. The overall anastomotic complication rate in the present study was 14%, and did not differ significantly between the short- and

long-interval groups (11.1% and 19%, respectively). These findings were comparable with the results of other studies reporting an anastomotic leakage rate of 10%-20%^[4,6,24,40,46]. Our early postoperative morbidity rates also showed no significant difference between the groups.

The current study had several limitations. First, the retrospective nature of the study did not allow a comprehensive appraisal of the reported findings, and may account for the significant effect of the interval time on poor response TRG (TRG 4) and its effect on DFS. The other limitation was that the study did not allow a full analysis of long-term oncological results in all patients, because of the patients who did not complete the 5-year follow-up period.

The findings of the present study suggest that delaying the neoadjuvant CRT-surgery interval provides nodal down-staging and improves pCR rates, as well as decreasing the rate of TRG poor response. TRG may be an important predictive factor for DFS. Extending the interval between CRT and surgery may improve survival through tumor down-staging without increasing the rate of surgical complications. Studies investigating the optimal time between neoadjuvant CRT and surgery and its effect on pre- and postoperative outcomes should be encouraged for better oncological outcomes and lowest morbidity.

COMMENTS

Background

Today, locally advanced distal and mid-rectal tumors are commonly managed with preoperative combined chemoradiotherapy (CRT) followed by a waiting time before total mesorectal excision (TME). Previous studies have shown that a 6-8-wk interval between CRT and surgery improves tumor down-staging and provides a higher pathological response rate. However, the optimum interval is still lacking in the medical literature. In addition, even though the effect of an extended interval on pathological complete response (pCR) is known, the impact of pCR on disease-free survival (DFS) and overall survival (OS) has not been clearly described. Thus, interval before surgery is important in terms of tumor response and survival. The aim of this study was to determine whether the interval time between preoperative neoadjuvant CRT and surgery affects the rates of pCR, perioperative surgical complications, sphincter-saving surgery, DFS and OS in locally advanced mid- or distal rectal cancer.

Research frontiers

Although the concept of interval between CRT and surgery in the management of locally advanced rectal cancer is generally accepted, a clear and explicit waiting period before surgery is not defined. However, previous studies have shown that an extended interval time between CRT and surgery improve survival and tumor response rates without increasing surgical complications. Based on the current results, there is a trend towards extension of the interval to > 8 wk before surgery for locally advanced rectal cancer.

Innovations and breakthroughs

In this study, longer interval time was related to nodal down-staging and better pathological tumor response. pCR rate was 19% in the longer-interval group, comparable with other studies. Also, patients who had an extended interval had higher OS and DFS rates. However, in this study, poor response tumor regression grade (TRG) 4 had a negative effect on DFS and was significantly lower in patients who had an extended interval before surgery. This finding emphasizes the importance of the negative effect of poor TRG on DFS.

Applications

The present study suggests that an interval > 8 wk between CRT and surgery provides tumor down-staging and higher tumor response rates, and improves survival. Poor TRG is associated with shorter interval before surgery and related to worse DFS. If a patient has TRG 4 in his/her histopathological examination of the resected specimen, the possibility of early recurrence should be considered in follow-up of the patient.

Terminology

TME is a gold standard surgical technique for treatment of rectal cancer, first described by Bill Heald in 1982. A significant length of the bowel around the tumor together with mesorectum involving metastatic lymph nodes is removed *en bloc*. Neoadjuvant CRT is the administration of chemotherapeutic agents before surgery for locally advanced rectal cancer. The goals of neoadjuvant CRT are to reduce tumor size before radical surgical intervention, and provide local control of the disease. DFS is the length of disease-free time until the first relapse of the disease after curative treatment. OS is the length of time that patient is still alive, from the date of diagnosis or the start of treatment. TRG is a scoring system evaluating the response of the primary tumor of the rectal wall to CRT in resected specimens.

Peer-review

The authors have concluded that studies investigating the optimal time between neoadjuvant CRT and surgery and its effect on pre and postoperative outcomes should be encouraged for better oncological outcomes and lowest morbidity.

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Retrospective Study

Questionnaire survey regarding the current status of super-extended lymph node dissection in Japan

Shinji Morita, Takeo Fukagawa, Hisataka Fujiwara, Hitoshi Katai

Shinji Morita, Takeo Fukagawa, Hisataka Fujiwara, Hitoshi Katai, Department of Surgical Oncology, National Cancer Center Hospital, Chuo-ku, Tokyo 104-0045, Japan

Author contributions: Morita S designed and performed the research and wrote the paper; Fukagawa T and Katai H supervised the report; Fujiwara H provided clinical advice; Katai H designed the research and contributed to the analysis.

Institutional review board statement: We have not included a statement of IRB review, since IRB approval is not required for questionnaire research that does not provide personal information.

Informed consent statement: Patients were not required to give informed consent prior to participation in the study because the analysis used anonymous clinical data that were obtained after each patient had agreed to receive treatment and had provided written consent.

Conflict-of-interest statement: We have no financial relationships to disclose.

Data sharing statement: No additional data are available.

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Correspondence to: Shinji Morita, MD, Department of Surgical Oncology, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. smorita@ncc.go.jp
Telephone: +81-3-35422511
Fax: +81-3-25422545

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Abstract

AIM

To verify the current status of super-extended lymph node dissection for advanced gastric cancer according to a questionnaire survey.

METHODS

One-hundred and five institutions responded to the questionnaire. The survey included the following items: Number of experiences, whether performed prophylactically and/or therapeutically, whether preoperative chemotherapy was provided, number of preoperative chemotherapy rounds, and therapeutic options after chemotherapy.

RESULTS

Eighty-seven of the 105 institutions (83%) had performed D3 gastrectomy in the past or continued to perform D3 gastrectomy at present. However, D3 gastrectomy was rarely performed prophylactically in clinical practice. Seventy-eight institutions (74%) indicated that preoperative chemotherapy with curative intent was required for patients suspected of having para-aortic node (PAN) metastases. After chemotherapy, a D3 gastrectomy was scheduled for patients with a complete or partial response, stable disease, and progressive disease at 36 (46%), 28 (36%), and 13 (17%) of the institutions, respectively.

CONCLUSION

For patients with apparent PAN metastasis, a D3 gastrectomy is typically planned if a few courses of

preoperative chemotherapy yield at least a stable disease condition.

Key words: Questionnaire survey; Advanced gastric cancer; Radical surgery; Para-aortic nodal dissection; Preoperative chemotherapy

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Core tip: In this paper, we discussed the current status of super-extended lymph node dissection (LND) for advanced gastric cancer based on the results of a questionnaire survey. A recent study has indicated that prophylactic super-extensive lymphadenectomy does not improve the survival rate in patients with curable gastric cancer. In another study, surgery with para-aortic dissection was effective with the addition of neoadjuvant chemotherapy. Although super-extended LND seems to be rarely accepted worldwide, the rigorous and careful selection of patients can provide long-term survival after systemic LND. Therefore, we present the current status of super-extended LND in Japan.

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INTRODUCTION

Gastrectomy with D2 lymph node dissection (LND) has been the standard operation for advanced gastric cancer (AGC)^[1,2]. Although the efficacy of LND has been proven, patients with extensive nodal metastasis have a poor prognosis, even after R0 resection.

Lymphatic flow from the stomach streams into the perigastric nodes and then passes to the nodes around the celiac axis and its main branches. Following its influx into the para-aortic nodes (PANs), it finally joins the systemic circulation *via* the thoracic duct. Thus, the PANs have been regarded as the final nodal station requiring dissection to remove the threat of systemic metastases originating from the lymphatic system^[3]. Therefore, Japanese surgeons pursued a path of lymphadenectomy expansion with curative intent in the 1970s and 1980s. Although a super-extended dissection can be safely performed with acceptable operative risks, the long-term outcomes of patients with PAN metastasis have been less than spectacular, with reported 5-year survival rates of less than 30% and an incidence of PAN involvement from AGC of 10%-30%^[4-8]. To investigate whether para-aortic nodal dissection (PAND) for gastric cancer has a survival benefit in a large prospective study, the Japan Clinical

Oncology Group (JCOG) conducted a multi-institutional, randomized, controlled trial of D2 vs D2 plus PAND for patients with curable gastric cancer. This trial obtained evidence that the recommendation for extended D2 lymphadenectomy plus PAND should be withdrawn for the treatment of T2b, T3, or T4 curable gastric cancers^[8]. Critics have pointed out that PAN involvement indicates a systemic level of metastasis that is already outside the reach of surgical management, and dissecting these lymph nodes is not necessarily correlated with a better prognosis, at least in a preventative manner. In response to this result, the new Japanese Classification of Gastric Carcinoma (JCGC) excluded PAN from the regional lymph nodes of the stomach^[9]. Positive No.16 lymph nodes are treated as distant metastases, resulting in a Stage IV classification. Therefore, Japanese surgeons have revised their treatment strategy by focusing on highly effective treatments, such as a multimodal approach that includes chemotherapy to reduce recurrences after radical surgery. At present, surgical resection is the only way to remove tumors completely. We present an overview of the current status of PAND in Japan, having gathered information through a questionnaire survey.

MATERIALS AND METHODS

At the 2012 annual meeting of the Japanese Society for Gastro-surgical Pathophysiology, 13 data items, including PAND for patients with primary AGC, were collected retrospectively using a questionnaire survey. In this study, D3 gastrectomy was defined as D2 plus PAND, and the further dissection of other group 3 nodes described in the 13th JCGC was not necessarily required. The PANs, which are defined as the third tier, correspond to the No.16-a2 areas (nodes between the level of the celiac axis and the left renal vein) and the No.16-b1 areas (nodes between the left renal vein and the inferior mesenteric artery), based on the JCGC^[9]. The questionnaire contained questions regarding experience level, indications for prophylactic or therapeutic use, and data pertaining to preoperative chemotherapy (Table 1). Unexamined cases and patients with missing data were excluded when calculating the incidence.

RESULTS

The questionnaire was prepared and sent to 254 institutions. One hundred and five institutions (41.3%) responded to the survey. The geographical and functional distributions of the responding 105 hospitals are shown in Figure 1A. Fifty-two were academic medical centers and seventeen were cancer centers. The other 36 institutions were general hospitals.

Do you have any experience performing PAND?

Eighty-seven (83%) of all the responding institutions had experience performing PAND, and 28 institutions (17%) were still performing this procedure (Figure

Table 1 List of questions regarding para-aortic nodal dissection

1. Do you have any experience performing D2 plus PAND (so-called D3 surgery)?
 - A. Yes (a: Presently; b: In the past)
Please indicate the number of patients who have received D3 surgery at your institution and go to question 2.
() patients.
 - B. No (end of questionnaire)
2. When performing a distal gastrectomy, do you dissect the left upper lateral nodes ("No.16-a2-lat")?
 - A. Yes
 - B. Sometimes (please specify:)
 - C. No
3. The following questions concern prophylactic PAND.
 - 3-1. Do you dissect PAN prophylactically, even if there is no obvious metastasis in the same area?
 - A. Yes
 - B. Sometimes (please specify:)
 - C. No
 - 3-2. Do you perform PAN sampling in the absence of an enlarged PAN?
 - A. Yes
 - B. Sometimes (please specify the site:)
 - C. No
 - 3-3. Do you dissect PAN additionally in cases that test positive after an intraoperative rapid diagnosis?
 - A. Yes
 - B. No
4. The following questions concern therapeutic PAND.
 - 4-1. Which treatment option do you select if there is obvious metastasis in the PAN area?
 - A. Chemotherapy administered before surgery (go to question 4-2)
 - B. Surgery (go to question 4-8)
 - C. Only chemotherapy
 - 4-2. Which chemotherapy regimen do you use?
 - A. S1 + CDDP
 - B. Docetaxel + CDDP + S1
 - C. Other (please specify:)
 - 4-3. How many courses of preoperative chemotherapy do you use?
 - A. 2 courses
 - B. 3 courses
 - C. Other (please specify:)
 - 4-4. Do you administer additional chemotherapy if a complete or partial response is obtained after the preoperative chemotherapy?
 - A. Yes (please specify:)
 - B. No
 - 4-5. Please select the extent of the lymph node dissection that you use for patients with a complete or partial response after preoperative chemotherapy.
 - A. D2
 - B. D2 plus PAN sampling
 - C. D3
 - 4-6. Please select the extent of lymph node dissection that you use for patients with stable disease after preoperative chemotherapy.
 - A. D2
 - B. D2 plus PAN sampling
 - C. D3
 - 4-7. Please select the extent of lymph node dissection that you use for patients with progressive disease after preoperative chemotherapy if there is no evidence of distant metastasis, except for PAN swelling, is present.
 - A. D2
 - B. D2 plus PAN sampling
 - C. D3
 - 4-8. Please select the extent of lymph node dissection that you use for patients who have not received preoperative chemotherapy.
 - A. D2
 - B. D2 plus PAN sampling
 - C. D3

PAND: Para-aortic nodal dissection.

1B). Fourteen institutions had each performed more than 100 procedures. Seven institutions had performed 50 or more procedures but less than 100 procedures. Forty-nine institutions did not have much experience, having performed less than 50 procedures (Figure 1C). Seventeen institutions did not provide a specific number of procedures on the questionnaire form.

When performing a distal gastrectomy, do you dissect the left upper lateral nodes ("No.16-a2-lat")?

For a distal gastrectomy, the dissection of the left upper lateral nodes ("No.16-a2-lat") has been regarded as optional because of the low incidence of metastasis. Twenty-six (30%) of the 86 respondents reported dissecting the a2 lat nodes even if the resected part was

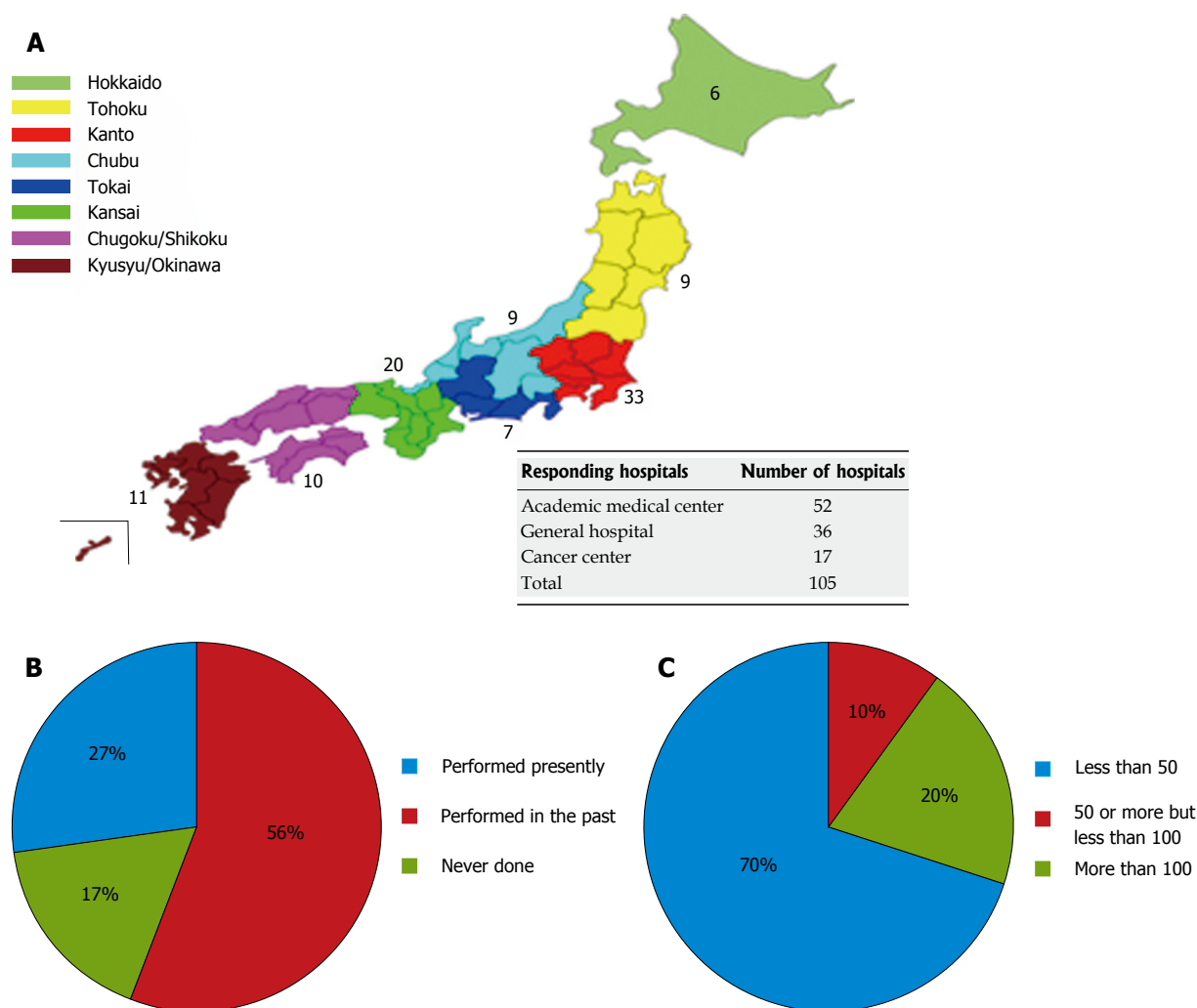


Figure 1 Geographical distribution, experiences and hospital volumes in the 105 responding hospitals. A: Geographical and functional distributions of the responding hospitals according to Japan's regions; B: Results for the question regarding experience performing PAND; C: Results for the question regarding the number of experiences performing PAND. PAND: Para-aortic nodal dissection.

distal to these nodes; 40 institutions (47%) reported that they sometimes dissected these nodes, and 20 institutions (23%) reported that they never dissected these nodes.

Prophylactic PAND dissection

For cases with no preoperative evidence of metastasis in the PAN, almost all the institutions never performed a prophylactic D3 dissection. Six (6%) of the 105 respondents performed prophylactic D3 dissections for staging purposes or in the case of nodal swelling in the supra-pancreatic area (Figure 2A). Only 21 institutions (20%) performed sampling at this site. Of the 21 institutions, 8 (38%) performed an additional dissection of the para-aortic area, if the examination of frozen samples was positive.

Therapeutic PAND dissection

For cases with apparent preoperative metastasis in the PAN, 78 (74%) of the 105 respondents reported performing preoperative chemotherapy, and 7 institutions

(7%) reported performing a D3 dissection without other therapies. The remaining institutions performed systemic chemotherapy in such cases (Figure 2B).

Preoperative chemotherapy

The most common preoperative chemotherapy regimen was a combination of S1 and cisplatin, which was selected by 57 (73%) of the 78 respondents. A triple-drug combination of S1, cisplatin and docetaxel was the second most common combination, selected by 20 institutions (26%). Two cycles of preoperative chemotherapy were selected by 62 institutions (79%), and three cycles were selected by 7 institutions (9%). If a complete or partial response was achieved after chemotherapy, 35 institutions (45%) provided additional chemotherapy for up to six cycles.

For cases with a PR or CR, the performance of an additional D3 dissection was selected by 36 (47%) of the 77 respondents, while a D2 dissection plus sampling was selected by 34 institutions (44%). Seven institutions (9%) limited the extent of LND to a D2 dissection. For

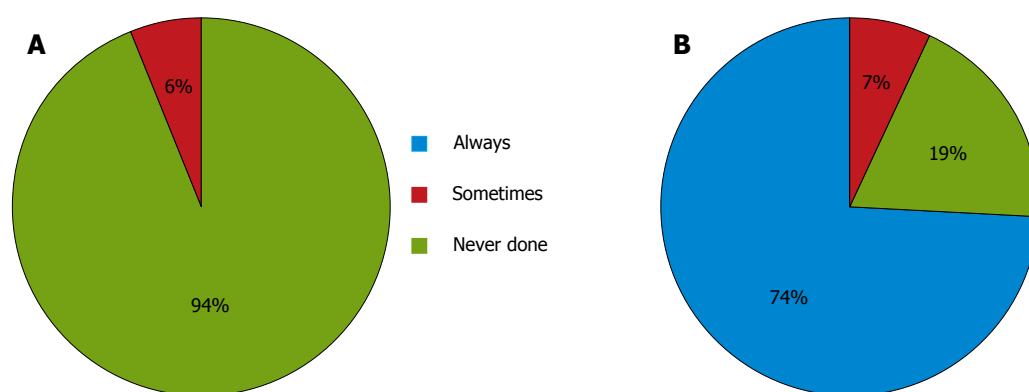


Figure 2 The answers to the questions related to prophylactical and therapeutical para-aortic nodal dissection. A: Results for the question regarding whether para-aortic nodal dissection is performed prophylactically; B: Results for the question regarding treatment options for patients with obvious metastasis in the para-aortic nodal area.

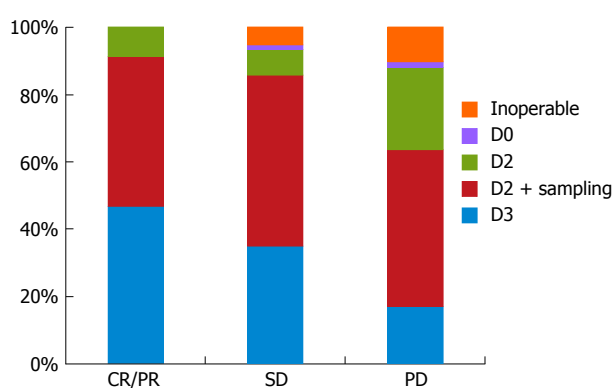


Figure 3 Results for the question regarding treatment options after preoperative chemotherapy in patients with obvious para-aortic nodal metastasis.

SD cases, the performance of an additional D3 dissection was selected by 27 institutions (35%), while a D2 dissection plus sampling was selected by 39 institutions (51%). Four institutions (5%) selected non-surgical treatment. For PD cases, 13 institutions (17%) still perform D3 dissections, although 36 institutions (47%) select a D2 dissection plus sampling and 19 institutions (25%) select a D2 dissection. Eight institutions (10%) have abandoned surgical treatment (Figure 3). Operations without preoperative chemotherapy were only performed at 7 institutions (7%) overall.

DISCUSSION

The 2008 annual report of the JGCA showed that D3 dissection was only performed in 7.2% of all surgically treated patients^[10]. In our data set, about 26% of the institutions that responded are still performing D3 gastrectomy. Overall, the number has been declining. The main reasons for this decrease are generally attributed to the following three aspects. First, the survival benefit for patients treated with a super-extensive LND has reached a plateau with the technical evolution and systemization of the surgical procedure. Basically, Japanese surgeons have adhered to the traditional

concept of a lymphadenectomy, which offers the best chance for the removal of involved lymph nodes and the treatment of AGC. However, metastasis to the para-aortic lymph nodes is correlated with a significantly poor prognosis, even after the complete surgical removal of these lymph nodes. Several reports have shown that the probability of PAN involvement is 30% at most^[11]. Several theories regarding the optimal level for extensive LND exist, and a uniform strategy has not yet been formulated.

The second reason is that only patients with PAN involvement and neither peritoneal seeding nor liver metastasis are candidates for this treatment. With advances in mass-screening and imaging equipment, early gastric cancer now accounts for nearly 50% of all gastric cancers in Japan^[10]. Moreover, obtaining multiple slices on computed tomography and/or diagnostic laparoscopy has further reduced the number of patients who meet the selection conditions^[12,13]. Thus, only a very few patients are likely to be candidates for this procedure, and such patients are rarely encountered in clinical practice.

Thirdly, a large randomized clinical trial had a considerable influence on the present results. A report by the JCOG showed that there was no survival benefit for a prophylactic D2 lymphadenectomy plus PAND (70.3%), compared with a D2 dissection alone (69.2%), in patients with AGCs^[8]. Our data shows that prophylactic dissection is not popular in Japan. The results of the above-mentioned clinical trial have contributed to the recent reduction in procedures. Some institutions perform PAND prophylactically, although they are few in number. However, the purpose of such procedures is only for staging, and not for curative intent. About two thirds of institutions (62%) do not perform complete PAND even if the frozen examination of PAN is positive. Similarly, a few institutions (7%) plan to dissect these nodes during their first attempt in cases with apparent PAN swelling preoperatively. Regardless of surgical treatment, the number of institutions performing chemotherapy as the initial treatment has been

increasing. This trend suggests that PAN involvement is regarded as being almost systemic, and the dissection of these lymph nodes is not necessarily related to a better prognosis, at least in a preventative manner. This situation emphasizes the importance of multidisciplinary therapies^[14-16].

Several selected phase I and II trials of preoperative chemotherapy in patients with resectable gastric cancer have been performed since the 1990s^[17-27]. The current practice for the treatment of patients with gastric cancer in Europe is now surgery with perioperative chemotherapy, after promising results were obtained in two major randomized control trials^[15,28]. In Japan, two selected phase II trials of neoadjuvant chemotherapy followed by a D2 gastrectomy with PAND for resectable gastric cancers have been reported^[27,29]. Although one trial was terminated because of treatment-related deaths, the other showed both the feasibility and the effectiveness of S-1 plus cisplatin followed by a D2 gastrectomy with PAND. In our data, around three-fourths of the institutions responded that they used the SP regimen for preoperative therapy. About one-fourth added one more agent, docetaxel, to the SP regimen. A neoadjuvant setting for tumor down-staging to improve resectability is considered to be required for the treatment of apparent PAN involvement, although some theoretical disadvantages do exist, such as tumor progression, oncological emergency, and a high perioperative morbidity^[30]. Further consideration will be needed to select the most effective regimen.

Finally, the JCOG9501 study also reported a paradoxical interaction in that the survival rates were better for those patients assigned to D2 plus PAND than for those assigned to D2 alone among cases with pathologically negative nodes^[8]. This result may indicate the effectiveness of an extended lymphadenectomy in cases that have responded well to preoperative chemotherapy. Our study showed a pronounced propensity for patients with a good clinical response (at least more than stable disease after chemotherapy) to have undergone a D3 dissection. The determination of the optimal extent of LND (D2 or D3) in cases with a complete response after chemotherapy is still a matter of debate. On the other hand, a considerable number of SD or PD cases had undergone D3 including PAND. Kurokawa *et al.*^[31] reported that the histological response rate seems to be a better surrogate endpoint for overall survival than radiologic response rate in studies of neoadjuvant therapy for gastric cancer. At the least, diagnostic laparoscopy should be a recommended preoperative examination for staging. Regarding the poor radiologic response to chemotherapy, careful handling is required after excluding apparent PD cases. To settle this important matter, further prospective validation is needed.

The main limitation of this study was that the intended group was confined almost exclusively to patients with PAN involvement. In addition, this highly targeted group needed to meet stringent requirements consisting of

the absence of peritoneal seeding and liver metastasis. Therefore, it should be noted that the response rate was comparatively low, given that mainly specialized institutions were included, and that biases in the data were unavoidable because of problems such as the limited number of patients from small and medium-sized hospitals.

In conclusion, preoperative chemotherapy with curative intent is almost essential for patients with PAN involvement. However, this does not deny that super-extended LND may play a certain role in the course of radical therapy. This procedure should be confined exclusively to specialized institutions, since eligibility is limited and a high surgical proficiency is required.

COMMENTS

Background

Super-extended lymph node dissection (LND), including the para-aortic area, is a controversial issue in the treatment of patients with advanced gastric cancer (AGC). The Japanese Society for the Study of Postoperative Morbidity after Gastrectomy conducted a nationwide questionnaire survey to clarify the current status of para-aortic LND for AGC.

Research frontiers

The long-term outcomes of patients with para-aortic node (PAN) metastasis have been less than spectacular. Positive No.16 lymph nodes are treated as distant metastases. The results of this study contribute to clarifying the current status of super-extended LND in Japan.

Innovations and breakthroughs

Platinum doublet chemotherapy, S1 plus CDDP, is widely used as a neoadjuvant chemotherapy for AGC with PAN metastasis. The study showed a pronounced propensity for patients with a good clinical response to have undergone a D3 dissection.

Applications

This large survey suggested that D3 dissections are not performed for prophylactic purposes. For patients with apparent PAN metastasis, a D3 gastrectomy is usually planned if a few courses of preoperative chemotherapy yield at least a stable disease condition.

Terminology

D3 dissection: Super-extended LND including perigastric, supra-pancreatic and para-aortic areas for AGC with a risk of extensive nodal involvement.

Peer-review

This manuscript deals with questionnaire survey on super-extended lymph-node dissection in patients with AGC. The conclusion of this manuscript seems to agree with the general consensus of current Japanese surgical oncologists, and could be acceptable for publication.

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**REVIEW**

- 715 Current debate in the oncologic management of rectal cancer
Millard T, Kunk PR, Ramsdale E, Rahma OE
- 725 Mucins in neoplasms of pancreas, ampulla of Vater and biliary system
Moschovis D, Bamias G, Delladetsima I
- 735 Nanomedicine strategies for sustained, controlled, and targeted treatment of cancer stem cells of the digestive system
Xie FY, Xu WH, Yin C, Zhang GQ, Zhong YQ, Gao J

MINIREVIEWS

- 745 Where does chemotherapy stands in the treatment of ampullary carcinoma? A review of literature
Ghosn M, Kourie HR, El Rassy E, Haddad FG, Hanna C, El Karak F, Nasr D

CASE REPORT

- 751 Arterial complication of irreversible electroporation procedure for locally advanced pancreatic cancer
Ekici Y, Tezcaner T, Aydın HO, Boyvat F, Moray G

Contents

World Journal of Gastrointestinal Oncology
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Current debate in the oncologic management of rectal cancer

Trish Millard, Paul R Kunk, Erika Ramsdale, Osama E Rahma

Trish Millard, Paul R Kunk, Erika Ramsdale, Division of Hematology-Oncology, Department of Medicine, University of Virginia Health System, Charlottesville, VA 22908, United States

Osama E Rahma, Center for Immuno-Oncology/GI Oncology, Dana-Farber Cancer Institute, Boston, MA 02215, United States

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Telephone: +1-617-6326954
Fax: +1-617-5827227

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Abstract

Despite the considerable amount of research in the

field, the management of locally advanced rectal cancer remains a subject to debate. To date, effective treatment centers on surgical resection with the standard approach of total mesorectal resection. Radiation therapy and chemotherapy have been incorporated in order to decrease local and systemic recurrence. While it is accepted that a multimodality treatment regimen is indicated, there remains significant debate for how best to accomplish this in regards to order, dosing, and choice of agents. Preoperative radiation is the standard of care, yet remains debated with the option for chemoradiation, short course radiation, and even ongoing studies looking at the possibility of leaving radiation out altogether. Chemotherapy was traditionally incorporated in the adjuvant setting, but recent reports suggest the possibility of improved efficacy and tolerance when given upfront. In this review, the major studies in the management of locally advanced rectal cancer will be discussed. In addition, future directions will be considered such as the role of immunotherapy and ongoing trials looking at timing of chemotherapy, inclusion of radiation, and non-operative management.

Key words: Chemoradiation; Immunotherapy; Non-operative management; Neoadjuvant chemotherapy; Rectal cancer

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Core tip: Numerous controversies exist within the treatment of locally advanced rectal cancer. This review article summarizes the relevant evidence for rectal cancer treatment and offers opinions on how to interpret the data in clinical practice. Additional information is provided on novel areas of interest that are being actively explored such as the role of immunotherapy, the need for biomarkers, and the non-operative management.

Millard T, Kunk PR, Ramsdale E, Rahma OE. Current debate in the management of rectal cancer. *World J Gastrointest*

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INTRODUCTION

Over the last several decades, the approach to treat locally advanced rectal cancer has become more complex. Surgical and pathologic advances as well as multimodality approaches combining surgery, chemotherapy, and radiation therapy (RT) have decreased recurrence rates and improved quality of care. The mainstay of treatment to date has been surgical resection, and total mesorectal excision (TME) is the current standard. The goal of adjuvant therapy (radiation and chemotherapy) is to further decrease the rate of local and distant recurrences. Fluoropyrimidine-based chemotherapy both sensitizes the tumor cells to radiation as well as eliminates micrometastatic disease. However, despite refinements in the treatment of locally advanced rectal cancer, substantial controversies remain: Among them, the optimal course of radiation, sequencing of therapy, and surgical approach to clinical complete responses to neoadjuvant therapy. In this article, we highlight several of the prominent controversies in the treatment of locally advanced rectal cancer and provide a platform for discussion of evolving areas of interest within the field.

TIMING OF RADIATION AND SHORT COURSE RADIATION VS LONG COURSE CHEMORADIATION

The use of radiation therapy prior to surgery, rather than after surgery, is currently the standard of care for locally advanced rectal cancer. This is based on two large studies showing decreased local recurrence when radiation is used in the neoadjuvant setting (Table 1). In the Dutch TME trial, patients were randomized to RT and TME or to TME alone. This study showed an overall 5-year local recurrence rate of 4.6% in patients treated with radiation and TME and of 11% in the TME group ($P < 0.0001$) with no statistical significant difference in the rate of distant metastases^[1,2]. In the German CAO/ARO/AIO-94 phase III trial, patients were randomly assigned to preoperative or postoperative chemoradiation (CRT). Although the 5-year local relapse rate was better in the preoperative RT group (13% vs 6%, $P = 0.006$)^[3], the updated follow-up showed no statistical difference in the 10-year cumulative incidence of local relapse, disease-free survival or distant metastases^[4].

Both short course radiation therapy and long course CRT have been found to be effective in this setting, but no agreement exists in regards to the ideal method of neoadjuvant radiation-based treatments despite the publication of randomized, prospective data.

Neoadjuvant short course RT and long course CRT have been compared in three randomized, prospective trials (Table 1). The Trans-Tasman Radiation Oncology Group randomized cT3N0-2M0 rectal cancer patients (within 12 cm from the anal verge) to pre-operative short course radiation of 5x5Gy/fraction or CRT to a total dose of 50.4 Gy (28 fractions). The patients in the short course randomization proceeded to surgery within 3-7 d after radiation completion and were treated with 6 monthly cycles of adjuvant fluorouracil (425 mg/m²) 4-6 wk after surgery. The patients in the CRT group were treated with continuous infusion FU (225 mg/m²) daily for the duration of the radiation, followed by surgery 6 wk later and four monthly cycles of adjuvant fluorouracil (425 mg/m²). Interestingly, there was no significant difference in three-year local recurrence rate (7.5% vs 4.4%, $P = 0.24$) or five-year distant recurrence rate (27% vs 30%, $P = 0.92$) between the two groups. However, pathologic downstaging was significantly more common in the CRT than in short course group (45% vs 28%, $P = 0.002$), but there was no difference in organ sparing surgeries, nor any difference in late toxicities (5.8% vs 8.2%, $P = 0.53$)^[5]. The authors concluded that long course CRT may be more effective than short course for distal tumors, based on a trend toward decrease in local recurrence. In a similar design, the Polish Colorectal Study Group randomized patients with cT3-4 resectable rectal cancer to either CRT (50.4 Gy in 28 fractions with bolus 5-fluorouracil and leucovorin) followed by surgery 4-6 wk later or to short course radiation (25 Gy in 5 fractions) followed by surgery 7 d later. As seen in the Trans-Tasman Radiation Oncology Group there was no statistical significant difference in the 4-year local recurrence rate (10.6% in the short course group vs 15.6% in the CRT group, $P = 0.21$). However, there was a significant difference in the pathologic complete response (pCR) in favor of the CRT group, and the short course group was significantly more likely to have a positive surgical margin^[6]. Although intriguing, this is likely due to the early surgery in the short course group rather than type of radiation as a longer interval before surgery has been shown to increase the rate of pCR^[7]. There was no difference in the rate of sphincter preservation, overall survival, or the incidence of late toxicity at a median follow up of 4 years incidence between the two groups^[6]. The Polish Colorectal Study Group most recently reported on a study randomizing patients to preoperative short course RT with consolidation FOLFOX4 for 3 cycles or to preoperative CRT. There was no statistical difference in rate of pCR between the short course consolidation and CRT groups (16% vs 11.5%, $P = 0.19$) and no difference at 3 years in overall survival, disease-free survival, and cumulative incidence of local failure^[8].

Although preoperative radiation is shown to be superior to postoperative radiation in terms of improving local recurrence rate, it remains unclear whether short course RT or long course CRT is preferable. The use of

Table 1 Landmark radiation trials in rectal cancer

Ref.	Study type	Treatment	Outcomes disease control	Overall survival	Comments
Dutch TME Trial van Gijn <i>et al</i> ^[1]	Phase III <i>n</i> = 1805	RT + TME <i>vs</i> TME alone	5-yr LR 4.6% <i>vs</i> 11% (<i>P</i> < 0.0001) 10-yr DR 25% <i>vs</i> 28% (<i>P</i> = 0.21)	48% <i>vs</i> 49% (<i>P</i> = 0.86) (10-yr)	
German CAO/ARO/ AIO-94 Sauer <i>et al</i> ^[3]	Phase III <i>n</i> = 823	Preoperative <i>vs</i> Postoperative CRT	5-yr LR 6% <i>vs</i> 13% (<i>P</i> = 0.006) 10-yr LR 7.1% <i>vs</i> 10.1% (<i>P</i> = 0.048) 10-yr DR 29.8% <i>vs</i> 29.6% (<i>P</i> = 0.9)	59.6% <i>vs</i> 59.9% (<i>P</i> = 0.85) (10-yr)	
TTROG Trial 01.04 Ngan <i>et al</i> ^[5]	Phase III <i>n</i> = 326	Preoperative RT <i>vs</i> CRT	3-yr LR 7.5% <i>vs</i> 4.4% (<i>P</i> = 0.24) 5-yr DR 27% <i>vs</i> 30% (<i>P</i> = 0.92)	74% <i>vs</i> 70% (0.62) (5-yr)	Short course RT with more pathologic downstaging (28% <i>vs</i> 45%b). No difference in organ sparing surgeries or late toxicities
Polish Colorectal Study Group Bujko <i>et al</i> ^[6]	Phase III <i>n</i> = 316	Preoperative RT <i>vs</i> CRT	4-yr LR 10.6% <i>vs</i> 15.6% (<i>P</i> = 0.21) 4-yr DR 31.4% <i>vs</i> 34.6% (<i>P</i> = 0.54)	67.2% <i>vs</i> 66.2% (<i>P</i> = 0.960) (4-yr)	CRT with improved pCR is attributed to longer interval before surgery. No difference in rate of sphincter preservation or late toxicities
Polish II Multicentre Bujko <i>et al</i> ^[8]	Phase III <i>n</i> = 515	Preoperative RT with adjuvant FOLFOX4 <i>vs</i> CRT	R0 77% <i>vs</i> 71% (<i>P</i> = 0.081) pCR 16% <i>vs</i> 11.5% (<i>P</i> = 0.19)	73% <i>vs</i> 64.5% (<i>P</i> = 0.055) (3-yr)	Published at GI ASCO 2016 with median follow up of 35 mo

LR: Local recurrence; DR: Distant recurrence; RT: Radiation therapy; TME: Total mesorectal excision; CRT: Chemotherapy radiation therapy.

CRT did improve downstaging in both of these studies, but this is likely due to the difference in timing between completion of radiotherapy and surgical resection between the two groups. The advantages of short course RT include reduced duration and cost of therapy, but there remains concerns about late toxicity. In addition, it remains unclear whether CRT provides any meaningful long term advantages given lack of benefit in controlling distant metastases or improving overall survival.

CHEMOTHERAPY COMPONENT OF CHEMORADIATION: WHICH AGENTS?

Standard neoadjuvant CRT includes a fluoropyrimidine as the chemotherapy component, primarily due to the radiation sensitization activity of these drugs. Capecitabine, an oral prodrug converted to 5-fluorouracil (5-FU) by intracellular thymidine phosphorylase, has been shown to be non-inferior to infusional 5-FU for this indication^[9,10]. It is unclear, however, whether multi-agent chemotherapy regimens combined with radiotherapy can improve outcomes. Several studies (Table 2) have investigated the addition of oxaliplatin to fluorouracil-based neoadjuvant CRT including the NSABP R-04^[9], the STAR-01^[11], the ACCORD 12/0405-ProDIGE 2^[12], and the PETACC-6^[13]. These studies added weekly oxaliplatin to either infusional fluorouracil or capecitabine in combination with preoperative radiation. The results of these studies showed no difference in therapeutic outcomes, including pathologic complete response, locoregional control, and survival outcomes, and there was an increase in grade 3-4 treatment toxicities in patients treated with oxaliplatin. In contrast, the results of the German CAO/ARO/AIO-04 suggest a

possible benefit with the addition of oxaliplatin to CRT. In this trial, patients with T3-4 or cN+ rectal cancer were randomized to preoperative long course CRT with infusional fluorouracil (1000 mg/m² days 1-5 and 29-33) followed by surgery and four cycles of adjuvant bolus fluorouracil (500 mg/m² days 1-5 and 29) or to preoperative CRT with infusional fluorouracil (250 mg/m² days 1-14 and 22-35) and oxaliplatin (50 mg/m² days 1, 8, 22, and 29) followed by surgery and eight cycles of adjuvant infusional fluorouracil (2400 mg/m² days 1-2 and 15-16), leucovorin (400 mg/m² days 1 and 15), and oxaliplatin (100 mg/m² days 1 and 15). Patients in the investigational arm had a higher pathologic complete response rate (17% *vs* 13%, *P* = 0.038) and a higher disease free survival (DFS) at 3 years (75.9% *vs* 71.2%, *P* = 0.03), without increases in overall toxicity^[14,15]. The results should be interpreted with caution given that different fluorouracil dosing and regimens were used in the two treatment arms. It is also unclear whether the improved DFS is attributable to the adjuvant inclusion of oxaliplatin. Moreover, the patients in the oxaliplatin arm were treated with eight cycles of adjuvant chemotherapy, but the control patients only received four cycle. Thus, this difference in protocol may contribute to the difference in disease free survival.

ADJUVANT CHEMOTHERAPY: WHAT AND WHEN?

Modern surgical techniques combined with radiotherapy have dramatically improved locoregional control of locally advanced rectal cancer, but systemic control remains a significant issue. Distant relapse is now the main driver of adverse survival outcomes in this disease. The rationale for adjuvant chemotherapy in surgically resected rectal

Table 2 Landmark chemotherapy studies in locally advanced rectal cancer

Ref.	Study type	Treatment	Outcomes	Comments
TIMING Garcia-Aguilar <i>et al</i> ^[22]	Phase II <i>n</i> = 144	All treated with CRT If evidence of response, given 2 cycles upfront FOLFOX6 then TME If no response, proceed to TME 6 wk after CRT	70 patients treated with upfront FOLFOX6 74 patients proceeded directly to TME pCR 25% <i>vs</i> 18% (<i>P</i> > 0.05)	No difference in toxicities
MSKCC Cercek <i>et al</i> ^[23]	Retrospective <i>n</i> = 61	Upfront FOLFOX (median 7 cycles) followed by CRT	Patients with TME (49): 100% R0 resections pCR 27%	Non-randomized, retrospective data
Calvo <i>et al</i> ^[24]	Prospective non-randomized <i>n</i> = 116	Upfront FOLFOX-4 (2 cycles) followed by CRT compared to historical controls treated with CRT	52 patients treated with upfront FOLFOX pCR 29% <i>vs</i> 8% (<i>P</i> = 0.006)	3 patients in FOLFOX had grade 3-4 toxicities (<i>vs</i> 0). No difference in surgical complications
Chau <i>et al</i> ^[25]	Prospective non-randomized <i>n</i> = 77	Upfront CAPOX (4 cycles) followed by CRT with TME 6 wk later	R0 resections in all but 1 patient pCR 24%, additional 48% with only microscopic tumor foci	10% with cardiac or thromboembolic toxicity during CAPOX. 12% with grade 3-4 diarrhea during CAPOX
Schou <i>et al</i> ^[26]	Prospective non-randomized <i>n</i> = 84	Upfront CAPOX (2 cycles) followed by CRT with TME 6 wk later	94% R0 resections pCR 23% 5-yr DFS 63%, 5-yr OS 67%	Grade 3-4 toxicities in 18%
Marechal <i>et al</i> ^[27]	Phase II, randomized <i>n</i> = 57	Randomized to preoperative CRT followed by TME <i>vs</i> upfront FOLFOX followed by CRT and TME	ypT0-1N0 34.5% <i>vs</i> 32.1% (<i>P</i> = 0.85) pCR 28% <i>vs</i> 25% (<i>P</i> = 0.92) pCR 16% <i>vs</i> 16% (<i>P</i> = 0.904)	A pre-planned interim analysis no difference in primary endpoints; study closed prematurely for futility
STAR-01 Aschele <i>et al</i> ^[11]	Phase III <i>n</i> = 747	Preoperative CRT with fluorouracil \pm oxaliplatin	pCR 16% <i>vs</i> 16% (<i>P</i> = 0.904)	Oxaliplatin group had more grade 3-4 adverse events (24% <i>vs</i> 8% <i>P</i> < 0.001)
ACCORD12/0405-Prodige 2 Gérard <i>et al</i> ^[12]	Phase III <i>n</i> = 598	Preoperative CRT with capecitabine \pm oxaliplatin	pCR 19.2% <i>vs</i> 13.9% (<i>P</i> = 0.09)	Oxaliplatin group had more grade 3-4 adverse events (25% <i>vs</i> 1%, <i>P</i> < 0.001)
NSABP R-04 O'Connell <i>et al</i> ^[9]	Phase III <i>n</i> = 1608	Preoperative CRT with fluorouracil \pm oxaliplatin <i>vs</i> Preoperative CRT with capecitabine \pm oxaliplatin	FU <i>vs</i> Cap: pCR 17.8% <i>vs</i> 20.7% (<i>P</i> = 0.14) Oxaliplatin <i>vs</i> No Oxaliplatin: pCR 19.5% <i>vs</i> 17.8% (<i>P</i> = 0.42) pCR 11.3% <i>vs</i> 13.3% (<i>P</i> = 0.31)	Patients treated with oxaliplatin experienced significantly more grade 3 or 4 diarrhea (<i>P</i> < 0.001)
PETACC-6 Schmoll <i>et al</i> ^[13]	Phase III <i>n</i> = 1094	Preoperative CRT with capecitabine \pm oxaliplatin (adjuvant chemo same drugs as preoperative)	pCR 11.3% <i>vs</i> 13.3% (<i>P</i> = 0.31)	Patients treated with preoperative oxaliplatin had significant more grade 3-4 adverse events (36.7% <i>vs</i> 15.1%)
German CAO/ARO/AIO-04 Rodel <i>et al</i> ^[15]	Phase III <i>n</i> = 1236	Preoperative CRT with fluorouracil \pm oxaliplatin (adjuvant chemo same drugs as preoperative)	pCR 17% <i>vs</i> 13% (<i>P</i> = 0.038) 3-yr DFS 75.9% <i>vs</i> 71.2%	Different fluorouracil dosing/schedule and different number of adjuvant cycles used across the arms
EORTC 22921 Bosset <i>et al</i> ^[19]	Phase III <i>n</i> = 1011	Preoperative RT + adjuvant FU/L <i>vs</i> preoperative RT <i>vs</i> CRT with adjuvant FU/L <i>vs</i> CRT	10-yr LR 14.5% <i>vs</i> 22.4% <i>vs</i> 11.7% <i>vs</i> 11.8% (<i>P</i> = 0.0017) ^a 10-yr DR 35.9% <i>vs</i> 39.6% <i>vs</i> 34.1% <i>vs</i> 33.4% (<i>P</i> = 0.52)	Any chemotherapy (neoadjuvant only, adjuvant only, or both) had significant reduction in local recurrence, but no difference in OS. Used concomitant bolus FU dosing not commonly utilized in the United States, and only four cycles of adjuvant chemotherapy

^a*P* < 0.01 any chemo *vs* no chemo. LR: Local recurrence; DR: Distant recurrence; TME: Total mesorectal excision; CRT: Chemotherapy radiation therapy.

cancer is largely extrapolated from colon cancer data^[16], but is also supported by a meta-analysis of randomized trials that compared surgery with or without adjuvant chemotherapy for rectal cancer^[17]. The European Organization for Research and Treatment of Cancer (EORTC) 22921 phase III trial randomized patients with clinical stage II or III resectable rectal cancer to preoperative radiation with or without concomitant fluorouracil (350 mg/m² IV bolus over 5 d) with leucovorin (20 mg/m² IV) followed by either surveillance or four cycles of adjuvant fluorouracil/leucovorin^[18]. There was no difference in the 10-year overall survival between patients getting adjuvant chemotherapy or not (51.8% *vs* 48.4%, *P* = 0.32). Additionally, the rate of

distant metastasis between all arms had no significant difference (34.1%-39.6%). However, the three arms receiving any chemotherapy (neoadjuvant only, adjuvant only, or both) had significant reduction in local recurrence (11.8%-14.5%) compared to those treated with radiation alone (22.4%, *P* = 0.0017), but this did not translate to an overall survival advantage^[19]. Notably, the EORTC 22921 trial incorporated a concomitant bolus fluorouracil dosing not commonly utilized in the United States, and only four cycles of adjuvant chemotherapy were given. Moreover, there were significant deviations from planned treatment, with 26.9% in the adjuvant group never initiating adjuvant therapy, most commonly due to post-operative complications, and less than 43% of

patients received the planned dose within the scheduled time interval^[18]. Despite the controversial results of the EORTC 22921, there remains substantial interest in the use of systemic chemotherapy, both as adjuvant therapy and as CRT, to decrease distant metastatic disease and improve survival^[20]. The theoretical advantages of systemic chemotherapy include eradicating distant micrometastases and providing ideal systemic treatment prior to a large and potential debilitating surgery (and thus a less fit patient)^[21].

Given the difficulties with adherence to postoperative chemotherapy, there is now significant interest in the utility of administering chemotherapy preoperatively (Table 2). In the phase II TIMING trial, stage II and III rectal cancer patients were treated with CRT, and those patients with evidence of clinical response 4 wk after CRT received two cycles of modified FOLFOX-6 followed by TME 3-5 wk later. The remainder of the patients proceeded to TME 6 wk after completion of CRT. The preliminary results from this study showed pathologic complete response of 25% in those treated with the upfront modified FOLFOX-6 compared to 18% in those who proceeded to surgery directly, but this result did not reach statistical significance^[22]. Memorial Sloan-Kettering Cancer Center (MSKCC) reported on its institutional experience offering upfront FOLFOX for patients with high-risk locally advanced rectal cancer. Sixty-one patients received induction FOLFOX (median 7 cycles), and of the 49 patients who proceeded to TME all had R0 resections and 47% had greater than 90% tumor response, with 27% pathologic complete responses^[23]. Despite the encouraging results of this trial, it should be interpreted with caution given the small sample size, the retrospective nature of the analyses, and the lack of randomization.

Several non-randomized studies have been conducted to investigate the use of neoadjuvant chemotherapy. Calvo *et al.*^[24] reported on patients with locally advanced rectal cancer treated with two cycles of induction FOLFOX-4 (oxaliplatin 85 mg/m² D1, 5-FU 400 mg/m² IV bolus and 600 mg/m² IV continuous in 22 h on D1 and D2, folinic acid 200 mg/d IV D1 and D2) followed by immediate CRT. Compared to patients treated with preoperative CRT alone, there was no significant difference in toxicities and patients treated with the induction chemotherapy had significantly more ypT0 (29% vs 8%, $P = 0.006$)^[24]. Chau *et al.*^[25] reported on 77 consecutive patients with MRI-defined poor-risk locally advanced rectal cancer treated with 12 wk of neoadjuvant chemotherapy (CAPOX: Oxaliplatin 130 mg/m² every 3 wk with capecitabine 2000 mg/m² 14 d on with 7 d off) followed by CRT with TME 6 wk after completion of CRT. All but one of the patients had R0 resections, pCR was seen in 16 patients, and an additional 32 patients had only microscopic tumor foci^[25]. Schou *et al.*^[26] report on 84 consecutive patients with locally advanced rectal cancer treated with two cycles of CAPOX before CRT followed by TME 6 wk later. An R0 resection was seen in 94% of patients, a pCR was seen in 23% of patients, and T downstaging occurred in

69%^[26]. Additional phase II studies examining the benefit of neoadjuvant chemotherapy have been reported. Marechal *et al.*^[27] reported on a phase II study of patients with T2-T4/N+ locally advanced rectal cancer who were randomly assigned to preoperative CRT (continuous infusion fluorouracil) or to oxaliplatin, folinic acid, and 5-FU followed by CRT and surgery. There was no difference in rate of patients with ypT0-1N0 (32.1% for patients with treated with neoadjuvant chemotherapy vs 34.5% for patients treated with CRT alone, $P = 0.85$), and there was no difference in pCR between the two groups. The patients treated with induction chemotherapy had significantly more grade 3-4 toxicities than those treated with CRT (35% vs 7%, $P = 0.017$). The results of these non-randomized studies show improved local control with neoadjuvant chemotherapy as well as good tolerance.

CAN RADIATION BE OMITTED?

Given potential improved pathological and clinical responses and good treatment tolerance with the incorporation of neoadjuvant systemic chemotherapy, some have questioned whether radiation can be omitted completely. The GEMCAD 0801 phase II trial included 46 patients with resectable T3N0-2 rectal adenocarcinoma and treated with four cycles of neoadjuvant capecitabine and oxaliplatin with bevacizumab (for the first 3 cycles) before TME. Pathologic CR occurred in 20% and T downstaging was observed in 48% of patients. It was noted that there was a 13% rate of anastomotic leak, which is higher than expected, and could be attributed to bevacizumab^[28]. Another phase II trial that was conducted at MSKCC administered six cycles of FOLFOX with bevacizumab for cycles 1-4 to newly diagnosed stage II-III rectal cancer. Patients with progression were treated with CRT followed by TME, and those that had stable disease or better proceeded directly to TME. All patients except 2 underwent surgery and they all had R0 resections with a 25% pCR rate, with no local recurrence at 4 years^[29]. Based on these results, the PROSPECT (Preoperative Radiation or Selective Preoperative Radiation and Evaluation Before Chemotherapy and TME) trial was launched^[30]. The PROSPECT trial is a phase II/III randomized trial designed to address whether preoperative radiation therapy can be used more selectively in locally advanced rectal cancer (Figure 1). Patients with T2N1 or T3N0-1 rectal cancer are randomized to either standard CRT or to six cycles of FOLFOX. Patients on the investigational arm undergo repeat staging prior to surgery, and those who fail to have a response of at least 20% per RECIST criteria proceed to treatment with CRT prior to surgery. Adjuvant chemotherapy with FOLFOX is suggested but not required. The outcomes of this study may change the paradigm of rectal cancer neoadjuvant treatment.

NON-OPERATIVE APPROACH

Neoadjuvant CRT results in significant downstaging of

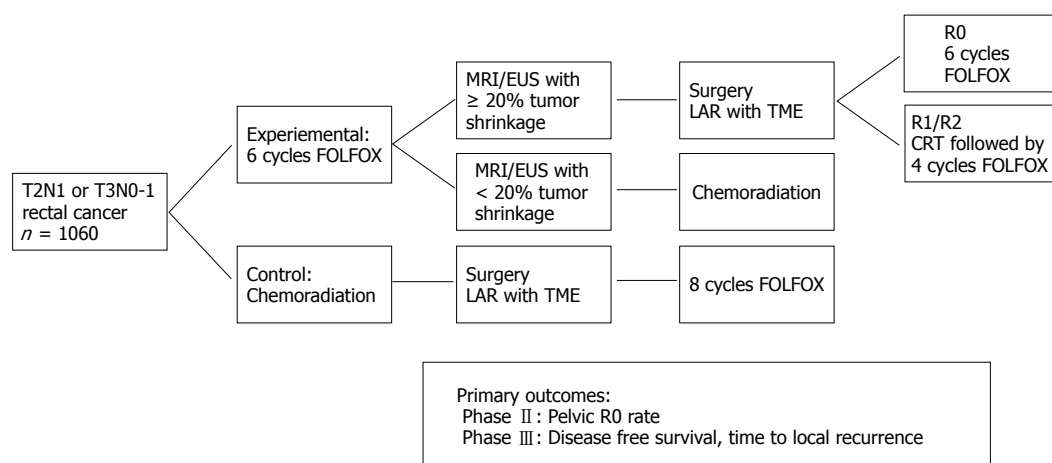


Figure 1 Preoperative radiation or selective preoperative radiation and evaluation before chemotherapy and total mesorectal excision trial investigating rectal cancer treatment without radiation. TME: Total mesorectal excision; MRI: Magnetic resonance imaging. Adapted from Clinicaltrials.gov.

locally advanced rectal cancer, and 15%-27% of patients will have a pathologic complete response (pCR)^[31]. The main question is whether patients who achieve pCR could be monitored rather than operated on in order to avoid the post-surgical comorbidities. Complete pathological response was found to correlate with 5-year DFS in a meta-analysis of patients with locally advanced rectal cancer treated with preoperative CRT (5 year DFS for patients with a pCR was 83.3% compared to 65.6% for those without pCR, $P < 0.0001$)^[31]. For those patients with a pCR to neoadjuvant treatment, a nonoperative approach has been investigated in several case series^[32-34]. Habr-Gama *et al.*^[33-35] reported on patients with clinical T2-4, N0-1 rectal cancer treated with CRT followed by assessment for clinical response 8 wk later. In this trial, 26.8% of the patients had a complete clinical response (cCR) based on exam, imaging, and endoscopy (including biopsies) and were subsequently monitored expectantly. The first year of surveillance included monthly physical exam, digital rectal exam, proctoscopy, and serum CEA levels as well as CT scans of the abdomen and pelvis every six months. Follow up visits were spaced to every two months for the second year and to every six months for the third year. Two patients developed local recurrence 56 and 65 mo after completion of CRT and were treated successfully with transanal full-thickness excision and salvage brachytherapy, respectively. Three patients developed systemic metastases for an overall recurrence rate of 7%, and no cancer related deaths occurred with a median follow up of 57.3 mo^[33]. Subsequent publications from this group showed similar results^[34]. A large phase II trial by MSKCC (Figure 2) is underway to further investigate the efficacy and safety of a non-operative approach. Patients with T3-4N0-1 locally advanced rectal cancer by MRI all receive neoadjuvant CRT and 4 mo of chemotherapy (CAPOX or FOLFOX), with randomization of the sequence of receipt. Patients with a cCR or near-cCR on follow-up examination will proceed to nonoperative expectant

management every 3 mo for two years and every 6 mo thereafter^[36,37]. This investigated approach may save patients the comorbidities associated with surgical intervention. However, if this approach is proven to be effective there needs to be a standard approach to surveillance. A small study reported data on the utility of combining clinical assessment (DRE and endoscopy) with T2W-MRI and diffusion-weighted MRI as having a 98% posttest probability for a cCR^[38].

Clinical downstaging from neoadjuvant therapy has instigated interest in other surgical management options; though TME has significantly improved outcomes, it is associated with considerable morbidity, including long term consequences such as fecal incontinence, urinary dysfunction, sexual dysfunction, and permanent ostomy^[39]. These issues can significantly impair patients' quality of life. Case series and retrospective data have indicated that carefully selected patients may have good oncologic outcomes from less aggressive surgical procedures^[40,41]. A prospective study of 10 patients with T3 rectal cancer who were deemed unfit for a radical surgical excision with TME or refused large surgery, were treated with CRT followed by transanal excision with no recurrence at 24 mo follow up^[42]. Another small but prospective and randomized study compared 100 patients with cT2N0 rectal cancer treated with CRT and randomized to either TME or endoluminal locoregional resection by transanal endoscopic microsurgery. There was no difference in local recurrence or metastatic recurrence, but these findings are limited by the small number of patients and that the patients were early stage and not being the definition of locally advanced disease^[43]. More recent evidence comes from a non-randomized, phase II trial of 79 patients with cT2N0 distal rectal cancer treated with neoadjuvant CRT followed by local excision. The primary endpoint, 3-year DFS, was 88.2%, and eight (10%) patients had recurrence (5 distant and 3 local)^[44]. This study shows that patients with cT2N0 distal rectal cancer may be treated with local excision, but is limited by lack of comparative results.

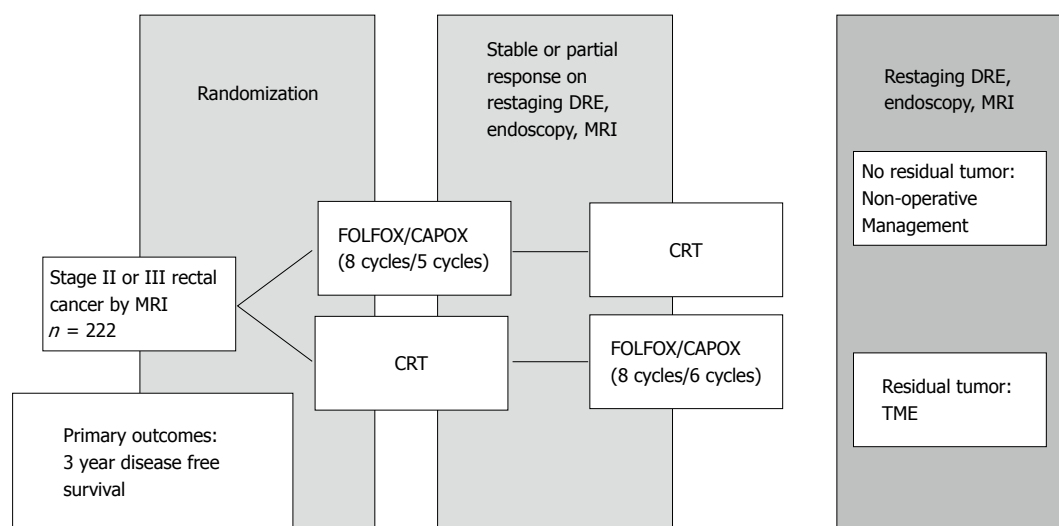


Figure 2 Memorial Sloan-Kettering Cancer Center phase II study of non-operative approach. TME: Total mesorectal excision; MRI: Magnetic resonance imaging. Adapted from Clinicaltrials.gov.

Local excision is an option for patients who cannot tolerate a major surgery, but it remains uncertain if this approach results in similar oncologic outcomes particularly in locally advanced stages of disease.

CONCLUSION

Despite considerable advances in rectal cancer treatment over the last two decades, controversies remain. The current standard is to treat patients with preoperative radiation, but the optimal course (CRT or 5×5 Gy) is not known. Studies suggest that the addition of oxaliplatin to CRT does not provide significant additional benefit and adds toxicity. As new approaches to chemotherapy are investigated, the inclusion of RT is being questioned. However, the optimal approach and timing for systemic chemotherapy in locally advanced rectal cancer has not been answered. Even the role of surgery, the traditional sine qua non of rectal cancer therapy, is being questioned, with non-operative approaches to management demonstrating excellent outcomes without the consequences of surgery.

Further refinements in approach may rely on the identification of predictive biomarkers in this disease. Advances in genomic and molecular tumor profiling have facilitated this search. Biopsy specimens from 52 patients with rectal cancer planned for preoperative radiotherapy were submitted to gene expression profiling using DNA microarray analysis (U95Av2 Gene Chip). The profiles of radiation responders and non-responders were compared, and thirty-three novel genes were identified with differential expression; these genes could predict response with an accuracy of 88.6%^[45]. Similar studies have been conducted to predict response to CRT^[46-49]. DNA microanalysis has identified genes predictive of response, but no genes were reported consistently across the studies. Nishioka *et al.*^[48] attempted to identify a candidate gene using immunohistochemistry (IHC) to

validate the DNA microarray results. Among 20 patient biopsies, 17 potentially predictive genes were identified. Matrix metalloproteinase-7 (MMP7) was identified as the gene with the largest difference between responders and non-responders and was analyzed with IHC. MMP7 was found to be overexpressed by IHC in 4 out of 10 responders and 0 out of 7 non-responders. Watanabe *et al.*^[49] used DNA microanalysis to identify four genes (LRRIQ3, FRMD3, SAMD5, and TMC7) expressed in responders to CRT with 89.1% prediction accuracy and validated the gene expression by RT-PCR analyses. While these results are intriguing, widespread clinical application remains limited by lack of reproducibility and the need for independent validation across separate cohorts.

Another area of investigation is the tumor micro-environment and how it interacts with the immune system. Immunologic features of tumor microenvironment may predict tumor response to therapy as well as form the basis for innovative treatment strategies. Studies of colorectal cancer showed that the patterns of immune cell infiltration within tumors had prognostic significance and lead to the development of the Immune Score^[50-52]. The densities of CD45RO⁺ memory T-cells and CD8⁺ cytotoxic T-cells tumor infiltrating lymphocytes (TILs) were determined for patients with stage I and II colorectal cancer. Higher densities at both the center of the tumor and the invasive margin were predictive of superior patient outcomes including disease free, disease specific, and overall survival ($P < 0.001$)^[52]. Not only does the number of CD8⁺ TILs correlate with survival, but it has been shown to have superior prognostication than the TNM staging^[53]. Although it is evident that colorectal tumors are infiltrated by the immune cells, mainly T-cells, this infiltration is not sufficient to induce tumor response. This may be due in part to the immune suppression provided by the activation of immune checkpoints, such as PD-L1/PD-1 pathway. Examination of colorectal tumors showed

PD-L1 expression in 37% of mismatch repair (MMR)-proficient tumors and in 29% of MMR-deficient tumors, with a strong correlation between PD-L1 expression and infiltration with CD8⁺ T-cells in the MMR-proficient tumors ($P = 0.0001$)^[54]. However, treatment with PD-L1 monoclonal antibody nivolumab showed no objective responses in 18 patients with colorectal cancer^[55]. More recent data revealed that high microsatellite instable tumors selectively exhibit upregulated expression of immune checkpoints including those being studied as drug targets: PD-1, PD-L1, CTLA4, LAG-3, and IDO^[56]. Accordingly, microsatellite stability may be a biomarker for immunotherapy in colorectal cancer, given the fact that tumors with microsatellite instabilities tend to have higher mutational loads leading to a better recognition by the immune system. Another area of active investigation is combination therapy of immunotherapy and CRT. Based on preliminary data showing that neoadjuvant CRT can lead to increase T-cells in rectal cancer^[57], we are currently in the process of conducting a phase II study investigating neoadjuvant CRT and pembrolizumab (anti-PD-1) in patients with stage II and III rectal cancer cancer^[58]. Furthermore, novel clinical trial designs need to be implemented in the neoadjuvant setting in colorectal cancer. The National Cancer Institute is planning to launch a large clinical trial to evaluate 3-year DFS in patients managed with total neoadjuvant therapy and TME or non-operative management, compared with standard historical controls managed according to standard of care (CRT and TME followed by adjuvant chemotherapy). The historical control arm continues to enroll patients while the adaptive trial design allows the addition of other investigational arms. This novel trial design and others will bring more advances to the field that may change the paradigm of rectal cancer treatment.

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Mucins in neoplasms of pancreas, ampulla of Vater and biliary system

Dimitrios Moschovis, Giorgos Bamias, Ioanna Delladetsima

Dimitrios Moschovis, Department of Gastroenterology, Agios Panteleimon General Hospital, 18453 Nikea, Greece

Giorgos Bamias, Academic Department of Gastroenterology, Medical School, National and Kapodistrian University of Athens, Laikon Hospital, 11527 Athens, Greece

Ioanna Delladetsima, First Department of Pathology, Medical School, National and Kapodistrian University of Athens, 11527 Athens, Greece

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Correspondence to: Ioanna Delladetsima, Associate Professor, First Department of Pathology, Medical School, National and Kapodistrian University of Athens, 75 Mikras Asias Street, Goudi, 11527 Athens, Greece. jokadelladetsima@hotmail.com
Telephone: +30-210-7456485
Fax: +30-213-2061794

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Abstract

Tumors of the pancreas, the ampulla of Vater, and the extrahepatic and intrahepatic bile ducts have significant histological similarities due to the common embryonic origin of the pancreatobiliary system. This obviates the need for discovery of biomarkers with diagnostic and prognostic value for these tumors. Mucins, especially MUC-1, -2, -4 and -5AC, are important candidates for developing into such reliable biomarkers. Increased expression of MUC1 occurs in pancreatic ductal adenocarcinomas and is associated with increased degrees of dysplasia in pancreatic intraepithelial neoplasia (PanIN). Positive expression of MUC2 in intraductal papillary mucinous neoplasms (IPMN) of the intestinal type indicates high potential progression to invasive carcinoma with *de novo* expression of MUC1, while absence of MUC2 expression in IPMNs of gastric type implies low potential to malignant evolution. *De novo* MUC4 expression correlates to the severity of dysplasia in PanIN and is associated with a poor prognosis in patients with pancreatic ductal adenocarcinomas. In biliary intraepithelial neoplasia (BilIN), increased expression of MUC1 is associated with higher degrees of dysplasia. Intrahepatic cholangiocarcinomas (ICC) are characterized by increased expression of all glycoforms of MUC1. Positive MUC2 expression in intraductal papillary neoplasm of the bile ducts (IPNB) of the intestinal type indicates high malignant potential with *de novo* expression of MUC1 in the invasive element. Absent MUC2 expression in any degree of BilIN may prove useful in differentiating them from IPNB. *De novo* expression of MUC4 is associated with poor prognosis in patients with ICC or carcinoma of the extrahepatic bile ducts (EHBDC). High *de novo* expression of MUC5AC is found in all degrees of BilIN and all types of IPNB and ICC. The MUC5AC is useful in the detection of neoplastic lesions of the bile duct at an early stage. Increased expression of mucin MUC1 in carcinoma of the ampulla of Vater associated with unfavorable behavior of the tumor, such as lymph node metastasis, infiltration of the pancreas and duodenum, advanced TNM classification and worse prognosis. Patients with

intra-ampullary papillary-tubular neoplasm (IAPN) of the pancreatobiliary immunophenotype did not show MUC2, while those of the intestinal immunophenotype are MUC2 positive. The expression of MUC4 is associated with poor prognosis in patients with carcinoma of the ampulla of Vater favoring metastasis and making them resistant to apoptosis. Moreover, it appears that MUC4 positivity correlates with recurrence of the tumor. Expression of MUC5AC is associated with the invasive potential of the tumor.

Key words: Ampulla Vater neoplasms; Biliary system neoplasms; Mucins; Pancreatic neoplasms

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Core tip: The combined status of mucin expression may be useful in the differential diagnosis of pancreatobiliary neoplasms, the detection of pre-malignant lesions and the evaluation of their malignant behavior. Besides their diagnostic and prognostic role, their involvement in carcinogenesis reveals their importance as putative therapeutic targets in the future.

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INTRODUCTION

All mucosal surfaces of the human body are covered by a thick viscous layer which protects them from external insults. A major component of this defensive barrier is a class of epithelial-derived glycoproteins known as "mucins". The structural signature of a mucin is a dense network of oligosaccharide side chains comprising of N-acetyl-galactosamine, which bind *via* an O-glycosidic linkage to specific amino acid residues (serine, threonine and proline), that occur in repetitive short stretches in the protein backbone termed "tandem repeats"^[1,2]. The side chains make up to 50%-90% of the weight of a mucin. Mucins are distinguished from proteoglycans (another major class of glycoproteins) by the lack of uronic acid and xylose and the presence of the aforementioned O-glycosidic links.

Genes encoding for the protein backbone of mucins are denoted by the three letter code "MUC", followed by a number (MUC1-21), which corresponds to the chronological order of discovery^[3]. Mucins are classified according to their structure and function as either "membrane bound" or "secreted"^[4]. The first class includes mucins MUC1, MUC3, MUC4, MUC12, MUC13, MUC16, MUC17, and MUC21. These mucins possess a large N-terminal extracellular portion, a transmembrane part

and a small C-terminal cytoplasmic one. The cytoplasmic segment is important for signal transduction, as it contains several phosphorylation sites that are important for interaction with other scaffolding and signaling proteins. The extracellular portion of mucins includes several specific domains of unknown function, which include epidermal growth factor (EGF)-like, AMOP (adhesion associated domain in MUC4 and other proteins), VWD (Factor Von Willebrand), SEA (sea-urchin sperm protein enterokinase and agrin) and NIDO (Nidogen-like)^[5]. Another unique feature of the membrane bound mucins is the existence of multiple isoforms with modified structure and function, which are generated by alternative splicing^[4].

Secreted or gel-forming mucins are encoded by genes located contiguously on chromosome 11 and include MUC2, MUC5AC, MUC5B, MUC6 and MUC7. This genetic clustering indicates a close functional association of the relevant proteins. Secreted mucins also have "tandem repeats", the number of which is a unique feature with potential diagnostic, prognostic and possibly therapeutic significance^[6]. Two unique domains that characterize secreted mucins are the VWD domain and the cysteine knot (CK) motif^[7]. The first appears to be important for connecting the mucins into oligomeric structures, while the second for the formation of mucin homo- or heterodimers.

EXPRESSION OF MUCINS IN NORMAL PANCREATOBILIARY SYSTEM

The mucin profile in normal pancreatobiliary system has been identified mostly through immunostaining studies with the use of mucin-specific antibodies. However, it should be noted, that different antibodies have been used across separate studies, leading to heterogeneous expression patterns. A typical example is the work of Yamashita *et al*^[8] who reported that immunoexpression of MUC1 in normal bile duct was dissimilar between three separate MAbs (DF3, MUSE11 and 139H2), that were tested. In particular, MUC1/139H2 was expressed in normal bile ducts of any size at the luminal surface of the biliary cells and/or in the cytoplasm. MUC1/MUSE11 was detected only in small-sized ducts (S < 100 μm), predominantly in the apical surface of the cells, while MUC1/DF3 was undetectable in normal biliary epithelium. In the same study, MUC2 staining in normal bile ducts also varied and was dependent upon the use of the specific antibody (polyclonal anti-MUC2/MRP or monoclonal CCP58 antibody). MUC2/anti-MRP was not expressed in normal bile duct epithelium, whereas MUC2/CCP58 exhibited supranuclear expression in 60% of the cases^[9]. Finally, MUC4 was undetectable in normal bile ducts, while MUC5AC immunostaining was present in a small number of cases^[9].

In another study, it was shown that in normal pancreatic tissue, MUC1 and all of its glycoforms were expressed at the apices of centroacinar cells, intercalated and intralobular ducts, and focally in the interlobular pancreatic ducts. MUC1 and MUC1 glycoforms were

Table 1 Expression of various glycoforms of mucin 1 in normal pancreatic tissue

Cells/structure	MUC1/ CORE	MUC1/ DF3	MUC1/ MY.1E12	MUC1/ HMFG1
Central areas of acini	-/+	-/+	++	++
Intercalated ducts	+	+	+	+
Intralobular ducts	+	+	+	-/+
Interlobular ducts	-	-/+	-/+	-
Main pancreatic ducts	-	-	-	-

MUC: Mucins.

undetectable in the main pancreatic duct^[10]. In the same study there was no detectable pancreatic expression of MUC2, MUC4, and MUC5AC. The expression of various glycoforms of MUC1 (MUC1/CORE, MUC1/DF3, sialylated MUC1/MY.1E12 and fully glycosylated MUC1/HMFG-1) in normal pancreatic tissue, is summarized in Table 1^[11].

EXPRESSION OF MUCINS IN PANCREATOBILIARY NEOPLASMS

Pancreatic carcinogenesis develops through the accumulation of several genetic and epigenetic lesions, some of which affect genes encoding for mucins. It has been shown that certain mucins are expressed *de novo* during neoplastic transformation of the epithelium, while specific mucin patterns have been recognized in different pre-malignant and malignant neoplasms of ductal origin. In recent years, mucins have gained significant value for the diagnostic approach to pancreatic neoplasms. At the same time, their high specificity has rendered them potential candidates for therapeutic interventions^[12]. Finally, mucin expression patterns have become a major criterion for the subclassification of intraductal papillary mucinous neoplasms (IPMN).

According to the WHO classification of 2010, IPMNs are histologically classified as pancreatobiliary, intestinal, gastric or oncocytic type^[13]. Distinction of IPMN subtypes is very important from the clinical perspective, because they demonstrate differences in their malignant potential. IPMN of pancreatobiliary type with MUC1 expression and intestinal type with MUC2 expression are located mainly in the main pancreatic duct and exhibit a high frequency of carcinoma development^[11]. In contrast, IPMN of gastric type with MUC5A expression is usually located in the pancreatic branch ducts and rarely shows malignant transformation. These findings are consistent with the clinicopathological description of the "International guideline for management of IPMN/MCN". According to this classification, IPMNs are classified as "IPMN-main duct type" and "IPMN-branch duct type"^[14].

A similar subclassification based on the aforementioned parameters (cell type, mucin profile and prognostic data), has been introduced for biliary and ampullary neoplasias. According to the WHO classification of 2010, intraductal papillary neoplasm of the bile ducts (IPNB)

are classified as pancreatobiliary, intestinal and gastric type, while the oncocytic type is referred as a variant of the pancreatobiliary^[15]. The most frequent histopathologic types are pancreatobiliary and intestinal.

MUC1

In 1993 it was reported for the first time, that pancreatic ductal adenocarcinomas (PDAC) with aggressive biological behavior usually express MUC1^[16]. Furthermore, the pattern of MUC1 expression in PDAC is different from that in the normal pancreas. In well differentiated PDAC, MUC1 is detected not only along the luminal cell surface of glandular formations, but also in the baso-lateral surface. In poorly differentiated PDAC, cytoplasmic expression of MUC1 is demonstrated^[17]. In addition, PDAC show high expression rates for MUC1 glycoforms (MUC1/CORE 66%, MUC1/DF3 96%, MUC1/MY.1E12 98%, MUC1/HMFG1 76%)^[18]. Regarding pancreatic intraepithelial neoplasia (PanIN), the expression profile of MUC1 is associated with the grade of dysplasia^[19]. Indeed, increase of MUC1 expression has been correlated with increase of PanIN grade^[20].

MUC1 is exclusively associated with pancreatobiliary type IPMN and its malignant variant^[13,21]. Moreover, underglycosylated MUC1 is absent or rarely seen in IPMN-intestinal type and IPMN-gastric type. On the other hand, glycosylated MUC1 is undetectable or occasionally detectable in IPMN-intestinal type, but is frequently observed in IPMN-gastric type^[11]. The invasive carcinomas, which develop in intestinal type IPMN, show *de novo* MUC1 expression^[20,22].

There are only few references to the expression of MUC1 in mucinous cystic neoplasms (MCN), oftentimes with conflicting results. In one study, MUC1 appeared to be rarely expressed in MCN^[23], while in another the majority of MCN was found positive for MUC1/DF3^[24]. On the other hand, MUC1 expression is detectable in up to 90% of intraductal tubulopapillary neoplasms (ITPN)^[25].

Regarding the mucin expression in neoplasms of the biliary system, MUC1 profile in biliary intraepithelial neoplasia (BiIN) was similar to that in PanIN, characterized by progressive increase with increasing severity of dysplasia^[26]. In the intraductal papillary neoplasm of the bile ducts (IPNB) of pancreatobiliary type, MUC1 expression rates were high in invasive, but low in non-invasive lesions^[9]. On the other hand, IPNB-intestinal type showed *de novo* expression of MUC1, particularly in cases with frequent invasive growth and significant poorer survival^[9].

Intrahepatic cholangiocarcinoma (ICC) with aggressive biological behavior and poor outcome usually presents a MUC1+/MUC2- profile, while bile duct cystadenocarcinoma (BDCC) with moderate aggressiveness and favorable prognosis demonstrates a MUC1-/MUC2+ pattern^[8]. The frequency of expression of MUC1 in ICC was significantly higher than that in BDCC^[27]. In general, positive staining for MUC1 has been associated with poor prognosis, regardless of the glycosylation status, in both ICC and

BDCC^[27].

The expression pattern of MUC1 in tumors of the ampulla of Vater appears to have predictive value based on results of recent studies^[28,29]. In particular, MUC1 positivity was associated with unfavorable behavior of the tumor, such as lymph node metastasis, vascular invasion, infiltration of the pancreas and duodenum, advanced TNM classification and worse prognosis^[29].

Utility of MUC1 as biomarker in pancreatobiliary neoplasia:

A strong association has been observed between elevated expression of MUC1 in pancreatic neoplasms and decreased survival. The negative predictive value was high (95%), as 19 out of 20 patients with no MUC1 expression survived more than 30 mo^[30]. Out of 13 candidate genes, only those of MUC1, mesothelin (MSLN), and MUC2 showed statistically significant differences in the expression pattern between the groups with aggressive and less aggressive carcinomas^[31]. Mesothelin/MSLN is a protein that is present on normal mesothelial cells and is overexpressed in mesothelioma and ovarian and pancreatic adenocarcinoma. Both MSLN and MUC1 appeared to be strong predictors of survival thus acquiring prognostic significance. In fact, the prognostic significance of MUC1 positivity exceeded that of conventional pathological features^[31]. Accordingly, the National Cancer Institute has identified MUC1 and MSLN among the most promising targets for cancer vaccine development^[32]. A radiolabeled monoclonal antibody against MUC1 was investigated recently in a phase I / II trial, and phase III study will follow^[33]. Finally, promoter-driven gene therapy, which exploits overactive MUC1 and MSLN promoters in various cancer types has been extensively studied in pre-clinical cancer models using viral vectors^[34].

Much of the oncogenic role of MUC1 may be attributed to the participation of the small cytoplasmic tail of MUC1 (MUC1.CT) in signal transduction and transcriptional events^[35]. MUC1.CT regulates the recruitment and activity of transcription factors, thereby regulating transcription of the corresponding genes^[36]. Other studies have implicated MUC1 in tumour growth, invasion and metastasis in pancreatic cancer^[37]. MUC1 participates in the regulation of pancreatic cancer cell metabolism^[38]. It was shown, that MUC1 physically occupies the promoter regions of genes involved in glycolysis and glucose metabolism in pancreatic cancer cells. The MUC1 selectively enhances the transcription of some of the glycolytic genes and this effect is more pronounced under conditions of hypoxia in a HIF-dependent manner. Furthermore, MUC1 expression is associated *in vivo* with increased glucose uptake in xenograft model of orthotopic implantation of pancreatic cancer. Increased tumour cell metabolism has been identified as a hallmark of cancer requirements for rapid cell growth and is consistent with previous studies which consider MUC1 as a modulator of growth and invasive properties in multiple cancer types^[39].

An association of MUC1 with Platelet-Derived Growth Factor-A (PDGF-A) has also been reported^[38]. PDGF-A

is one of the several regulators of tumor growth, angiogenesis and metastasis in pancreatic carcinoma. MUC1 regulates the expression and secretion of PDGF-A. In particular, the increase of MUC1 expression induces expression of PDGF-A in multiple human and mouse cell lines *in vitro* and *in vivo*, as well as in pancreatic cancer models with or without expression of MUC1. Both PDGF-A and MUC1 are considered unfavorable prognostic markers and potential targets for therapeutic intervention in pancreatic cancer. Moreover, expression of hypoxia-inducible factor 1- α (HIF1- α) as a marker of pancreatic cancer progression correlates with the expression of PDGFA and with poor prognosis^[40]. Therefore, it is hypothesized, that MUC1 promotes nuclear translocation of this transcription factor, which is a known regulator of PDGFA^[41].

MUC2

MUC2 is not expressed in PDAC^[16]. This was originally reported in 1993^[17] and was later confirmed by immunohistochemistry^[18]. Similarly, MUC2 was not detected in PanIN independently of the grade of dysplasia^[19]. In contrast, MUC2 expression has been reported in IPMNs. More specifically IPMN-intestinal type, that expresses MUC2, is primarily located at the main pancreatic duct and shows high frequencies of malignant transformation and invasive carcinoma (usually mucinous/colloid carcinoma)^[11]. On the other hand, MUC2 has not been detected in MCN^[23,24].

Regarding expression in biliary neoplasms, in an important study by Zen *et al.*^[26], MUC2 expression was absent in all BilIN, while it was expressed in the majority of IPNB. In a more recent study, it was also reported that MUC2 was detectable in 50% of IPNB^[15], although quantitative data on the relation to specific subtypes are missing. This was however addressed in another study, in which MUC2 expression was observed in 95% of IPNB intestinal type and in 50% of IPNB pancreatobiliary type^[9]. Expression of MUC2 in extrahepatic bile ducts carcinoma (EHBDC) was related to tumor progression and the expression of the mucins was associated with poor prognosis^[42]. ICC with aggressive biological behavior and poor outcome usually demonstrates a MUC2 negative profile while BDCC with moderate aggressiveness and favorable prognosis exhibits MUC2 positive profile^[8,26]. Overall, MUC2 expression is considered to be a favorable prognostic marker in neoplastic lesions of the bile ducts^[26,27]. Regarding the carcinoma of ampulla of Vater, expression of MUC2 is associated with non-invasive pancreatobiliary papillary neoplasia of intestinal subtype which is more frequently associated with invasive neoplasia^[43]. In a study of 105 adenocarcinomas of the ampulla of Vater, intestinal immunophenotype was characterized by MUC2 and caudal-related homeobox transcription factor-2 (CDX2) positivity, while pancreatobiliary immunophenotype was characterized by MUC2 and CDX2 negative expression^[43].

MUC4

Recently, it was found that high expression of MUC4 was an independent poor prognostic factor in adenocarcinomas of the pancreas and bile ducts^[9]. In a study of 135 PDAC, MUC4 was expressed in 32% of the cases while intense expression was associated with significantly poorer survival^[29]. In the studies of Swartz *et al* and Park *et al* MUC4 was detected in 89% and 79% of PDAC respectively^[44,45]. These discrepancies may be ascribed to the application of a lower threshold-value for MUC4 positivity in the latter two studies. There are no current data available regarding the expression of MUC4 in IPMN and MCN. In sharp contrast, PanIN is associated with *de novo* expression of MUC4. Moreover, MUC4 expression intensity increases in parallel with the degree of dysplasia^[44,45].

MUC4 is also expressed in neoplasms of the biliary system. In one study MUC4 was expressed in 63% of IPNB. The MUC4 showed more frequent expression in IPNB-intestinal relative to IPNB-pancreatobiliary type^[9]. In intrahepatic and in extrahepatic cholangiocarcinoma MUC4 is expressed *de novo* mainly in the cytoplasm of cancer cells and is correlated with poor prognosis. The survival rate of patients with MUC4 positivity is significantly poorer compared to patients with negative MUC4 expression^[9,46]. There is no information concerning MUC4 expression in BDCC.

MUC4 is less frequently expressed in the normal epithelium of the ampulla and is an independent marker of the pancreatobiliary type adenocarcinoma of the ampulla^[28] associated with poor prognosis and recurrence of the tumor^[28,47].

Utility of MUC4 as biomarker in pancreatobiliary neoplasia: MUC4 expression is associated with poor prognosis in patients with pancreatobiliary neoplasia. There has been a strong correlation between the expression of MUC4 and carcinoma recurrence^[28,47]. In a retrospective study of biliary tract carcinoma in 2006, it was observed that patients with positive MUC4 in bile had reduced survival (5.0 mo vs 11.5 mo)^[46], while a similar difference in survival rate has been reported in a Japanese population, which underwent surgery for extrahepatic biliary tract carcinoma^[46].

MUC4 is a membrane associated mucin which inhibits intercellular adhesion and adhesion between cells and stroma favoring extravasation and metastasis^[48,49]. It has also been implicated as promoter in infiltrative growth and metastasis of pancreatic cancer by facilitating cancer cell motility^[50-52]. In various *in vitro* and *in vivo* studies, selective suppression of MUC4 expression highlighted its role in cell adhesion and epithelial mesenchymal transition^[53] and in restriction of cancer cell growth and metastasis^[54]. In pancreatic tumor cells selective downregulation of MUC4 led to suppression of pERK1/2 and increased expression of E-cadherin. These findings suggest that MUC4 inhibits the function of E-cadherin as well as of N-cadherin and EGFR1 by activation of Akt and

JNK/2 signaling pathways, respectively^[55]. Decreased expression of matrix metalloproteinase-9 (MMP-9) has also been identified after downregulation of MUC4^[56]. Inversely, activation of ERK1/2 signaling pathway *via* MUC4, activates MMP-9, which in turn causes degradation of E-cadherin resulting in alteration of intercellular contacts. These data demonstrate that MUC4-mediated repression of E-cadherin may enhance the invasive capacity of tumor cells. Suppression of MUC4 is also correlated with changes in the shape of cancer cells indicating its involvement in epithelial-mesenchymal transition^[56,57]. Finally, cells expressing MUC4 are resistant to immune mediated apoptotic cell death by natural killer cells (NK)^[58]. Mucins, including MUC4, which are connected with cell membrane, have become a point of interest as they act as endogenous ligands and modulators of the tyrosine kinase receptor ErbB2^[59], which is part of an antiapoptotic pathway utilized by most common epithelial tumors^[60]. The therapeutic blockage of ErbB2 with the monoclonal antibody trastuzumab improves the effectiveness of chemotherapy and survival in breast cancer^[61]. In breast carcinoma cells, MUC4 has anti-aggregatory action and appears to promote tumor growth^[62]. These oncogenic properties of MUC4 point out its significance as a potential therapeutic target.

MUC5AC

High *de novo* expression of MUC5AC has been observed in many types of pancreatobiliary neoplasms including all grades of PanIN^[19,22] and BilIN^[26], PDAC^[11], ICC^[15], as well as all types of IPMN^[11] and IPMB^[9]. In particular, IPMN of gastric type with MUC5AC expression is usually located in the pancreatic branch ducts, and rarely shows malignant transformation^[14]. Since MUC5AC is not expressed in normal pancreatobiliary epithelium^[26], it seems to be a specific marker of early neoplastic lesions of the pancreatobiliary system^[11,26] which correlates with poor prognosis^[63]. Diverse expression rates (37.5% vs 100%) have been reported in MCN and are attributed to the use of different antibodies^[23,24].

MUC5AC expression was frequent in both BilIN and IPNB^[26] and showed high expression in both IPNB-intestinal and IPNB-pancreatobiliary type^[9,15]. In a study in Northern Thailand, a region with high incidence of BTC (biliary tract cancer), MUC5AC protein was detected in the serum of 63% of the patients with BTC, whereas it was absent in healthy controls^[64]. In the same study in benign biliary epithelium MUC4 and MUC5AC were undetectable. There are no literature data for MUC5AC expression in BDCC.

Expression of MUC5AC in neoplasias of the ampulla of Vater depends on the histological subtype. Positive expression of MUC5AC is related to the pancreatobiliary phenotype and participates in subsequent stages of carcinoma extension such as invasion and metastasis^[65,66]. In intestinal type carcinomas a weak expression of the neoplastic epithelium was observed. In very few cases it was also detected in the center of the tumor, while in

lymph node metastases and vascular infiltrates it was mostly absent^[67].

Utility of MUC5AC as biomarker in pancreatobiliary neoplasia: In a study from Thailand^[63], detection of MUC5AC in cholangiocarcinomas was associated with larger-sized tumors (> 5 cm), and advanced-stage of disease. Patients who had positive serum MUC5AC status had a significantly poorer prognosis compared to patients with undetectable serum MUC5AC ($P < 0.001$). Multivariate analysis showed that patients with positive serum MUC5AC status had a 2.5-fold higher risk of death compared to patients who had negative serum MUC5AC status ($P < 0.001$). Moreover, the diagnostic specificity of MUC4 and MUC5AC for BTC was found superior of CA19-9 (93% MUC4, 96% MUC5AC vs 65% CA19-9)^[68]. This high specificity, however, was weakened by the comparatively low sensitivity (27% MUC4 and 44% MUC5AC), which increased to 58% by combining MUC4 and MUC5AC^[68].

MUC5AC has been shown to be a major component of gastric and bronchial mucous, while in other normal tissues the expression is low or absent. MUC5A is known to be a marker of and associated with poor prognosis in diffuse type gastric carcinoma^[69].

According to a recent study in MUC5AC knockdown human pancreatic cancer cell lines that were generated by the introduction of siRNA, ectopic expression of MUC5AC did not affect cell proliferation *in vitro*, but was directly involved in tumor progression *in vivo* by dramatically reducing growth and metastatic potential^[70].

All the results described above concerning the expression of mucins 1, 2, 4 and 5AC in neoplasms of pancreas, biliary system and ampulla Vater are formatting in Tables 2-4 respectively.

Other mucins

MUC3: MUC3 is a predictor of poor prognosis in gastric and breast cancer. In pancreatobiliary neoplasms, a strong independent association was identified in the setting of periampullary carcinomas between cytoplasmic expression of MUC3 and favorable prognosis, in contrast to poor outcome of carcinomas showing membranous expression^[71]. The cysteine-rich domains within MUC3 are able to inhibit apoptosis and promote migration of MUC3-expressing cells, favouring cancer progression^[72]. However, neither vascular nor perineural invasion correlated with membranous expression of MUC3, suggesting that an alternative mechanism may be responsible for poor survival in these patients^[71].

MUC6: MUC6 had no effects on survival of patients with pancreatic cancer^[73].

MUC16 (CA 125): MUC16 (CA 125) is known as a tumor marker in ovarian and pancreatic cancer. Studies have investigated the expression of MUC16 in the initiation, progression and metastasis of pancreatic cancer^[74-76]. Its participation in the diagnosis, prognosis and treatment of

pancreatic cancer and its relation to the stage and degree of differentiation has also been explored. MUC16 is not expressed in the normal pancreatic ducts, but it was detected in pancreatitis^[74]. It was also detected in pre-cancerous lesions, suggesting that its overexpression is already detectable at the early stages of carcinogenesis. In one study 20% of PanIN- I, 28% of PanIN- II and 42% of PanIN- III were found positive^[75]. The expression of MUC16 was significantly higher in high-grade dysplasia PanIN- III compared to low grade dysplasia, while there was no significant difference in MUC16 expression between PanIN- I and II^[75]. The expression of MUC16 seems to be stronger in metastatic foci as compared to the primary tumor, thus, possibly playing an important role in metastasis of adenocarcinoma^[74].

In the same study MUC16 was detected in 65% of pancreatic carcinomas. Progressive increase of expression was found to be parallel to the loss of tumor differentiation with positivity being 50% in well differentiated, 59% in moderately differentiated and 66% in undifferentiated adenocarcinomas^[74]. Whereas the expression of MUC16 was not significantly different between the three degrees of differentiation, the expression was significantly higher in moderately differentiated and undifferentiated carcinomas compared with the well differentiated. The observation that MUC16 is overexpressed in pancreatic carcinoma may be associated with a similar upregulation of other mucins associated to the cell membrane such as MUC4 and MUC1^[1,76]. Overall, these results suggest a possible involvement of MUC16 in the pathogenesis of pancreatic adenocarcinoma and provide the basis for future studies aimed at unraveling the role of this glycoprotein.

CONCLUSION

Mucins in epithelial neoplasias of the pancreatobiliary system, in particular MUC-1, 2, 4 and 5AC are gaining importance due to their potential roles as diagnostic, predictive and prognostic markers.

In pancreatic neoplasms, increased expression of MUC1 occurs in pancreatic ductal adenocarcinomas and correlates with increasing degree of dysplasia in PanIN. Positive expression of MUC2 in IPMN-intestinal type indicates high biological potential for progression to invasive carcinoma with *de novo* expression of MUC1. IPMN-gastric type with negative MUC2 expression implies low probability for malignant evolution. *De novo* MUC4 expression seems to increase with enhancement of dysplasia in PanIN and is related to poor prognosis in patients with pancreatic ductal adenocarcinomas.

In biliary carcinogenesis expression of MUC1 correlates with the degree of BilIN dysplasia while increased expression of all MUC1 glycoforms was observed almost uniformly in ICC. Positive MUC2 expression in IPNB-intestinal type indicates high malignant potential associated with *de novo* expression of MUC1 in the invasive component. Absence of MUC2 in BilIN may be useful in the differential diagnosis from IPNB. Appearance of MUC4 in patients with ICC or EHBDC is associated

Table 2 Mucins expression in pancreatic neoplasms

	Normal	PanIN			PDCA	IPMN				MCN	ITPN
		PanIN- I	PanIN- II	PanIN- III		Gastric-type		Intestinal-type			
						Non-invasive	Invasive	Non-invasive	Invasive		
MUC1	2	2	3	4	4	1	4	1	4	CR	4
MUC2	1	1	1	1	1	1	1	4	4	1	1
MUC4	1	2	3	4	4	NA	NA	NA	NA	NA	NA
MUC5AC	1	4	4	4	4	4	4	4	4	CR	1

NA: Non available data; CR: Conflicting results; PanIN: Pancreatic intraepithelial neoplasia; IPMN: Intraductal papillary mucinous neoplasms; MCN: Mucinous cystic neoplasms; ITPN: Intraductal tubulopapillary neoplasms; MUC: Mucins. 1: No expression; 2: Low expression; 3: Moderate expression; 4: High expression.

Table 3 Mucins expression in neoplasms of biliary system

	BilIN			EHBDC	IPNB			
	BilIN- I	BilIN- II	BilIN-III		Pancreatobiliary-type		Intestinal-type	
					Non-invasive	Invasive	Non-invasive	Invasive
MUC1	2	3	4	4	2	4	1	3
MUC2	1	1	1	3	NA	4	NA	3
MUC4	NA	NA	NA	4	NA	4	NA	3
MUC5AC	4	4	4	4	4	4	4	4

NA: Non available data; BilIN: Biliary intraepithelial neoplasia; EHBDC: Extrahepatic bile ducts; IPNB: Intraductal papillary neoplasm of the bile ducts; MUC: Mucins. 1: No expression; 2: Low expression; 3: Moderate expression; 4: High expression.

Table 4 Mucins expression in ampulla Vater neoplasms

	Ampulla vater	
	Pancreatobiliary-type	Intestinal-type
MUC1	4	4
MUC2	1	4
MUC4	4	4
MUC5AC	4	3

MUC: Mucins. 1: No expression; 2: Low expression; 3: Moderate expression; 4: High expression.

with poor prognosis. *De novo* expression of MUC5AC occurs in all grades of BilIN and in all types of IPNB and ICC. MUC5AC is useful in detection of bile duct neoplastic lesions at early stage.

Finally, in carcinoma of the ampulla of Vater, increased expression of mucin MUC1 is associated with unfavorable behavior of the tumor, such as lymph node metastasis, infiltration of the pancreas and duodenum, advanced TNM classification and worse prognosis. IAPN with pancreatobiliary-immunophenotype show no MUC2 expression while those with intestinal-immunophenotype are positive. The expression of MUC4 is linked with poor prognosis in patients with carcinoma of the ampulla of Vater favoring metastasis and tumor recurrence. Expression of MUC5AC correlates with carcinoma invasive capacity.

In conclusion, the combined status of mucin expression may be useful in the differential diagnosis of pancreatobiliary neoplasms, the detection of pre-

malignant lesions and in the evaluation of their malignant behavior. Besides their diagnostic and prognostic role, their involvement in carcinogenesis reveals their importance as putative therapeutic targets in the future. Further studies are needed to clearly define the exact positioning of these evaluations in the diagnostic and therapeutic algorithms of neoplastic lesions of the pancreas and biliary system.

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Nanomedicine strategies for sustained, controlled, and targeted treatment of cancer stem cells of the digestive system

Fang-Yuan Xie, Wei-Heng Xu, Chuan Yin, Guo-Qing Zhang, Yan-Qiang Zhong, Jie Gao

Fang-Yuan Xie, Guo-Qing Zhang, Department of Pharmacy, Shanghai Eastern Hepatobiliary Surgery Hospital, Shanghai 200438, China

Fang-Yuan Xie, Yan-Qiang Zhong, Jie Gao, Department of Pharmaceutical Sciences, School of Pharmacy, the Second Military Medical University, Shanghai 200433, China

Wei-Heng Xu, Department of Biochemical Pharmacy, School of Pharmacy, the Second Military Medical University, Shanghai 200433, China

Chuan Yin, Department of Gastroenterology, Changzheng Hospital, Second Military Medical University, Shanghai 200003, China

Author contributions: Xie FY, Xu WH and Yin C contributed equally to this work; all the authors contributed to this article.

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Correspondence to: Jie Gao, PhD, Associate Professor, Associated Director of Department of Pharmaceutical Sciences, School of Pharmacy, the Second Military Medical University, 325 Guohe Road, Shanghai 200433, China. gaojie@smmu.edu.cn
Telephone: +86-21-81871286
Fax: +86-21-81870801

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Abstract

Cancer stem cells (CSCs) constitute a small proportion of the cancer cells that have self-renewal capacity and tumor-initiating ability. They have been identified in a variety of tumors, including tumors of the digestive system. CSCs exhibit some unique characteristics, which are responsible for cancer metastasis and recurrence. Consequently, the development of effective therapeutic strategies against CSCs plays a key role in increasing the efficacy of cancer therapy. Several potential approaches to target CSCs of the digestive system have been explored, including targeting CSC surface markers and signaling pathways, inducing the differentiation of CSCs, altering the tumor microenvironment or niche, and inhibiting ATP-driven efflux transporters. However, conventional therapies may not successfully eradicate CSCs owing to various problems, including poor solubility, stability, rapid clearance, poor cellular uptake, and unacceptable cytotoxicity. Nanomedicine strategies, which include drug, gene, targeted, and combinational delivery, could solve these problems and significantly improve the therapeutic index. This review briefly summarizes the ongoing development of strategies and nanomedicine-based therapies against CSCs of the digestive system.

Key words: Nanomedicine; Cancer stem cells; Digestive system; Drug delivery; Gene delivery

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Core tip: There are reviews in the literature contributed to the applications of nanotechnology for the detection and treatment of gastrointestinal diseases. However this is a first review to report the current development of strategies and nanomedicine-based therapies against cancer stem cells of the digestive system.

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INTRODUCTION

Currently, gastrointestinal cancer is the second leading cause of cancer-related deaths worldwide. Despite some progress achieved in cancer treatment, the current therapies have limitations with respect to their ability to prevent tumor metastasis and relapse. Recent scientific studies have found that a small proportion of cancer cells (0.01%-4%) can proliferate indefinitely. These cells are similar to adult stem cells with respect to their proliferation, self-renewal, and differentiation into other cells; therefore, they were named cancer stem cells (CSCs)^[1]. CSCs were first isolated from acute myeloid leukemia by Bonnet *et al*^[2] in 1997. It was not until 2003 that CSCs in solid tumors were studied when Al-Hajj *et al*^[3] identified CSCs with a phenotype of CD44⁺/CD24^{-/low}/Lineage⁻ in breast cancer. This provided strong evidence for the existence of CSCs in solid tumors and theoretically supported the possible identification of CSCs in other solid tumors. Subsequently, CSCs were identified in a variety of tumors, including tumors of the digestive system, such as gastric cancer^[4], liver cancer^[5], and colon cancer^[6-9].

CSCs have many characteristics similar to those of stem cells, for example, the self-renewal and differentiation abilities, and some common signaling pathways, including the Wnt/ β -catenin, Notch, and Hedgehog pathways^[10-13]. However, CSCs also exhibit some unique characteristics because of abnormally regulated genetic mechanisms: (1) quiescence, conventional anticancer therapies always kill rapidly proliferating cancer cells, but have less effect on quiescent CSCs^[1,14,15]; (2) high tumorigenicity, only a handful of CSCs can lead to tumor development, whereas the same number of non-CSCs are unable to form clones or tumors *in vivo*^[16,17]; (3) resistance, CSCs highly express membrane transport proteins of the ATP binding cassette (ABC) family, which can transport and efflux a variety of materials, including metabolites, drugs, toxic substances, endogenous lipids, peptides, nucleotides, and sterols, which accounts for the drug efflux and drug resistance of CSCs^[18,19]; (4) high levels of anti-apoptotic molecules^[1]; and (5) enhanced DNA repair ability^[20-23]. Conventional

therapies including chemotherapy, radiotherapy, biotherapy, and thermal therapy mainly focus on the differentiation and proliferation of cancer cells rather than those of CSCs, resulting in an increase in the CSCs fraction, which can lead to metastasis and recurrence^[24,25]. Consequently, the development of effective therapeutic strategies against CSCs plays a key role in increasing the efficacy of cancer therapy.

In recent years, the applications of nanomaterials and nanotechnology in CSC-targeted therapy have received more and more attention. As an emerging interdisciplinary field, nanotechnology can provide materials and tools with unique physical and chemical properties and biological functions for CSC-targeted therapy. In this review, we briefly discussed the properties of CSCs and the conventional strategies against CSCs of the digestive system, as well as a summary of the latest achievements in the nanomedicine approaches for CSC therapy in the digestive system.

CANCER STEM CELLS OF THE DIGESTIVE SYSTEM

Normal gastrointestinal tissues comprise a specific class of stem cells, named gastrointestinal stem cells, which are adult stem cells, with a capacity to self-renew and replicate. They can differentiate into any type of cells in the gastrointestinal tract and play an important role in the regeneration of gastrointestinal mucosa and maintenance of tissue homeostasis. It seems that gastrointestinal stem cells may undergo mutation and can transform into CSCs, which, in turn, participate in the initiation and progression of the gastrointestinal tumors^[26]. However, Houghton found that *Helicobacter pylori* induced chronic inflammation in the gastric tissue of C57BL/6 mice and this inflamed tissue included bone marrow-derived cells, which could develop into intraepithelial carcinoma through dysplasia^[27]. The exact origin of gastrointestinal CSCs is unknown: They may be derived directly from the mutation of normal stem cells, or it may be that mature cells acquire tumor formation potential and transform into CSCs.

The identification of CSCs plays an important role in the evaluation of the prognosis of patients and serves to guide treatment. Currently, the main method used for the isolation of CSCs is based on the surface markers (such as membrane proteins, adhesion molecules, and receptors) that distinguish CSCs from non-CSCs, which can be sorted by flow cytometry or magnetic-activated cell sorting (MACS). In recent years, great progress has been made in the study of gastrointestinal CSCs and their markers, which can theoretically support the diagnosis and treatment of gastrointestinal tumors. In addition, the identification of specific cellular markers of gastrointestinal CSCs has become a research focus. So far, some possible markers of gastrointestinal CSCs have been evaluated, such as CD24, CD133, CD44, CD166, stage-specific embryonic antigen (SSEA), Oct-4,

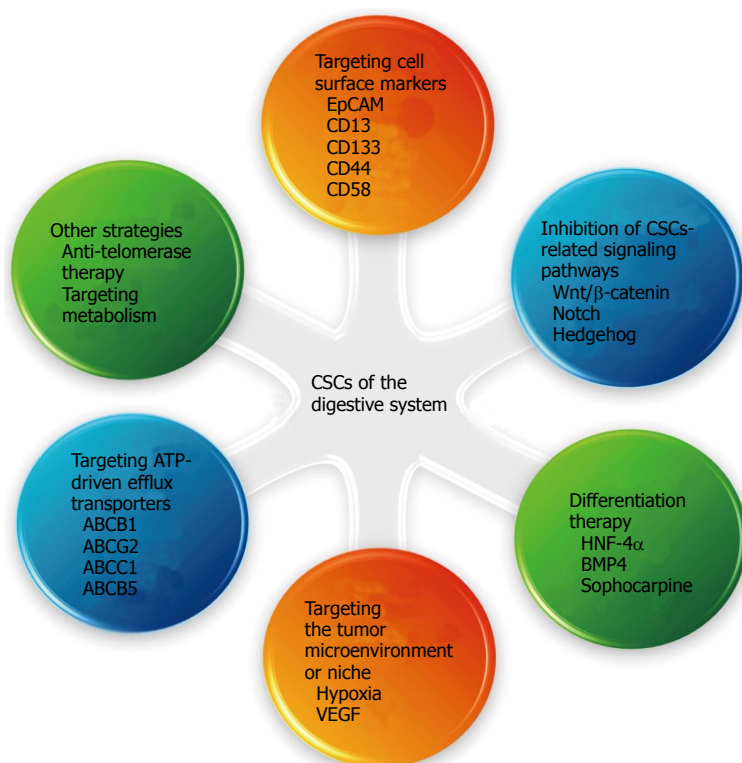


Figure 1 Possible therapeutic strategies that can eliminate cancer stem cells of the digestive system. CSC: Cancer stem cells; VEGF: Vascular endothelial growth factor; HNF-4 α : Hepatocyte nuclear factor-4 α ; BMP: Bone morphogenetic protein.

and Sox-2. CD133 and CD44 are the main markers of gastrointestinal CSCs. However, recent studies of CSC phenotypes have presented a new challenge^[28,29]; CSCs are of different phenotypes and it is urgent and necessary to target all subsets of CSCs within the tumor to prevent a relapse. Thus, there is a need to investigate more cell surface markers in addition to CD133 and CD44, for the identification of gastrointestinal CSCs.

STRATEGIES AGAINST CSCs OF THE DIGESTIVE SYSTEM

The current failure in the treatment of gastrointestinal cancer is attributable to drug resistance and recurrence after therapy in most cases, in which CSCs are thought to play a crucial role. Therefore, strategies targeting CSCs may bring new hope for the treatment of gastrointestinal cancer^[19,30]. Currently, several strategies have been proposed to target CSCs of the digestive system (Figure 1). For example, specific surface markers and altered signaling pathways are attractive therapeutic targets. Induction of the differentiation of CSCs and targeting of the tumor microenvironment or the niche supporting the CSCs are also efficient strategies. Inhibition of ATP-driven efflux transporters that are overexpressed on the CSCs surface, is believed to increase the sensitivity of the tumor to chemotherapeutic drugs. Other strategies, such as anti-telomerase therapy and modulation of abnormal metabolism, are also worth evaluating.

Targeting cell surface markers

CSCs express some unique surface markers that

distinguish them from other cells; therefore, strategies targeting these specific surface markers can eradicate CSCs of the digestive system, which is an effective approach for the treatment of gastrointestinal cancer. Aptamers, which are oligonucleotide or peptide molecules that can specifically bind to a desired site and penetrate the cancer cells, have been found to target the CSCs surface markers. Shigdar *et al.*^[31] isolated the first RNA aptamer against epithelial cell adhesion molecule (EpCAM), a putative marker of gastric, colorectal, and liver CSCs. In addition, monoclonal antibodies have been developed to block CSC surface markers. Haraguchi *et al.*^[32] demonstrated that CD13 is a marker for liver CSCs and treatment with anti-CD13 antibody suppressed the self-renewal and tumor-initiating ability of dormant CSCs. Smith *et al.*^[33] reported that a murine anti-human CD133 antibody conjugated to a potent cytotoxic drug, monomethyl auristatin F, selectively targeted CD133⁺ cells, which is a marker for gastric and liver CSCs. Other surface markers, including CD44^[34] and CD58^[35], have been also utilized to specifically eradicate the CSCs of the digestive system.

Inhibition of CSCs-related signaling pathways

The normal function of stem cells depends on the normal regulation of a variety of signaling pathways. Dysregulation of the signaling pathways results in abnormal proliferation and differentiation. Therefore, inhibition of CSCs-related signaling pathways is an effective method for cancer therapy. The common CSCs-related signaling pathways include: (1) Wnt/ β -catenin pathway: Implicated in the maintenance and proliferation of CSCs^[36]. Cai *et al.*^[37]

suggested that the Wnt/ β -catenin pathway is essential for the self-renewal of cancer stem-like cells in human gastric cancer. Another study also suggested the same role for the Wnt/ β -catenin pathway in gastric CSCs and showed that salinomycin (SAL) could inhibit gastric tumor growth by suppressing Wnt/ β -catenin signaling in CSCs^[38]. The Wnt/ β -catenin pathway plays an important role not only in gastric CSCs, but also in colon and liver CSCs^[39,40]. Song *et al.*^[39] demonstrated that small molecules could target the Wnt signaling pathways in CSCs for the treatment of colorectal cancer; (2) notch pathway: Required for the maintenance of gastrointestinal stem cells^[41]. Luo *et al.*^[42] suggested that the Notch pathway promotes CSCs activity in hepatocellular carcinoma (HCC). Wang *et al.*^[40] demonstrated that the Wnt/ β -catenin and Notch signaling pathways play important roles in the activation of liver CSCs; and (3) hedgehog pathway: Implicated in the unchecked self-renewal and the development of metastatic tumors^[43]. Song *et al.*^[44] suggested that the Sonic Hedgehog pathway is essential for the maintenance of the cancer stem-like cells in human gastric cancer. A study that investigated the molecular mechanisms of curcumin and curcumin analogs against colorectal CSCs suggested the involvement of signaling pathways, including Wnt/ β -catenin, Sonic Hedgehog, Notch, and PI3K/Akt/mTOR^[45].

Differentiation therapy

Differentiation therapy of tumors refers to treatment of malignant tumors *via* induction of cell differentiation. The abnormal differentiation of CSCs is one of the important causes of cancer development, thus, inducing the differentiation of CSCs is an important method for cancer therapy^[46]. Hepatocyte nuclear factor-4 α (HNF-4 α), a central regulator of differentiated hepatocyte phenotype, suppresses tumorigenesis and tumor development by inducing the differentiation of the hepatocarcinoma cells, especially CSCs, into mature hepatocytes^[47]. Lombardo *et al.*^[48] found that bone morphogenetic protein 4 (BMP4) induces the differentiation of colorectal CSCs and increases the antitumor effects of 5-fluorouracil and oxaliplatin. Zhang *et al.*^[49] verified that sophocarpine has the ability to suppress HCC and CSCs and could act as a differentiation therapy drug.

Targeting the tumor microenvironment or niche

The microenvironment is an important condition for the survival of cells, which plays an important role in the regulation of the proliferation and differentiation of cells. The stem cell microenvironment, called niche, includes the niche cells, extracellular matrix, and soluble factors derived from the niche cells. CSCs are also believed to reside in niches, which maintain the principle properties of CSCs, preserve their phenotypic plasticity, protect them from the immune system, and facilitate their metastatic potential^[50]. Targeted therapy against this microenvironment is of great significance for the treatment of cancer. Vermeulen *et al.*^[51] proposed that

colon cancer stemness was partly orchestrated by the microenvironment. Hypoxia, which influences the liver CSC microenvironment, has been identified as a major cause of hypervascularization in HCCs^[52]. Targeting hypoxia is an effective strategy to manipulate the niche of the quiescent, drug-resistant cells. Several studies have indicated that angiogenesis can be related to CSC survival and drug resistance and shown that vascular endothelial growth factor (VEGF) is one of the most specific and critical regulators of angiogenesis, which promotes CSC activity by governing both the microvasculature formation and the intrinsic self-renewal pathways^[53-55]. Targeting VEGF with inhibitors or antibodies can lead to normalization of the tumor vasculature, disruption of the CSC niche, and inhibition of tumor growth^[56-58].

Targeting the ATP-driven efflux transporters

CSCs express high levels of ABC transporters, such as ABCB1, ABCG2, and ABCC1, which represent the three principal multidrug-resistance (MDR) genes that have been identified in tumor cells^[19,59]. These transporters actively efflux the drugs outside the cells, conferring resistance to chemotherapeutic drugs^[59]. Xie *et al.*^[60] suggested that the overexpression of ABCG2 is responsible for chemotherapy failure in colon cancer. Inhibition of ABCB1 (MDR1) expression, which encodes P-glycoprotein (Pgp), can increase the sensitivity of HCC cells to anticancer drugs, such as doxorubicin and daunorubicin^[61]. Pgp's cousin, ABCB5, is another ABC transporter implicated in the drug resistance of CSCs in different tumor types. For example, Cheung *et al.*^[62] found that the expression of granulins-epithelin precursor (GEP) and ABCB5 in liver CSCs was associated with chemoresistance and reduced the survival rates of patients with HCC. However, inhibition of ABC transporters is likely to have significant side effects^[63], and the ability to overcome MDR clinically is rather limited^[64]. Therefore, targeted and combined therapy may be required to circumvent drug resistance and nanomedicine may show tremendous potential to overcome MDR.

Other strategies

Anti-telomerase therapy: Telomerase activation leads to telomere maintenance, which plays an important role in the immortality of CSCs. Compared to the normal cells, the telomerase activity in CSCs is higher and the length of the telomere is shorter. Anti-telomerase therapy can specifically shorten the CSCs telomere, causing replicative senescence, apoptosis, and cell cycle arrest with little damage to the normal cells. Several anti-telomerase agents, such as the antisense oligonucleotide inhibitor GRN163L and immunotherapies that use dendritic cells (GRVAC1), hTERT peptide (GV1001), or cryptic peptides (Vx-001), are currently in clinical trials for treatment of various tumors and are speculated to efficiently target CSCs^[65,66]. A recent study has implied that co-inhibition of telomerase and tankyrase 1, which elongates the telomere, may be a rational strategy for telomere-directed gastric cancer therapy^[67].

Targeting the metabolism: Recently, there is growing evidence that metabolism and stemness are highly intertwined processes in tumors^[68]. For example, gastrointestinal CSCs showed higher inducible nitric oxide synthase (iNOS) expression, lower reactive oxygen species (ROS) production, and a different metabolic profile with respect to non-CSCs. Aerobic glycolysis blockade, oxidative stress-based therapies, and nitric oxide synthase inhibition target the gastrointestinal CSCs and could have profound anticancer effects^[69].

NANOMEDICINE-BASED THERAPIES AGAINST CSCs OF THE DIGESTIVE SYSTEM

As discussed above, conventional therapies may not successfully eradicate CSCs owing to various problems, including solubility, stability, rapid clearance, poor cellular uptake, and unacceptable cytotoxicity. Thus, more and more attention has been drawn to the application of nanomedicine^[70-72]. Nanomedicine can be defined as the application and further development of nanotechnology to solve the problems faced in medicine, *i.e.*, to diagnose, treat, and prevent diseases at the cellular and molecular levels^[73-75]. Nanomedicine is characterized by a size of less than 200 nm in general, which is smaller than the traditional medicine, thus, it has the advantages of large specific surface area, high surface reaction activity, and high adsorption capacity. Moreover, it can be optimized in the aspects of drug loading, pharmacokinetic properties, and biocompatibility by different modifications of the particle surface. In conclusion, nanomedicine has the following characteristics: Sustained, controlled, and targeted drug delivery, improved drug stability, prolonged half-life of drugs, good biocompatibility, *etc.* Consequently, nanomedicine strategies for sustained, controlled, and targeted treatment of CSCs of the digestive system may offer superior outcomes leading to efficient cancer therapy (Figure 1).

Drug delivery

One important application of nanomedicine is the transport of chemotherapeutic drugs with poor solubility, stability, or severe side effects. For example, the monocarboxylic polyether antibiotic, SAL, which primarily functions as a highly selective potassium ionophore, has been shown to affect various CSCs, including liver, gastric, and colorectal CSCs^[76-79]. However, it exhibits poor aqueous solubility and severe nervous and muscle toxicity, which hinder its clinical applications^[80,81]. Therefore, various studies incorporate SAL into nanocarriers to address these issues. For instance, Yao *et al.*^[82] developed a gastric CSC-targeted drug delivery system (SAL-SWNT-CHI-HA complexes), which could enhance the bioavailability and cytotoxic activity of SAL. In our previous study, we developed novel iRGD (internalizing Arg-Gly-Asp peptide)-conjugated DSPE-PEG2000

nanomicelles (M-SAL-iRGD) for delivery of SAL to both liver cancer cells and CSCs. M-SAL-iRGD possessed a small size of around 10 nm and a drug encapsulation efficacy higher than 90%. It showed a superior tumor penetrating ability and therapeutic efficacy^[83]. Similarly, curcumin has extraordinary anticancer properties; however, it has limited application in the treatment of cancer owing to its insolubility, instability, and poor pharmacokinetics, which greatly hamper its *in vivo* efficacy^[84-86]. Wang *et al.*^[87] developed a novel nanoparticle formulation in which curcumin was encapsulated in stearic acid-g-chitosan oligosaccharide (CSO-SA) polymeric micelles to overcome these hurdles. Curcumin-loaded CSO-SA micelles could increase curcumin accumulation in cancer cells and were effective in inhibiting colorectal CSCs both *in vitro* and *in vivo*. In another study, Wang *et al.*^[88] used the CSO-SA micelles to deliver a standard chemotherapy for colorectal cancer treatment (oxaliplatin). This could also increase oxaliplatin accumulation in both colorectal cancer cells and tissues and could effectively eradicate colorectal CSCs.

Gene delivery

Nucleic acids, especially small interfering RNAs (siRNAs) and microRNAs (miRNAs), can effectively target genes overexpressed in CSCs and involved in the maintenance of stemness and tumorigenicity. However, their characteristics, such as negative charge, high molecular weight, and low stability, limit their application. Therefore, nanomedicines have been developed to condense them for effective delivery. For instance, a novel non-cytotoxic and pH-sensitive anti-EpCAM monoclonal antibody-labeled CSCs-targeted block copolymer vesicle was synthesized as a nanocarrier for anticancer drugs and siRNA. The polymer vesicles showed good pH-regulated drug release capability and excellent stability in water, PBS, and 40% fetal bovine serum. The EpCAM-positive CSC-targeted vesicles showed a high delivery efficacy of both the anticancer drug, doxorubicin hydrochloride (DOX-HCl), and siRNA to the CSCs^[89]. Similarly, Kim *et al.*^[90] developed a tumor-targeted nanodelivery platform (scl) and showed that systemic administration of scl carrying the wtp53 gene was able to induce tumor growth inhibition and promote the death of both CSCs and non-CSCs in subcutaneous colorectal cancer xenografts. Nanomedicine for siRNA delivery can also sensitize CSCs to chemotherapeutic drugs. For example, Liu *et al.*^[91] designed a novel siRNA delivery carrier system with multidrug resistance gene (MDR1)-targeted siRNA (siMDR1) and showed that it effectively reduced the expression of MDR1 in human colon CSCs, resulting in a significant increase in the chemosensitivity to paclitaxel.

Recent studies have indicated that miRNAs are important regulators of CSCs^[92]. For example, miR-34, a transcriptional target of p53, inhibits the biological properties of gastric CSCs. Restoration of miR-34 expression in gastric CSCs inhibits sphere formation *in vitro* and tumor regeneration *in vivo*^[93]. Liu *et al.*^[94] developed gelatinase-stimulated PEG-Pep-PCL nanoparticles to

Table 1 Nanomedicine-based therapies against cancer stem cells of the digestive system

Tumor types	Nanomedicine	Ref.
Drug delivery		
Gastric cancer	SAL-loaded carbon nanotubes functionalized with HA	[82]
Liver cancer	SAL-loaded iRGD-conjugated DSPE-PEG2000 nanomicelles	[83]
Colorectal cancer	Curcumin-loaded CSO-SA micelles	[87]
	Oxaliplatin-loaded CSO-SA micelles	[88]
Gene delivery		
Liver cancer	anti-EpCAM-monoclonal-antibody-labeled block copolymer vesicle	[89]
Colorectal cancer	Wtp53 gene loaded scL nanocomplex	[90]
Colon cancer	MDR1 siRNA loaded lipid nanoparticles	[91]
Gastric cancer	miR-200c loaded gelatinase-stimuli PEG-Pep-PCL nanoparticles	[94]
Targeted delivery		
Gastric cancer	SAL-loaded carbon nanotubes functionalized with HA	[82]
Liver cancer	anti-CD44 antibody-mediated liposomal nanoparticle loaded of doxorubicin	[96]
	CD90-targeted thermosensitive magnetoliposomes-encapsulated 17-AAG	[97]
Combinational delivery		
Gastric cancer	Nanoparticle co-loaded miR-200c and DOC	[99]
Liver cancer	micellar nanoparticle co-delivering platinum (IV) prodrug and siNotch1	[100]
Colorectal cancer	Liposomes co-encapsulated irinotecan and floxuridine	[101]
Colon cancer	Nanoliposomes co-encapsulated vincristine and topotecan	[102]

deliver miR-200c, which were reported to inhibit CSC-like properties. The miR-200c nanoparticles enhanced the radiotherapy efficacy, reduced the expression of CD44, and the percentage of CD44⁺ gastric cancer cells. Meanwhile, other CSCs properties, including invasiveness and resistance to apoptosis, could be suppressed by miR-200c nanoparticles.

Targeted delivery

In addition to its ability to improve drug stability and biocompatibility, nanomedicine can also be modified to direct or guide the therapeutic agents to CSCs. Since CSCs express specific cell surface biomarkers, it may be a promising strategy to use these biomarkers for targeted drug delivery. Hyaluronic acid (HA), a glycosaminoglycan widely found in the extracellular matrix, can specifically recognize its receptors, CD44, and has been identified as a potent targeting ligand to tumors possessing CD44-overexpressing cells^[95]. Yao *et al.*^[82] developed SAL-loaded chitosan (CHI)-coated single-walled carbon nanotubes (SWNTs) functionalized with HA, which facilitated the uptake of SWNTs into the gastric CSCs *via* CD44 receptor-mediated endocytosis. In addition, anti-CD44 antibodies could also be used for CSC-targeted therapy. Wang *et al.*^[96] developed doxorubicin-loaded anti-CD44 antibody-functionalized liposomal nanoparticles, which specifically targeted CD44⁺ cells of HCC to mitigate the side effects of conventional chemotherapy. In a recent study, CD90⁺ LCSCs were isolated by magnetic-activated cell sorting from HCC cells. Therefore, Yang *et al.*^[97] prepared a CD90-targeted thermosensitive magnetoliposomes (TMs)-encapsulated 17-allylamino-17-demethoxgeldanamycin (17-AAG), which is a heat-shock protein 90 (HSP90) inhibitor, to sensitize the CD90⁺ LCSCs to magnetic hyperthermia and enhance its antitumor effects *in vitro* and *in vivo*.

Combinational delivery systems

As described above, nanomedicine-based single drug delivery systems are effective in targeting the CSCs in the digestive system. However, various CSCs-targeted drugs that are not highly cytotoxic as compared to the conventional chemotherapeutic drugs, are not very effective in reducing the bulk cancer cells, which can spontaneously and stochastically turn into CSCs again^[98]. Therefore, combinational delivery of chemotherapeutics and CSC-specific agents for eliminating both the cancer cells and CSCs is a promising method to improve cancer treatment. Liu *et al.*^[99] co-loaded miR-200c and docetaxel (DOC) into an intelligent gelatinase-stimulated nanoparticle, which exhibited synergetic effects on the inhibition of both CSCs and non-CSC cancer cells. The miR-200c/DOC nanoparticles prominently suppressed the *in vivo* tumor growth. Shen *et al.*^[100] developed a micellar nanoparticle to deliver platinum (IV) prodrug and siNotch1 into both non-CSCs and CSCs of SMMC7721. The combined drug delivery system could remarkably augment drug delivery into tumor tissues, thus, substantially suppressing the tumor growth (Table 1).

Additionally, nanomedicine is crucial for the delivery of dual drugs with predictable ratios at the tumor site to achieve a synergistic effect. Mayer *et al.*^[101] co-encapsulated irinotecan and floxuridine at a 1:1 molar ratio inside 100-nm-diameter liposomes composed of distearoylphosphatidylcholine/distearoylphosphatidylglycerol/cholesterol (7:2:1 molar ratio). The liposomes maintained the drug ratio in the plasma after injection, and delivered the formulated drug ratio directly to the tumor tissue of the colorectal cancer. In another study, vincristine and topotecan were successfully co-encapsulated at therapeutically relevant levels in the same nanoliposome (LipoViTo). The nanoliposomes controlled the drugs' "biofate" and maintained a fixed drug ratio *in*

vivo, displaying an enhanced therapeutic efficacy against colon cancer^[102].

CONCLUSION

The CSCs theory revealed more facts about cancer, but the CSCs in the digestive system are still not fully understood. There is a need for further investigation of the new markers, abnormal metabolism, and signal transduction pathways of CSCs, which will improve our strategies to target CSCs. In this review, we summarized the current strategies against CSCs of the digestive system. Nanomedicines have been shown to effectively deliver drugs and genes to target CSCs of the digestive system. A number of studies have shown that there is a significant increase in the therapeutic outcome with nanomedicine. However, there are still great challenges limiting the effective application of nanomedicine in clinical practice. One of the most important challenges is the biological safety issues. There is still no clear evidence that the nanomaterials can be effectively metabolized *in vivo* and will not accumulate to cause side effects. In addition, it is difficult to determine the safe dose of nanomedicine because of the lack of clear evaluation criteria.

Although there are still some difficulties preventing the wide application of nanomedicine in clinical practice, there is a reason to believe that, with the progress of nanotechnology and the in-depth research of CSCs, the unique advantages of nanomedicine will create good conditions for the development of personalized therapy for cancer patients and will finally be capable of conquering cancer of the digestive system.

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Where does chemotherapy stands in the treatment of ampullary carcinoma? A review of literature

Marwan Ghosn, Hampig Raphael Kourie, Elie El Rassy, Fady Ghassan Haddad, Colette Hanna, Fadi El Karak, Dolly Nasr

Marwan Ghosn, Hampig Raphael Kourie, Elie El Rassy, Fady Ghassan Haddad, Colette Hanna, Fadi El Karak, Department of Oncology, Faculty of Medicine, Saint Joseph University, Beirut 1104-2020, Lebanon

Hampig Raphael Kourie, Department of Oncology, Jules Bordet Institute, Free University of Brussels (ULB), B-1070 Brussels, Belgium

Dolly Nasr, Department of Radiation Oncology, Faculty of Medicine, Saint Joseph University, Beirut 1104-2020, Lebanon

Author contributions: Ghosn M initiated the review; Ghosn M, Kourie HR, El Rassy E performed the review, analyzed the data and wrote first draft; Ghosn M, Kourie HR, El Rassy E, Haddad FG, Hanna C, El Karak F and Nasr D reviewed and commented on the paper and provided final approval.

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Correspondence to: Marwan Ghosn, MD, Department of Oncology, Faculty of Medicine, Saint Joseph University, Monot St, Beirut, PO Box 166830, Beirut 1104-2020, Lebanon. mghosn.hdf@usj.edu.lb
Telephone: +961-1-3226842
Fax: +961-1-1613397

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Abstract

Ampullary carcinoma (AC) is a rare gastrointestinal tumor without clear treatment recommendations. The management of this tumor is usually extrapolated from the treatment of pancreatic, biliary duct and intestinal cancers. Few papers have studied the AC as an independent entity and yet succumbs to several limitations. These studies were retrospective single institutional experiences with limited sample sizes recruited over a long period of time. Unlike metastatic ACs where chemotherapy is the only recommended option, localized AC once excised may be approached by either chemotherapy alone or concomitant chemoradiation therapy. In this review, we report the overall survival and recurrence factors of more than 1000 patients from all the studies treating exclusively ACs. We also review the medical treatment of this tumor and conclude to the necessity of multi-institutional randomized controlled studies for AC exclusively.

Key words: Ampullary cancer; Prognostic factors; Treatment; Review; Novel therapies

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Core tip: This paper is a minireview outlining the actual knowledge concerning the treatment of ampullary carcinoma. After a brief review of the prognostic factors and current treatment options for localized and advanced ampullary carcinoma, we discuss the new molecular targets and report on the potential novel therapies.

Ghosn M, Kourie HR, El Rassy E, Haddad FG, Hanna C,

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INTRODUCTION

Ampullary carcinoma (AC) is an uncommon tumor accounting for approximately 0.2% of gastrointestinal malignancies and 7% of periampullary tumors^[1]. It is continuously increasing in frequency and actually is the second most common of periampullary tumors after pancreatic cancers^[1,2]. Adenocarcinomas are the most common tumors of the ampulla and may be subdivided pathologically into intestinal and pancreaticobiliary subtypes for potential prognostic purposes^[3]. Few trials have studied the AC as an independent entity. It is frequently seen as a subgroup of pancreatic and biliary tract cancer trials even though ACs have a better prognosis and constitute a confounding factor in these studies. In comparison to pancreatic adenocarcinomas, prognostic factors are in favor of the ampullary tumors. The tumor size and staging at diagnosis, the positivity of lymph nodes (LN), the vascular and neural invasions were lower in ACs^[4]. Nevertheless, trials treated ACs as pancreatic cancers. This dilemma probably stands essential for the absence of any guidelines from both the National Cancer Network (NCCN) and the European Society for Medical Oncology (ESMO) concerning the treatment of advanced ACs^[5,6]. In this paper we report on the recurrence factors and overall survival (OS) of patients with AC. We also review the position of chemotherapy in this setting.

PROGNOSTIC FACTORS IN AC

Although localized AC is known for its high rates of resectability and good long term OS, most of the series report a high proportion of recurrent disease. However, these series are of small numbers which disables any statistical OS analysis^[1]. LN spreading and number of resection LV^[7-12], the vascular, nervous and pancreatic invasion^[7,11-15] along with the unresectability of the tumor and positive margin status after resection^[9,10,13,16], and intraoperative transfusions^[7,11,17] are the most consistent survival factors throughout the studies of localized AC.

Several studies tried to establish the risk factors for the recurrences of excised ACs. Todoroki *et al.*^[14] in 2003 did not experience locoregional failure with pancreaticoduodenectomy. Recurrences occurred distally and were affected by lymphatic and venous invasion with a mean time to relapse of 13 mo. Perioperative blood transfusion, LN spreading and pancreatic invasion increased the risk of recurrence^[7,11,17].

Very few studies elaborated the prognostic factors of

advanced ACs. These factors can be extrapolated from studies of unresectable pancreatic and periampullary cancers. Negative prognostic factors include weight loss, abdominal pain, peritoneal dissemination and liver metastasis. Older age is also a negative prognostic factor except in white younger women characterized by a worse prognosis than older ones^[18,19].

EVOLUTION OF TREATMENTS

Tumor resection is the mainstay in the treatment of localized AC. Current surgical options prefer radical pancreaticoduodenectomy over local resection despite its higher morbidity^[7]. The conventional local regional resection technique considers a transduodenal approach. The extraduodenal technique is a potential alternative that offers a complete removal of the tumor with concurrent excision of retropancreatic LN^[20]. Preoperative endoscopic biliary drainage is not widely acceptable among pancreatic surgeons in view of the increased morbidity and delays of definite treatment^[21]. However, the only study involving exclusively AC showed that preoperative biliary drainage reduces postoperative wound infection without influencing mortality^[22].

The role of chemotherapy for both local and advanced AC is not yet clearly established in view of the rarity of the disease. The only relevant data is commonly found in series combining patients with small bowel, pancreatic or biliary tract tumors. Tables 1 and 2 report the response rate, time to progression and OS of 10 retrospective single institutional experience of small sample sizes varying between 26 and 186 patients with AC that were recruited over periods ranging from 5 to 33 years.

While reviewing the localized AC studies, most of the series used a pancreatic cancer chemotherapy regimen that consisted of fluorouracil and radiotherapy to treat ACs^[23-29]. Regimens also combined gemcitabine and radiotherapy after the introduction of the first in 1997^[30]. The ESPAC-3 trial by Neoptolemos *et al.*^[31] in 2012 included the largest sample of AC patients; 297 of the 428 patients enrolled in this trial had AC. Participants were divided into three subgroups: The control group consisted of 144 patients, the fluorouracil and the gemcitabine subgroups contained 143 and 141 patients respectively. Overall, the increase in median OS in the chemotherapy group was not statistically significant (43.1 mo vs 35.2 mo; $P = 0.25$)^[31]. By analyzing exclusively AC data, the median OS of the gemcitabine and the fluorouracil subgroups were 71 mo and 57.8 mo respectively in comparison to the 41 mo of the control arm group^[31]. In opposition, Jiang *et al.*^[30] in 2013 showed a trend toward increased OS in the fluorouracil group.

Papers reporting treatments of advanced ACs are fewer, only two papers were published to date^[32,33]. The first introduced in 2010 platinum for the first time in the treatment of AC; the regimens consisted of a combination of cisplatin with either gemcitabine or

Table 1 Response rate, time to progression and survival in patients with localized ampullary carcinoma

Ref.	n	Patient characteristics	Protocols	OS	RR/TTP
Lee <i>et al</i> ^[23]	39	1988-1997 33% CRT	RT (48.7 Gy) with continuous/ concurrent infusion of 5-FU	3 yr: 55%	3 yr: 54% DFS
Sikora <i>et al</i> ^[24]	113	1989-2000 104 patients remained alive after surgery	RT (50.4 Gy) with concurrent 5-FU	OS: 30 mo 1 yr: 79% 3 yr: 43% 5 yr: 33%	NC
Bhatia <i>et al</i> ^[25]	125	1977-2005	29 patients: RT (50.4 Gy) with 5-FU 96 surgery	3.4 yr 1.6 yr	NC
Krishnan <i>et al</i> ^[26]	96	1990-2006 56% CRT	RT (45 Gy preop or 50.4 Gy postop) with 5-FU (42%) or capecitabine (43%)	25.2 mo in patients with CRT <i>vs</i> 16.5 mo in control arm	NC
Kim <i>et al</i> ^[27]	118	1991-2002 35% CRT	RT (40 Gy) with 5-FU (day 1, 3) every split course	5 yr: 52.8% <i>vs</i> 66.9% in the control arm	NC
Narang <i>et al</i> ^[28]	186	1992-2007	RT with 5-FU	39.9 mo 2 yr: 62.4% 5 yr: 39.1%	NC
Palta <i>et al</i> ^[29]	137	1976-2009	61 CRT 43 adjuvant 18 neoadjuvant	3 yr: 62% in CRT and 46% in adjuvant	Neoadjuvant: 28% pCR
Jiang <i>et al</i> ^[30]	64	1992-2009	5-FU-based <i>vs</i> gemcitabine based	5-FU trend toward benefit for OS (<i>P</i> = 0.007)	5-FU significant improvement for TTP

CRT: Chemoradiotherapy; NC: Not calculated; OS: Overall survival; RT: Radiotherapy; TTP: Time to progression.

Table 2 Response rate, time to progression and survival in patients with advanced

Ref.	n	Patient characteristics	Protocols	OS	RR/TTP
Kim <i>et al</i> ^[32]	29	2003-2008	31% Cis + Gem 69% Cis + 5-FU	12.5 mo (no significant difference between the two groups)	NC
Shoji <i>et al</i> ^[33]	26	1997-2010	5-FU-based gemcitabine-based 5-FU based gemcitabine-based	OS = 9.1 mo 8 mo 12.3 mo	RR = 7.7%

Cis: Cisplatin; Gem: Gemcitabine; NC: Not calculated; OS: Overall survival; RR: Response rate; TTP: Time to progression.

fluorouracil but failed to establish any OS difference between the two protocols^[32]. In opposition, Shoji *et al*^[33] showed more OS benefit in the gemcitabine group. This study reported 26 advanced AC patients receiving chemotherapy without tumor resection. The fluorouracil and gemcitabine based protocols had a response rate of 7.7% and an OS of 9.1 mo (OS = 9 and 12.3 mo respectively). It is of particular importance to note a phase II trial by Overman *et al*^[34] that recruited 30 patients among which 40% had advanced AC. Patients received a treatment with capecitabine and oxaliplatin (CAPOX) and had an overall response rate of 33% (95%CI: 10%-65%)^[34].

TREATMENT MODALITIES IN LOCALIZED AC

In the absence of solid data, neither NCCN nor ESMO established standard chemotherapy regimens for patients with ACs^[5,6]. Effectively, the Americans approach this tumor differently than the Europeans (Figure 1)^[35].

In discordance with the European treatment regimens that extrapolate chemotherapy protocols from pancreatic tumor trials^[30,31,36], the American treatment regimen is supported by the result of RTOG 9704 trial^[37]. As of stage IB of AC, the treatment approach is identical to resectable pancreatic adenocarcinomas with a sequence of gemcitabine and concurrent infusional fluorouracil and radiotherapy. Though the optimal sequencing is not clear, an acceptable protocol includes gemcitabine 1000 mg/m² for 3 weekly followed by conformal radiotherapy with concurrent infusional fluorouracil 250 mg/m² daily, and after 3 to 5 wk gemcitabine is reintroduced at 1000 mg/m² for 3 of every 4 wk for 3 mo^[38]. As with pancreatic cancer, the infusion protocol of fluorouracil is not clear yet.

CHEMOTHERAPY REGIMENS

TREATMENTS IN ADVANCED AC

As with localized AC, the optimal chemotherapy is not yet elucidated. The concurrent chemotherapy regimen recommended in advanced AC is an association of

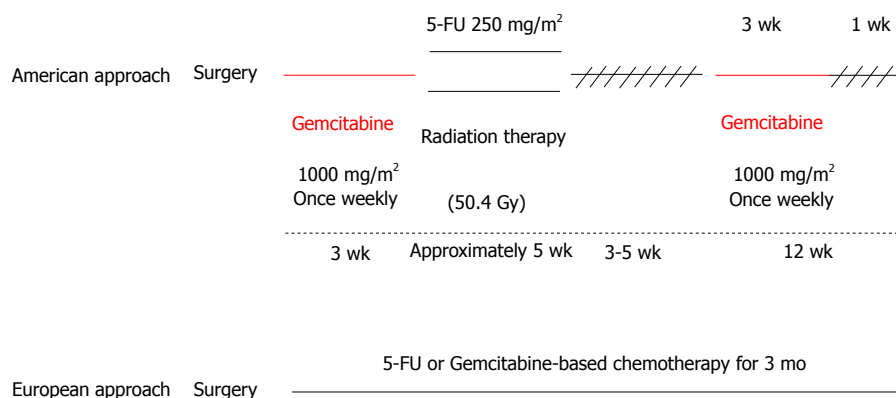


Figure 1 Concurrent American and European approach for the treatment of localized ampullary carcinoma^[35]. 5-FU: 5-fluorouracil.

cisplatin and gemcitabine^[38]. Other acceptable regimens adopted from the pancreatic chemotherapy treatment panel are fluorouracil or gemcitabine associated with oxaliplatin^[37-40]. An interesting approach in this context considers the pathologic subtype as an indicator for a potential chemotherapy regimen where fluorouracil-based therapy is used for intestinal ACs and gemcitabine-based therapy for pancreaticobiliary ACs^[34].

NOVEL THERAPIES

Given the rarity of the disease, the performance of well-powered randomized controlled clinical trials is very difficult. Multiple phase II trials including targeted therapies are actually ongoing among which a combination of CAPOX and bevacizumab (NCT01208103), CAPOX and panitumumab (NCT01202409), gemcitabine-oxaliplatin (GEMOX) and erlotinib (NCT00832637). The only study ongoing in the adjuvant setting is evaluating the role of high volume washing of the abdomen in increasing survival after surgery in patients with pancreatic and peripancreatic tumors (NCT02757859).

The ongoing studies seem promising but recruit also other peripancreatic tumors besides AC. A recent-genomic sequencing study of AC identified severe genetic aberrations with deleterious mutations and deletions in KRAS, SMAD4 and PTEN. This genomic profile suggests that the oncogenesis of ACs differs from both biliary tract and pancreatic cancers. The combination of these genomic aberrations suggests a therapeutic approach by mTOR/PI3K inhibition for patients with AC^[41]. Moreover, another genomic analysis revealed mutations in the WNT signaling pathway with high frequency inactivating mutations of ELF3 and a high rate of microsatellite instability. Such findings coupled with small-molecule inhibitors of β -catenin would be of particular interest to be evaluated in clinical trials^[42]. The only ongoing genetic analysis-guided dosage treatment study of patients with advanced gastrointestinal cancer include a combination of nab-paclitaxel, fluorouracil, leucovorin and irinotecan (FOLFIRABAX) (NCT02333188).

CONCLUSION

Given the rarity of the ACs, the published literature lacks well-powered randomized controlled trials. Effectively, the published data is limited to single institutional retrospective studies with small sample sizes. These studies recommend gemcitabine monotherapy or in combination with conformal radiotherapy for the treatment of localized AC and the combination of gemcitabine and cisplatin for the treatment of advanced AC. While analyzing these data, one should be aware to the selection bias of retrospective studies. Moreover, the results of single institutional studies are not to be extrapolated to community hospitals where the surgeons are less experienced in the management of this rare disease. Any effort for future therapeutic development should consider multi-institutional randomized controlled studies recruiting exclusively AC.

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Arterial complication of irreversible electroporation procedure for locally advanced pancreatic cancer

Yahya Ekici, Tugan Tezcaner, Hüseyin Onur Aydın, Fatih Boyvat, Gökhan Moray

Yahya Ekici, Tugan Tezcaner, Hüseyin Onur Aydın, Gökhan Moray, Department of General Surgery, Medical Faculty of Baskent University, 06490 Ankara, Turkey

Fatih Boyvat, Department of Radiology, Medical Faculty of Baskent University, 06490 Ankara, Turkey

Author contributions: Ekici Y, Tezcaner T and Boyvat F performed the operation; Aydın HO analyzed the data; Ekici Y and Tezcaner T wrote the paper; and Moray G done the critical view.

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Correspondence to: Yahya Ekici, MD, Professor, Department of General Surgery, Medical Faculty of Baskent University, 06490 Ankara, Turkey. dryahyaekici@gmail.com
Telephone: +90-533-3665676
Fax: +90-312-2234909

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Abstract

Irreversible electroporation (IRE) is a non-thermal ablation technique used especially in locally advanced pancreatic carcinomas that are considered surgically unresectable. We present the first case of acute superior mesenteric artery (SMA) occlusion secondary to pancreatic IRE procedure that has not been reported before in the literature. A 66-year-old man underwent neoadjuvant chemoradiotherapy for locally advanced pancreatic ductal adenocarcinoma. IRE procedure was applied to the patient during laparotomy under general anesthesia. After finishing the procedure, an acute intestinal ischemia was detected. A conventional vascular angiography was performed and a metallic stent was successfully placed to the SMA and blood flow was maintained. It is important to be careful in such cases of tumor involvement of SMA when evaluating for IRE procedure of pancreatic tumor.

Key words: Irreversible electroporation; Mesenteric artery occlusion; Locally advanced pancreatic cancer; Superior mesenteric artery

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Core tip: Irreversible electroporation (IRE) is a non-thermal ablation technique is a hope for patients who have unresectable locally advanced pancreatic carcinomas; especially, when any vascular surgical approach is impossible because of the tumor involvement. We encountered superior mesenteric artery occlusion after IRE procedure for locally advanced pancreatic cancer. We suggest that treating physicians should keep in mind that kind of mortal vascular complications of IRE. Stent placement *via* angiography could be the lifesaving treatment choice for vascular occlusions due to IRE procedure.

Ekici Y, Tezcaner T, Aydın HO, Boyvat F, Moray G. Arterial complication of irreversible electroporation procedure for

locally advanced pancreatic cancer. *World J Gastrointest Oncol* 2016; 8(10): 751-756 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v8/i10/751.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v8.i10.751>

INTRODUCTION

The majority of the pancreatic adenocarcinomas are diagnosed at an advanced stage, with resectable tumors being identified in only 10%-20% of the cases at the time of first admission^[1]. Involvement of the celiac trunk, superior mesenteric artery or portal vein without distant metastasis is observed in 40% of cases with stage 3 disease^[2,3]. For these patients, a 5-year survival rate of 6.8% has been reported following surgical resection^[4]. These patients are now classified as locally advanced stage pancreatic cancer (LAPC), and their survival rates are reportedly better compared to patients with distant metastasis. The probability of surgical resection following neoadjuvant treatment is higher in these patients^[5]. Nowadays, different ablation techniques are being studied in combination with neoadjuvant treatment^[6]. Some thermal ablation techniques such as radiofrequency ablation (RFA), cryoablation and microwave ablation have been developed and defined for tumor ablation. Thermal ablation methods are reported to cause necrosis during treatment for LAPC through thermal injury in the peripancreatic fatty tissue, main vascular structures, pancreatic tissue, duodenum and biliary tract^[7]. These morbid complications are limitations of these techniques for pancreatic tumors.

Irreversible electroporation (IRE) creates pores in cell membrane by transmitting short electrical currents at high voltage^[8]. As a result, the phospholipid membrane of the cell is permanently damaged, and the apoptosis and necrosis complex is observed due to the impairment of cellular homeostasis. Since IRE is not a thermal ablation technique, it is assumed that it is affected to a lesser extent from the vascular flow surrounding the tissue^[9]. No decrease in ablation energy is observed, due to the effect on the main vascular blood flow. Also, due to the shorter duration of treatment, a larger affected tissue volume, and a lower pain response, this technique is considered to be superior to other ablation techniques^[10]. Previous studies demonstrated that IRE use in the treatment of locally advanced pancreatic cancer was safe, since it resulted in no harm to the peripancreatic tissues and did not affect the superior mesenteric artery, porta hepatis and biliary tract^[11]. Metallic stent removal is recommended prior to the use of IRE in cases where a metallic stent is applied in the biliary tract for biliary obstruction. It is believed that the presence of a metallic stent in the field of electrical flow within the duodenum may result in damage to the adjacent structures^[12].

Studies conducted in various centers around the world have reported the short and long term protocol of IRE to be effective in the treatment of LAPD. In these



Figure 1 Coronal plain computerized tomography. Locally advanced pancreatic malignant mass of 45 mm in diameter surrounded and narrowed superior mesenteric artery (arrow).

studies no important morbid complication was reported like SMA occlusion. However, in this case report, we draw attention to a previously unpublished complication of IRE in LAPC.

CASE REPORT

A 66-year-old man with locally advanced pancreatic head adenocarcinoma diagnosed by fine-needle aspiration biopsy 6 mo ago was admitted to our institution. Tumor markers were measured, with CA 19-9 = 4801 IU/mL and CA 125 = 161 IU/mL. After diagnosis, a plastic stent was placed in ductus choledocus with ERCP. Immediately afterwards, chemoradiation was administered to the patients [5 cycles of chemotherapy consisting of Folfox + 5-FU + Oxaliplatin every 14 d, for 28 d RTA (28 fractions 50, 4 gray IMRT received)]. After admission to our hospital, computerized tomography (CT) examination was performed to the patient, and a malignant mass of 45 mm in diameter was observed at the pancreatic head level (Figure 1). It was noted that the tumor had surrounded the superior mesenteric artery 360 degrees, the affected segment of SMA was 40 mm. The tumor had apparently reduced the SMA diameter by 3 mm (Figure 1). Main pancreatic duct was markedly dilated, and the diameter was measured as approximately 7 mm. There was no metastasis and the tumor was stable after neoadjuvant chemotherapy.

The patient was referred to our hospital with a diagnosis of LAPC. Irreversible electroporation for pancreatic tumor and simultaneous gastrojejunostomy was planned. The abdomen was opened with a median incision above the umbilicus and abdominal exploration was performed. During the exploration, no distant metastases were identified anywhere in the abdomen. Intraoperative liver ultrasonography was used to detect possible metastasis to liver. The patient was under general anesthesia, surgery was performed in the supine position. The gastro-colic omentum was opened to reach and expose the pancreatic tumor. Electroporation was performed in accordance with the procedural instructions.



Figure 2 Conventional angiography of superior mesenteric artery immediately after irreversible electroporation. Angiography revealed that there is occlusion in superior mesenteric artery (red arrow) and also occlusion in hepatic artery (white arrow) originated from superior mesenteric artery.

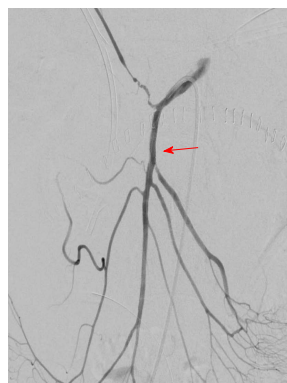


Figure 3 Conventional angiography of superior mesenteric artery after stent placement. After the stent placement superior mesenteric artery recanalized and intestinal blood flow was maintained (arrow shows the recanalized superior mesenteric artery).

IRE was performed using the Nanoknife System (Angiodynamics, Lanthan), as described in the previous manuscript on IRE for the pancreas. High definition intraoperative ultrasound imaging was used in all cases, which is required to demonstrate non-traumatic precise needle placement and also for continuous ablation assessment during IRE. In sum, 3 monopolar probes with 2-cm spacing will deliver an electroporation defect of approximately 3.5 cm axial, 2.5 cm anterior-posterior, and 2.5 cm cranial-caudal. This electroporation procedure is achieved through a maximum of 1.5 cm exposure, 1500 V/cm, and 100 s wavelength. The procedure was performed in 45 min. After IRE procedure a gastrojejunostomy completed. After completing gastrojejunostomy anastomosis, a color change was concurrently observed in the small intestine. Based on a suspicion of SMA occlusion, arterial pulse was checked in the small bowel mesentery. No pulse was obtained in this area. The abdomen of the patient was closed, and he was taken to the angiography unit because SMA occlusion developed in the tumor surrounded segment. In angiography images, it was observed that a short segment after the origin of SMA, right hepatic artery was arising. Even after 1 cm distal to right hepatic artery origin there was no blood flow in the SMA (Figure 2). First of all, occluded segment was dilated with a 3 mm balloon. After dilatation a 4 mm and 3 cm length metallic stent was placed to this dilated segment (Figure 3). There were no complications in the postoperative follow-up. The patient was discharged without any complication the sixth day postoperatively.

DISCUSSION

Nearly 30% to 40% of patients with pancreatic adenocarcinoma have a locally advanced disease with stage III at the time of diagnosis and half of the tumors involved celiac axis and SMA. Autopsy studies have demonstrated that 30% of the patients with pancreatic cancer are in locally advanced stage with no distant metastasis^[13]. Chemoradiotherapy used in the conventional treatment

of such patients is of limited efficacy. In the past, hepaticojejunostomy or gastrojejunostomy were commonly used in the palliative surgical treatment of patients with LAPC. IRE is a new hope for the patients' with LAPC in locally control of pancreatic cancer with survival benefits. We perform IRE for LAPC invaded or encapsulated superior mesenteric artery in the absence of distant metastasis. We perform 3 to 5 per annually IRE since 2013 in our institution.

In the classical surgical approach, pancreaticoduodenectomy (PD) is preferred in resectable pancreatic carcinomas. Although acute occlusion or thrombosis in the hepatic artery, portal vein, SMA or celiac artery are rarely observed following this operation, they are still considered as the most significant causes of mortality. Gaujoux *et al*^[14] preoperatively evaluated 545 patients who underwent PD, by arterial reconstruction, examining SMA and celiac artery stenosis with CT, and detecting hemodynamically significant stenosis is 27 (5%) of the patients. On the other hand, five patients died due to undetectable stenosis and ischemic complications. Stent placement and bypass procedures were preoperatively performed in those patients, with an effort being made to prevent postoperative ischemic complications. Overall, the rate of complications was reported as 2.6%. It was observed that 36% of these complications were ischemic complications associated with undetectable stenosis^[14]. Other studies in the literature recommend the application of CT, angiography or intraoperative Doppler ultrasonography, and clamping the gastroduodenal artery for the diagnosis of SMA occlusion^[15]. Median arcuate ligament compression, atherosclerotic stenosis or fibromuscular dysplasia and tumor involvement of SMA might be the cause of acute occlusion of SMA in patients with pancreatic cancer^[16]. Recommended treatment modalities in acute SMA occlusion include thrombectomy, revascularization with side-to-side or end-to-side anastomosis, and stent application by angiography^[17]. While SMA occlusion is rarely observed during PD, acute SMA occlusion has never been previously reported in the literature

in patients who underwent IRE. The impaired blood supply in the jejunal segments, which was noticed intraoperatively in our case presented here, enables the detection of SMA occlusion. Since access to the occluded segment in our case would be easier by vascular angiography, stent placement was preferred for treatment choice. Accessing the SMA surgically, on the other hand, was not possible because of tumor involvement. Therefore, by using angiography, diagnosis and treatment were both performed at the same time.

Owing to the imaging techniques used today, LAPC can be diagnosed preoperatively. Endoscopically applied biliary and/or duodenal stents provide a chance for palliative treatment by eliminating the risk of surgical morbidity for the patient^[18]. Currently defined thermal ablation techniques are known to be inefficient for adequate ablation of tumor cells adjacent to vascular structures due to the heat sink effect. IRE provides non-thermal ablation in tumor cells through the short electrical currents with high amplitude. Therefore, it can be applied in LAPC without being affected from the flow in portal vein, celiac artery, biliary tract or SMA. However, IRE modulates vascular smooth muscle cells to apoptosis; but it has been demonstrated that elastin and collagen in particular, which are present in vascular structures, have been affected from this ablation technique^[19]. After IRE blood flow is intact and vascular smooth muscle cells repopulate in a short time period^[19]. The method's efficacy was demonstrated in studies performed on large tumor areas, where its application was associated with shorter times and lesser complications. Folfirinox or gemcitabine based chemotherapy is used for approximately 4 mo in the first line treatment of LAPC, and its efficacy is approved by the National Comprehensive Network Guideline^[20].

In a study by Martin *et al*^[21], 54 patients who underwent IRE were compared with 85 patients who were admitted for standard chemotherapy. At the end of the study, no significant differences were found in overall survival and progression-free survival between patients who underwent systemic chemotherapy for 4 mo and who underwent IRE treatment^[21]. The overall survival results of the IRE applied to 200 patients revealed no significant differences with patients receiving chemoradiotherapy and it was recommended that chemoradiotherapy, is used along with IRE as part of a triple treatment protocol. In this multicenter study, minor complications were observed among those 200 patients, but no case of acute vascular occlusion was encountered^[22]. Other studies with large series demonstrated that application of IRE was safe and efficient around vascular structures^[23].

Unexpected complications are being reported as clinical studies are continuing. Following application of this technique, various complications have been reported secondary to electrical transmission, especially in patients with metallic biliary stents or enteric stents^[12].

In the review of the literature, we did not encounter any study on SMA occlusion following IRE application, as

was the case with our patient presented herein. A recent systematic review reported an IRE-related complication rate of 13%, with an IRE-related mortality of 2%^[24]. Martin *et al*^[22], in a recent study with a population of 200 patients suffering from LAPC treated with IRE, showed an overall rate of adverse events of 37% (74 patients with 149 overall complications) and a mortality rate of 2%. The most common complications (including both percutaneous and open techniques) described after the use of IRE on pancreas are pancreatitis, pneumothorax, hematoma, abdominal pain, bile leakage, pancreatic leakage, duodenal leakage, duodenal ulcer, deep vein thrombosis/pulmonary embolism and superior mesenteric venous occlusion.

In healthy individuals average diameter of SMA was reported between 7.3 and 5.9 mm^[24]. The measured diameter of SMA in this case with CT scan was 3 mm. The tumor involved the artery and decreased its diameter nearly 50%. After the IRE procedure edema may developed around the ablation area or at the wall of the artery may especially cause an SMA occlusion. SMA occlusion and intestinal ischemia was noticed earlier in the present case, since IRE was applied simultaneously with open surgery, and this condition was treated by an angiographically applied vascular stent before necrosis developed in the jejunal segments. It is more important to be careful in percutaneous IRE procedures for LAPC because it is difficult to diagnose intestinal ischemia such a short time period.

The anatomy of the hepatic artery varies substantially. Reported rate of replaced right hepatic artery from SMA ranges from 5% to 25%^[25]. A variant artery is an important risk factor when the surgeon unaware of this anomaly. It is possible to progress the thrombosis to the stump of right hepatic artery in such cases. This is another possible fatal complication of SMA occlusion when hepatic artery arises from SMA. The liver and gut will become necrotic within a few hours.

Recently, percutaneous IRE application has been tried and its results in terms of overall survival were observed to be positive^[26]. Placement of probes could be both percutaneous and surgical; it depends on localization of the lesion and experience of the team. One should pay attention to acute vascular occlusions that might develop in such cases. It is also necessary to bear in mind that acute vascular occlusions might develop during percutaneous or surgical IRE application in LAPC.

IRE expands the scope of treatment of lesions that is near to major vascular/biliary/urinary structures. Because of this adjacency, these lesions are not suitable for local ablative therapies and could only be try to treat with some forms of external beam radiation therapy. In order to prevent injury of these major structures by displacement of electrodes, there is a need for general anesthesia (deep paralysis)^[23]. The only contraindication of IRE is patients with pacemakers or with cardiac arrhythmias^[27]. IRE is mostly applied for locally advanced tumors invaded to adjacent structures.

As in our case, if there is a severe vascular invasion is decreasing the diameter more than 50%, a coated vascular stent could be placed to the narrowed segment before applying IRE.

In conclusion, we present a new and fatal complication of IRE procedure for LAPC. In our patient, the diameter of SMA was decreased over 50% when compared with a healthy individuals SMA. We think that both arterial narrowing and IRE depend tissue edema are affective factors for acute SMA occlusion. However, it might also be speculated that the monopolar probes itself caused direct SMA injury or induced, *e.g.*, a small hematoma in the vicinity of the SMA, resulting in occlusion of the already narrowed vessel.

COMMENTS

Case characteristics

A 66-year-old man presented with locally advanced pancreatic head adenocarcinoma.

Differential diagnosis

Chronic inflammation, desmoids reaction around the tumor.

Laboratory diagnosis

Tumor markers were measured, with CA 19-9 = 4801 IU/mL and CA 125 = 161 IU/mL.

Imaging diagnosis

Computerized tomography examination revealed a malignant mass of 45 mm in diameter was observed at the pancreatic head level and the tumor had surrounded the superior mesenteric artery 360 degrees.

Treatment

The patient received systemic chemotherapy following irreversible electroporation.

Related reports

This case is a unique case of superior mesenteric artery occlusion as a complication of irreversible electroporation.

Term explanation

Irreversible electroporation creates pores in cell membrane by transmitting short electrical currents at high voltage. As a result, the phospholipid membrane of the cell is permanently damaged, and the apoptosis and necrosis complex is observed due to the impairment of cellular homeostasis.

Experiences and lessons

While a general clinical decision making algorithm would not be appropriate and each clinical situation needs to be individualized. As in this case, if there is a severe vascular invasion is decreasing the diameter more than 50%, a coated vascular stent could be placed to the narrowed segment before applying irreversible electroporation.

Peer-review

The manuscript by Ekici *et al* describes a case of arterial complication following irreversible electroporation (IRE) for locally advanced pancreatic cancer. IRE is one of the new methods for local therapy of non-metastatic but locally advanced pancreatic cancers. The present case report is both timely and important, highlighting a potential adverse event using this technique.

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- 757 Robotic rectal surgery: State of the art

Staderini F, Foppa C, Minuzzo A, Badii B, Qirici E, Trallori G, Mallardi B, Lami G, Macrì G, Bonanomi A, Bagnoli S, Perigli G, Cianchi F

- 772 Molecular predictive markers in tumors of the gastrointestinal tract

Papadopoulou E, Metaxa-Mariatou V, Tsaousis G, Tsoulos N, Tsirigoti A, Efstathiadou C, Apessos A, Agiannitopoulos K, Pepe G, Bourkoulas E, Nasioulas G

MINIREVIEWS

- 786 Clinical impact of chemotherapy to improve tumor microenvironment of pancreatic cancer

Tsuchikawa T, Takeuchi S, Nakamura T, Shichinohe T, Hirano S

- 793 Current noninvasive tests for colorectal cancer screening: An overview of colorectal cancer screening tests

Song LL, Li YM

CASE REPORT

- 801 Case of pseudo-Meigs' syndrome caused by gastric cancer-related metastatic ovarian tumor with prolonged survival

Okamoto M, Maeda K, Yanagitani A, Tanaka K

Contents

World Journal of Gastrointestinal Oncology
Volume 8 Number 11 November 15, 2016

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Fabio Staderini, Caterina Foppa, Alessio Minuzzo, Benedetta Badii, Etleva Qirici, Giuliano Perigli, Fabio Cianchi, Center of Oncological Minimally Invasive Surgery, Department of Surgery and Translational Medicine, University of Florence, 50134 Florence, Italy

Giacomo Trallori, Department of Experimental and Clinical Biomedical Sciences, University of Florence, 50134 Florence, Italy

Beatrice Mallardi, Istituto per lo Studio e Prevenzione Oncologica, 50134 Florence, Italy

Gabriele Lami, Giuseppe Macri, Department of Experimental and Clinical Biomedical Sciences, University of Florence, 50134 Florence, Italy

Andrea Bonanomi, Siro Bagnoli, Unit of Gastroenterology, AOU Careggi, 50134 Florence, Italy

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Correspondence to: Fabio Cianchi, MD, Center of Oncological Minimally Invasive Surgery, Department of Surgery and Translational Medicine, University of Florence, Largo Brambilla 3, 50134 Florence, Italy. fabio.cianchi@unifi.it
Telephone: +39-33-9307447

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Abstract

Laparoscopic rectal surgery has demonstrated its superiority over the open approach, however it still has some technical limitations that lead to the development of robotic platforms. Nevertheless the literature on this topic is rapidly expanding there is still no consensus about benefits of robotic rectal cancer surgery over the laparoscopic one. For this reason a review of all the literature examining robotic surgery for rectal cancer was performed. Two reviewers independently conducted a search of electronic databases (PubMed and EMBASE) using the key words "rectum", "rectal", "cancer", "laparoscopy", "robot". After the initial screen of 266 articles, 43 papers were selected for review. A total of 3013 patients were included in the review. The most commonly performed intervention was low anterior resection (1450 patients, 48.1%), followed by anterior resections (997 patients, 33%), ultra-low anterior resections (393 patients, 13%) and abdominoperineal resections (173 patients, 5.7%). Robotic rectal surgery seems to offer potential advantages especially in low anterior resections with lower conversions rates and better preservation of the autonomic function. Quality of mesorectum and status of and circumferential resection margins are similar to those obtained with conventional laparoscopy even if robotic rectal surgery is undoubtedly associated with longer operative times. This review demonstrated that robotic rectal surgery is both safe and feasible but there is no evidence of its superiority over laparoscopy in terms of postoperative, clinical outcomes and incidence of complications. In conclusion robotic rectal surgery seems to overcome some of

technical limitations of conventional laparoscopic surgery especially for tumors requiring low and ultra-low anterior resections but this technical improvement seems not to provide, until now, any significant clinical advantages to the patients.

Key words: Robotic surgery; Robotic rectal surgery; DaVinci rectal surgery; Robotic rectal cancer; Robotics for rectal cancer; Robotic rectal resection

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Core tip: Laparoscopic rectal surgery has progressively expanded. However it has some technical limitations. The need to overcome these limitations leads to the development of robotic platforms. Although the positive feedback is by the surgeons, there is still no evidence in literature about the superiority of robotic rectal surgery when compared to traditional laparoscopy.

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INTRODUCTION

Laparoscopic colorectal surgery has progressively expanded since a number of randomized controlled trials (RCTs)^[1-3], review articles^[4,5], meta-analysis^[6] and case series^[7] have demonstrated its better postoperative outcomes when compared to open surgery. However, laparoscopic surgery has some technical limitations such as poor ergonomics, 2-dimension view, coning and fulcrum effect, that may influence surgery in narrow anatomical fields such as in the pelvis during rectal surgery. The need to overcome these limitations leads to the development of robotic platforms. The da Vinci robotic surgical system is the only totally robotic platform available. After approval by Food and Drug Administration in 2000, its use progressively spreaded as demonstrated by the increasing number of publications. Three-D high definition vision, wrist-like movement of instruments (endowrist™), stable camera holding, motion filter for tremor-free surgery and improved ergonomics for the surgeon are the advantages of the robotic system that may make rectal surgery more affordable and theoretically should provide better outcomes for the patient. Although the positive feedback is by the surgeons, there is still no evidence in literature about the superiority of robotic rectal surgery when compared to traditional laparoscopy. The aim of this study was to review the rapidly expanding literature in order to focus on the current state and assess any

benefits of robotic rectal cancer surgery.

RESEARCH AND LITERATURE

A review of the literature examining robotic surgery for rectal cancer during the period from 2000 to 2015 was performed. Two reviewers independently conducted a search of electronic databases (PubMed and EMBASE) using the key words "rectum", "rectal", "cancer", "laparoscopy", "robot". The reference lists provided by the identified articles were additionally hand-searched to prevent article loss by the search strategy. This method of cross-references was continued until no further relevant publications were identified. The last search was performed on December 2015. Inclusion criteria were prospective, retrospective, randomized, comparative studies about robotic rectal surgery for cancer including anterior resections, low anterior resections, ultralow anterior resections, abdominoperineal resections, proctectomies, proctocolectomies. Exclusion criteria were: Abstracts, letters, editorials, technical notes, expert opinions, reviews, meta-analysis, studies reporting benign pathologies, studies in which the outcomes and parameters of patients were not clearly reported, studies in which it was not possible to extract the appropriate data from the published results, overlap between authors and centers in the published literature, non-English language papers.

The literature search yielded 266 papers, the process is listed in Figure 1. After the 1st filtering, the remaining 60 studies were 33 comparative, 26 case series, and 1 RCT. Then 17 studies were excluded due to duplicated data. They were 7 comparative and 9 case series. After this process a total of 43 papers, 27 comparative including only 1 RCT and 16 case series were included and reviewed.

STUDIES OVERVIEW

The number of publications about robotic rectal surgery for cancer has been constantly increasing. Among the papers we included there was only 1 paper per year published in 2006, 2007, 2008, 3 papers in 2009, 2 in 2010, 5 per year in 2011 and 2012, 10 in 2013 and 15 in 2014. With regard to the nationality of the 1st author there were 16 studies in the South Korea (37.2%), 11 in the United States (25.5%), 4 in Italy (9.3%), 2 in Turkey (4.6%), 2 in the Singapore (4.6%), 1 in Japan (2.3%), 1 in Denmark (2.3%), 1 in Spain (2.3%), 1 in Romania (2.3%), 1 in Brazil (2.3%), 1 in Canada (2.3%), 1 in Taiwan (2.3%), 1 in China (2.3%) (Table 1).

Surgical technique

A total of 3013 robotic operations were performed. Sixteen studies^[10,12,14,16,17,22,23,25,27,28,37,38,40-42,48] (1257 patients) reported a totally robotic procedure which was carried out with either a single^[10,16,17,22,23,25,27,28,37,38,40-42,48] or a double docking^[12,28] technique. In 22 studies^[8,13,15,18,20,21,25,26,30-34,36,39,43-47,49,50] (1384 patients) an

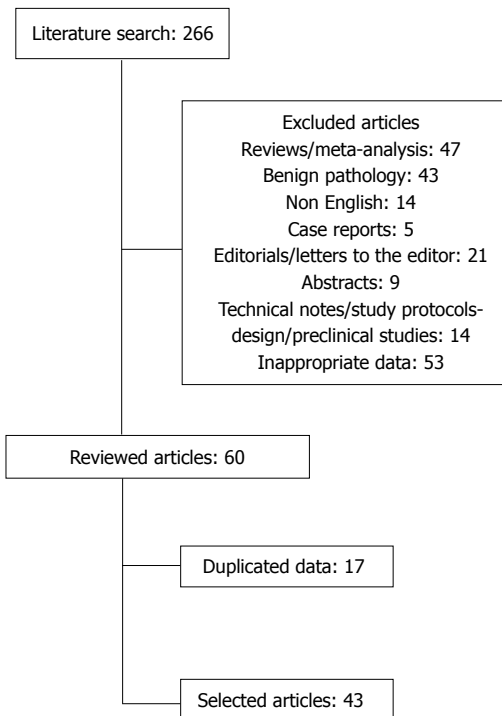


Figure 1 Flow diagram of literature search.

hybrid robotic technique was performed: The inferior mesenteric vessels ligation and splenic flexure mobilization were performed laparoscopically whereas pelvic dissection and total mesorectal excision were performed robotically. In 5 studies^[9,11,19,29,35] (372 patients) the robotic technique was not specified. Laparoscopic procedures described in the 27 comparative studies^[8-33] were performed in the same manner as robotic surgery using laparoscopic instruments (Table 1).

Demographics and preoperative data

Most of patients were male (1911, 63%), the mean age was 58, the mean BMI was 26.6. Nine hundred-eight patients (20%) underwent a neoadjuvant chemotherapy, 71 (2.3%) a neoadjuvant chemo-radiotherapy and 8 (0.2%) radiotherapy only. With regard to the type of operation, 1450 (48.1%) were low anterior resections, 997 (33%) were anterior resections (AR), 393 (13%) ultra-low anterior resections (ULAR) and 173 (5.7%) abdominoperineal resections (APR). In the studies where the type of operation was not specified and where it was stated that a TME was performed^[27,29,41] we assumed that all operations were low anterior resections (LAR) (Table 2)

Operative data

The mean robotic operative time ranged from 202 min^[31] to 485.8 min^[17]. For the 1345 laparoscopic patients in the selected comparative studies the mean operative time ranged from 158.1^[30] to 374.3 min^[17]. This difference was statistically significant in 12 comparative studies^[10,14,17-24,27,28,30] with a longer time for robotic surgery. Levic *et al.*^[9] were the only authors that reported

a longer laparoscopic operative time ($P = 0.055$), but all interventions were performed with a single port technique (Table 3).

The estimated blood loss (EBL) was not reported in 14 studies. The mean value ranged from 17 mL^[36] to 280 mL^[14] with the robotic approach and from 59.2^[18] to 271.4^[15] in the laparoscopic group. Among 16 comparative studies^[8-10,12-15,17,19-21,23,24,29,31,33] that evaluated the EBL only Kang *et al.*^[23] and Erguner *et al.*^[21] reported a significantly lower EBL with the robotic approach when compared to the laparoscopic one.

Thirty seven studies reported the conversion rate to open surgery. Three^[8,22,31] out of 22 comparative studies^[8-15,17,19-25,28-33] showed a significantly lower conversion rate in the robotic series when compared to laparoscopy. The difference in overall conversion rate reported by Ielpo *et al.*^[14] was not statistically significant. However, when data were analyzed according to the tumor location (upper, mid, lower rectum), the conversion rates between robotic and laparoscopic procedures for lower rectal cancers were respectively 1.8% and 9.2% ($P = 0.04$).

The rate of patients that underwent a protective ileostomy creation ranged from 0%^[30] to 100%^[10] both in the robotic and laparoscopic group. The difference in protective ileostomy creation was statistically significant in 5 studies. Kuo *et al.*^[17] reported a lower rate in the robotic vs the laparoscopic group whereas Saklani *et al.*^[19], Erguner *et al.*^[21], Kim *et al.*^[25], Baek *et al.*^[29] showed a lower rate in the laparoscopic vs the robotic group.

Postoperative data

The mean postoperative day to first flatus ranged from 1.9^[48] to 3.2^[30] d in the robotic cases and from 2.4^[23] to 3.4^[17] in the laparoscopic ones. No statistically significant difference between robotic and laparoscopic cases was reported in any of the articles reviewed (Table 4).

The day of first postoperative liquid diet was available in 11 studies^[6,22,27,29,34,36,43,45,47,48,50] ranging from 1^[16] to 3.9^[45] d in the robotic cases. Only two^[22,29] comparative studies reported the first postoperative liquid diet in their robotic and laparoscopic series, in one^[22] of these the difference was statistically significant in favour of robotic surgery (3 d vs 5 d, $P = 0.005$).

The day of first postoperative solid diet was available in 11 studies^[8,10,13,17,19,23-25,30,34,37] ranging from 2.58^[10] to 7.5^[18] d in the robotic cases and from 2.48^[10] to 7.7^[18] d in laparoscopic cases. Among 9 comparative studies^[8,10,13,17,19,23-25,30] only Kang *et al.*^[23] reported a significant earlier oral intake in the robotic group (4.5 d vs 5.2 d, $P = 0.004$) when compared to the laparoscopic one.

The mean length of hospital stay ranged from 4.5^[33] to 14.2^[17] and from 3.6^[33] to 15.1^[17] d after robotic and laparoscopic surgery respectively. Among 8 comparative studies, Tam *et al.*^[15], Levic *et al.*^[9] and Park *et al.*^[30] reported a shorter length of stay in their laparoscopic series whereas 5^[8,22-24,32] studies reported a significant

Table 1 Studies overview

Ref.	Year	Country	Study design	Surgical technique	Platform	No. of pts Robot	No. of pts Lap	No. of pts Open
Park <i>et al</i> ^[8]	2015	South Korea	Comparative	Hybrid	DV	133	84	
Levic <i>et al</i> ^[9]	2014	Denmark	Comparative	NS	DV	56	36	
Yoo <i>et al</i> ^[10]	2014	South Korea	Comparative	Tot rob	NS	44	26	
Koh <i>et al</i> ^[11]	2014	Singapore	Comparative	NS	NS	19	19	
Melich <i>et al</i> ^[12]	2014	Canada	Comparative	Tot rob	DV	92	106	
Barnajian <i>et al</i> ^[13]	2014	United States	Comparative	Hybrid	DV-S	20	20	20
Ielpo <i>et al</i> ^[14]	2014	Spain	Comparative	Tot rob	NS	56	87	
Tam <i>et al</i> ^[15]	2014	United States	Comparative	Hybrid	DV	21	21	
Ghezzi <i>et al</i> ^[16]	2014	Brazil	Comparative	Tot rob	DV-S	65		109
Kuo <i>et al</i> ^[17]	2014	Taiwan	Comparative	Tot rob	DV	36	28	
Park <i>et al</i> ^[18]	2014	South Korea	Comparative	Hybrid	DV	32	32	
Saklani <i>et al</i> ^[19]	2013	South Korea	Comparative	NS	NS	74	64	
Fernandez <i>et al</i> ^[20]	2013	United States	Comparative	Hybrid	DV-S	13	59	
Erguner <i>et al</i> ^[21]	2013	Turkey	Comparative	Hybrid	NS	27	37	
D'Annibale <i>et al</i> ^[22]	2013	Italy	Comparative	Tot rob	DV-S	50	50	
Kang <i>et al</i> ^[23]	2013	South Korea	Comparative	Tot rob	NS	165	165	165
Park <i>et al</i> ^[24]	2013	South Korea	Comparative	Hybrid	DV	40	40	
Kim <i>et al</i> ^[25]	2012	South Korea	Comparative	Tot rob	DV	62	147	
Kim <i>et al</i> ^[26]	2012	South Korea	Comparative	Hybrid	DV	30	39	
Bertani <i>et al</i> ^[27]	2011	Italy	Comparative	Tot rob	DV	52		34
Kwak <i>et al</i> ^[28]	2011	South Korea	Comparative	Tot rob	DV	59	59	
Baek <i>et al</i> ^[29]	2011	United States	Comparative	NS	NS	41	41	
Park <i>et al</i> ^[30]	2011	South Korea	Comparative	Hybrid	DV	52	123	88
Patriti <i>et al</i> ^[31]	2009	Italy	Comparative	Hybrid	DV	29	37	
Baik <i>et al</i> ^[32]	2008	South Korea	Comparative	Hybrid	DV	18	18	
Pigazzi <i>et al</i> ^[33]	2006	United States	Comparative	Hybrid	DV	6	6	
Parisi <i>et al</i> ^[34]	2014	Italy	Case series	Hybrid	DV Si	40		
Baek <i>et al</i> ^[35]	2014	South Korea	Case series	NS	NS	182		
Shiomi <i>et al</i> ^[36]	2014	Japan	Case series	Hybrid	DV	113		
Kim <i>et al</i> ^[37]	2014	South Korea	Case series	Tot rob	DV-S	200		
Stănculea <i>et al</i> ^[38]	2013	Romania	Case series	Tot rob	DV-Si	100		
Zawadzki <i>et al</i> ^[39]	2013	United States	Case series	Hybrid	DV	77		
Sng <i>et al</i> ^[40]	2013	South Korea	Case series	Tot rob	DV-S	197		
Du <i>et al</i> ^[41]	2013	China	Case series	Tot rob	DV	22		
Alimoglu <i>et al</i> ^[42]	2012	Turkey	Case series	Tot rob	DV	7		
Akmal <i>et al</i> ^[43]	2012	United States	Case series	Hybrid	DV	80		
Park <i>et al</i> ^[44]	2012	United States	Case series	Hybrid	DV-S	30		
Kang <i>et al</i> ^[45]	2011	South Korea	Case series	Hybrid	DV	389		
deSouza <i>et al</i> ^[46]	2010	United States	Case series	Hybrid	DV	44		
Pigazzi <i>et al</i> ^[47]	2010	United States	Case series	Hybrid	DV	143		
Choi <i>et al</i> ^[48]	2009	South Korea	Case series	Tot rob	DV	50		
Ng <i>et al</i> ^[49]	2009	Singapore	Case series	Hybrid	DV	8		
Hellan <i>et al</i> ^[50]	2007	United States	Case series	Hybrid	DV	39		

Tot rob: Totally Robotic; DV: Da Vinci; NS: Not specified.

shorter length of stay after robotic surgery.

No statistically significant differences in the overall 30 d mortality between the robotic and laparoscopic approach was found among 15 comparative studies^[8-11,13,14,19-24,29-31] (0.10% and 0.45% respectively).

Twenty-three studies reported the reintervention rate. In the robotic series it ranged from 0%^[8,22,32,33,42,48] to 15%^[20] whereas it ranged from 0%^[32,33] to 15.7%^[11] after laparoscopic surgery. The most common cause of reintervention was anastomotic leak in both the robotic and laparoscopic groups. No statistically significant differences were found in any of the 12 comparative studies^[11-15,20-24,32,33].

The overall complication rate in the robotic and laparoscopic groups was 24.5% and 27.7% respectively. No significant differences in this parameter

were reported between the robotic and laparoscopic series^[8-11,13-15,19-25,28-33]. The most frequent complication in both the robotic and laparoscopic cases was anastomotic leak followed by bowel obstruction and urinary complications (Table 5). Thirteen studies^[10,18,19,22-24,26,31,37,38,40,44,45] reported urinary and sexual dysfunction after rectal surgery, 9 of these were comparative. Park *et al*^[18] reported an earlier and significant restoration of erectile function after robotic surgery when compared to the laparoscopic one. Kim *et al*^[26] observed an earlier recover of urinary function after robotic intervention within six months from the operation ($P = 0.001$). After 6 mo the difference was no more statistically significant.

Table 6 shows the studies which classified complications according to the Clavien Dindo Scoring System. Clavien-Dindo 1 and 2 were the most frequent

Table 2 Demographics and preoperative data

Ref.	M/F	Age	BMI	ASA				Preop CHT	Type of operation			
				1	2	3	4		AR	LAR	ULAR	APR
Park <i>et al</i> ^[8]	86/47	59.2 (32-86)	23.1 (14.6-32.8)	94	31	8	0	15	100	33	0	0
Levic <i>et al</i> ^[9]	34/22	65 (23-83)	24.8 (16-34.5)	17	35	4	0	15	0	41 ¹	0	15
Yoo <i>et al</i> ^[10]	35/9	59.77 (+ 12.33)	24.13 (+ 3.33)	26	17	1	0	24	0	0	44	0
Koh <i>et al</i> ^[11]	15/4	62 (47-92)	-	5	14	0	0	8	0	0	17	2
Melich <i>et al</i> ^[12]	52/40	60 (57.7-62.2)	23.1 (22.5-23.7)		1 (1-3)			13	0	92	0	0
Barnajian <i>et al</i> ^[13]	12/8	62 (44-82)	22 (18-31)	0	4	16	0	10	0	15	0	5
Ielpo <i>et al</i> ^[14]	25/31	43.4 (+ 11)	22.8 (+ 2.5)	11	32	11	0	46	0	40	1	15
Tam <i>et al</i> ^[15]	10/11	60 (41-73)	25 (20-37)	-	-	-	-	18	11	1	4	5
Ghezzi <i>et al</i> ^[16]	41/24	61	24.7	12	49	4	0	47	0	44	11	10 ²
Kuo <i>et al</i> ^[17]	21/15	55.9 (30-89)	-	0	33	3	0	28	0	0	36	0
Park <i>et al</i> ^[18]	32/0	-	23.8	-	-	-	-	15 (+ RT)	0	22	9	1
Saklani <i>et al</i> ^[19]	50/24	59.6 (32-85)	23.4 (16.9-29.8)	50	24	0	0	74	0	46	26	2
Fernandez <i>et al</i> ^[20]	13/0	67.9 (+ 2.1)	-	0	0	11	2	10	0	5	0	8
Erguner <i>et al</i> ^[21]	14/13	54 (24-78)	28.3 (19.8-30.8)	-	-	-	-	4	0	27	0	0
D'Annibale <i>et al</i> ^[22]	30/20	66 (+ 12.1)	-	-	-	-	-	34 (+ RT)	17	33	0	0
Kang <i>et al</i> ^[23]	104/61	61.2 (+ 11.4)	23.1 (+ 2.8)	109	56	0	0	39	165 ³	0	0	0
Park <i>et al</i> ^[24]	41/21	56	24.2	33	28	1	0	9	0	51	10	1
Kim <i>et al</i> ^[25]	28/12	57.3	23.9	27	9	4	0	32	0	0	40	0
Kim <i>et al</i> ^[26]	18/12	54.13 (+ 8.52)	24.36 (+ 2.4)	29	1	0	0	10	29	1 ³	0	0
Bertani <i>et al</i> ^[27]	31/21	59.6 (+ 11.6)	24.8 (+ 3.62)	49			3	24	0	52	0	0
Kwak <i>et al</i> ^[28]	39/20	60 (53-68)	23.3 (21.8-25.2)	28	27	4	0	8 (RT)	0	54	5	0
Baek <i>et al</i> ^[29]	25/16	63.6 (48-87)	-	0	18	22	1	33	0	33	2	6
Park <i>et al</i> ^[30]	28/24	57.3	23.7	21	26	5	0	12 (+ RT)	52	0	0	0
Patriti <i>et al</i> ^[31]	11/18	68	24	2	13	14	0	7 (+ RT)	29	0	0	0
Baik <i>et al</i> ^[32]	14/4	57.3 (37-79)	22.8 (19.4-31.7)	12	6	0	0	-	18	0	0	0
Pigazzi <i>et al</i> ^[33]	2/4	60 (42-78)	31 (25-36)	0	2	4	0	2	0	6	0	0
Parisi <i>et al</i> ^[34]	19/21	67 (39-86)	25.22 (18.36-33.20)	20	14	6	0	17	0	35	0	5
Baek <i>et al</i> ^[35]	117/65	57.6 (26-78)	23.4 (14.8-30.5)	111	65	6	0	50	0	182	0	0
Shiomi <i>et al</i> ^[36]	78/35	64 (23-84)	23.4 (16.7-30.6)	39	74	0	0	3	11	71	23	8
Kim <i>et al</i> ^[37]	134/66	58.15	23.85	-	-	-	-	43	0	200	0	0
Stănciulea <i>et al</i> ^[38]	66/34	62 (32-84)	26 (16.4-38)	-	-	-	-	58	30	39	8	23
Zawadzki <i>et al</i> ^[39]	45/32	60.1 (34-82)	28 (18-43)	62	15	0	48	0	68	9	0	0
Sng <i>et al</i> ^[40]	131/66	60 (20-89)	23.5 (16.9-33.1)	117	71	9	0	54	3	126	55	13
Du <i>et al</i> ^[41]	14/8	56.4 (+ 7.8)	22.5 (+ 2.1)	-	-	-	-	-	0	22	0	0
Alimoglu <i>et al</i> ^[42]	5/2	52.9 (32-88)	-	-	-	-	-	4	0	0	0	7
Akmal <i>et al</i> ^[43]	50/30	60.35 (24-85)	27.2 (18-44)	0	37	39	4	62	0	40	21	19
Park <i>et al</i> ^[44]	16/14	58	27.6	0	12	18	0	20	0	5	19	6
Kang <i>et al</i> ^[45]	252/137	59 (26-86)	-	280	107	2	0	72	382	1 ³	0	6
deSouza <i>et al</i> ^[46]	28/16	63	-	4	27	13	0	31	0	30	6	8
Pigazzi <i>et al</i> ^[47]	87/56	62 (26-87)	26.5 (16.5-44)	0	0	57	93 (+ RT)	0	80	32	31	0
Choi <i>et al</i> ^[48]	32/18	58.5 (30-82)	23.2 (19.4-29.2)	27	19	4	0	3 (+ RT)	0	40	8	2
Ng <i>et al</i> ^[49]	5/3	55 (42-80)	-	-	-	-	-	-	2	0	6	0
Hellan <i>et al</i> ^[50]	21/18	58 (26-84)	26 (16-44)	0	0		17	33	0	22	11	6

¹9 hartmann; ²1 Posterior pelvic exenteration; ³1 hartmann. AR: Anterior resections; ULAR: Ultra-low anterior resections; APR: Abdominoperineal resections; CHT: Chemotherapy; BMI: Body mass index; ASA: American society anesthesiologists.

complications in both groups (13.8% robotic vs 12.4% laparoscopic).

Oncological outcome

The mean number of harvested nodes ranged from 10^[14] to 20.6^[48] and from 9^[14] to 21^[10] in the robotic and laparoscopic cases respectively. Three of 22 comparatives^[8-15,17,19-25,28-33] studies reported a statistically significant difference in the number of harvested nodes between the robotic and laparoscopic approach: Levic *et al*^[9] and D'Annibale *et al*^[22] showed an higher number of examined nodes after robotic surgery whereas Yoo *et al*^[10] showed an higher number of examined nodes after laparoscopic surgery (Table 7).

The mean length of distal resection margins

after robotic rectal surgery was available in 20 studies^[8-10,13,15-17,19,21-38,40,41,43,45,48,50]. It ranged from 13.3 mm^[10] to 460 mm^[15]. Tumor involvement rate of distal margins was available 21 studies^[8,9,11,12,15,17,20,21,23,25,26,28-30,34,36,37,39,46,48,50] and ranged from 0%^[8,15,17,20,21,25,26,28-30,34,36,37,48,50] to 2.6%^[39] of patients. An involvement of distal resection margin was found in 6 (0.47%) out of 1257 patients operated on with the robotic technique.

The mean length of distal resection margins after laparoscopic rectal surgery was available in 19^[8-10,13,15,17,19,21-26,28-33] studies. It ranged from 13 mm^[25] to 510 mm^[15]. The involvement of distal margins was available in 14 studies^[8,9,11,12,15,17,20,21,23,25,26,28-30] and ranged from 0%^[8,9,11,12,15,21,23,25,26,28-30] to 5%^[15] of patients. A distal margin positivity was reported in 3 (0.3%) out of 857

Table 3 Operative data

Ref.	Patients	Mesorectum	Technique	Mean operative time (min)	EBL (mL)	Conversion to open (%)	Stoma (%)
Park <i>et al</i> ^[8]	133	RME	Hybrid	205.7 (109-505)	77.6 (0-700)	0 (0)	29 (21.8)
	84	LME	Tot lap	208.8 (94-407)	82.3 (0-1100)	6 (7.1)	20 (23.8)
Levic <i>et al</i> ^[9]	56	RME	NS	247 (135-111) ¹	50 (0-400) ¹	3 (5.4)	31 (55.3)
	36	LME	SP	295 (108-465) ¹	35 (0-400) ¹	0 (0)	9 (25)
Yoo <i>et al</i> ^[10]	44	RME	Tot rob	316.43 (+ 65.11)	239.77 (+ 278.61)	0 (0)	44 (100)
	26	LME	Tot lap	286.77 (+ 51.46)	215.38 (+ 247.29)	0 (0)	26 (100)
Koh <i>et al</i> ^[11]	19	RME	NS	390 (289-771) ¹	-	1 (5.2)	17 (89)
	19	LME	HAL	225 (130-495) ¹	-	5 (26.3)	0 (0)
Melich <i>et al</i> ^[12]	92	RME	Tot rob	285 (266-305)	201 (165-237)	1 (1.1)	-
	106	LME	Tot lap	262 (252-272)	232 (191-272)	4 (3.8)	-
Barnajian <i>et al</i> ^[13]	20	RME	Hybrid	240 (150-540) ¹	125 (50-650) ¹	0 (0)	11 (55)
	20	LME	Tot lap	180 (140-480) ¹	175 (50-900) ¹	2 (10.5)	11 (55)
	20	OME	Open	240 (115-475) ¹	250 (50-800) ¹	na	12 (60)
Ielpo <i>et al</i> ^[14]	56	RME	Tot rob	309 (150-540)	280 (0-4000)	2 (3.5)	28 (50)
	87	LME	Tot lap	252 (180-420)	240 (0-4000)	10 (11.5)	53 (60.9)
Tam <i>et al</i> ^[15]	21	RME	Hybrid	274.8 (189-449)	252.6 (30-2000)	1 (4.7)	13 (62)
	21	LME	Tot lap	236.3 (171-360)	271.4 (50-1200)	0 (0)	11 (52)
Ghezzi <i>et al</i> ^[16]	65	RME	Tot rob	299 (+ 58)	0 (0-175) ¹	1 (1.5)	51 (91.1)
	109	OME	Open	207 (+ 56.5)	150 (0-400) ¹	na	66 (63.3)
Kuo <i>et al</i> ^[17]	36	RME	NS	485.8 (315-720)	80 (30-200)	0 (0)	7 (19.4)
	28	LME	Tot lap	374.3 (210-570)	103.6 (30-250)	0 (0)	13 (46.4)
Park <i>et al</i> ^[18]	32	RME	Hybrid	-	-	-	3 (9.4)
	32	LME	Tot lap	-	-	-	3 (9.4)
Saklani <i>et al</i> ^[19]	74	RME	NS	365.2 (150-710)	180 (0-1100)	1 (1.4)	53 (71.6)
	64	LME	Tot lap	311.6 (180-530)	210 (0-1200)	4 (6.3)	35 (54.7)
Fernandez <i>et al</i> ^[20]	13	RME	Hybrid	528 (416-700) ¹	157 (50-550) ¹	1 (8)	-
	59	LME	HAL	344 (183-735) ¹	200 (25-1500) ¹	10 (17)	-
Erguner <i>et al</i> ^[21]	27	RME	Hybrid	280 (175-480)	50 (20-100)	0 (0)	19 (70.3)
	37	LME	Tot lap	190 (110-300)	125 (50-400)	0 (0)	13 (35.1)
D'Annibale <i>et al</i> ^[22]	50	RME	Tot rob	270 (240-315) ¹	-	0 (0)	-
	50	LME	Tot lap	280 (240-350) ¹	-	6 (12)	-
Kang <i>et al</i> ^[23]	165	RME	Tot rob	309.7 (+ 115.2)	133 (+ 192.3)	1 (0.6)	41 (25)
	165	LME	Tot lap	277.8 (+ 81.9)	140.1 (+ 216.4)	3 (1.8)	43 (27.2)
	165	OME	Open	252.6 (+ 88.1)	275.4 (+ 368.4)	na	47 (31.8)
Kim <i>et al</i> ^[25]	62	RME	Tot rob	390 (+ 97)	-	3 (4.8)	22 (35.5)
	147	LME	Tot lap	285 (+ 80)	-	5 (3.4)	34 (23.1)
Park <i>et al</i> ^[24]	40	RME	Hybrid	235.5 (+ 57.5)	45.7 (+ 40)	0 (0)	14 (35)
	40	LME	Tot lap	185.4 (+ 72.8)	59.2 (+ 35.8)	0 (0)	6 (15)
Kim <i>et al</i> ^[26]	30	RME	Hybrid	-	-	-	-
	39	LME	Tot lap	-	-	-	-
Bertani <i>et al</i> ^[27]	52	RME	Tot rob	260 (190-570)	100 (50-1000)	-	-
	34	OME	Tot lap	164 (100-350)	120 (50-2000)	-	-
Kwak <i>et al</i> ^[28]	59	RME	Tot rob	270 (241-325) ¹	-	0 (0)	25 (42.4)
	59	LME	Tot lap	228 (177-254) ¹	-	2 (3.4)	26 (44.1)
Baek <i>et al</i> ^[29]	41	RME	NS	296 (150-520)	200 (20-2000) ¹	3 (7.3)	33 (94.3)
	41	LME	NS	315 (174-584)	300 (17-1000) ¹	9 (22)	14 (40)
Park <i>et al</i> ^[30]	52	RME	Hybrid	232.6 (+ 54.2)	-	0 (0)	1 (1.9)
	123	LME	Tot lap	158.1 (+ 49.2)	-	0 (0)	5 (4.1)
	88	OME	Open	233.8 (+ 59.2)	-	na	4 (4.5)
Patrity <i>et al</i> ^[31]	29	RME	Hybrid	202 (+ 12)	137.4 (+ 156)	0 (0)	0 (0)
	37	LME	Tot lap	208 (+ 7)	127 (+ 169)	7 (19)	0 (0)
Baik <i>et al</i> ^[32]	18	RME	Hybrid	217.1 (149-315)	-	0 (0)	-
	18	LME	Tot lap	204.3 (114-297)	-	2 (11)	-
Pigazzi <i>et al</i> ^[33]	6	RME	Hybrid	264 (192-318)	104 (50-200)	0 (0)	-
	6	LME	Tot lap	258 (198-312)	150 (50-300)	0 (0)	-
Parisi <i>et al</i> ^[34]	40	RME	Hybrid	340 (235-460) ¹	50 (20-250) ¹	0 (0)	22 (55)
Baek <i>et al</i> ^[35]	182	RME	NS	-	-	-	-
Shiomi <i>et al</i> ^[36]	113	RME	Hybrid	302 (135-683) ¹	17 (0-690) ¹	0 (0)	-
Kim <i>et al</i> ^[37]	200	RME	Tot rob	308.3	-	1 (0.5)	9 (4.5)
Stănciulea <i>et al</i> ^[38]	100	RME	Tot rob	-	150 (0-250) ¹	4 (4)	64 (64)
Zawadzki <i>et al</i> ^[39]	77	RME	Hybrid	327 (178-510) ¹	189 (30-1000) ¹	3 (3.9)	53 (69)
Sng <i>et al</i> ^[40]	197	RME	Tot rob	278.7 (145-515)	< 50 (50-1500) ¹	0 (0)	-
Du <i>et al</i> ^[41]	22	RME	Tot rob	220 (152-286)	33 (10-70)	0 (0)	-
Alimoglu <i>et al</i> ^[42]	7	RME	Tot rob	-	-	0 (0)	-
Akmal <i>et al</i> ^[43]	80	RME	Hybrid	303.5	-	4 (5)	46 (57.5)
Park <i>et al</i> ^[44]	30	RME	Hybrid	369 (306-410) ¹	100 (75-200) ¹	-	-
Kang <i>et al</i> ^[45]	389	RME	Hybrid	322.35	-	3 (0.7)	93 (24)

deSouza <i>et al</i> ^[46]	44	RME	Hybrid	347 (155-510) ¹	150 (50-1000) ¹	-	34 (77.2)
Pigazzi <i>et al</i> ^[47]	143	RME	Hybrid	297 (90-660)	283 (0-6000)	7 (4.9)	71 (50)
Choi <i>et al</i> ^[48]	50	RME	T Tot rob	304.8 (190-485)	-	0 (0)	16 (32)
Ng <i>et al</i> ^[49]	8	RME	Hybrid	278.7 (145-515)	-	0 (0)	6 (75)
Hellan <i>et al</i> ^[50]	39	RME	Hybrid	285 (180-540) ¹	200 (25-6000) ¹	1 (2.5)	4 (10.2)

Tot rob: Totally robotic; Tot lap: Totally laparoscopic; HAL: Hand assisted laparoscopy; SP: Single port; NS: Not specified. ¹Median. EBL: Estimated blood loss; RME: Robotic mesorectal excision; LME: Laparoscopic mesorectal excision; OME: Open mesorectal excision.

Table 4 Postop data

Ref.	Pts	Mesorectum	Flatus (POD)	Liquid diet (POD)	Solid diet (POD)	Length of stay (d)	30 d mortality (%)	Reinterventions (%)	
Park <i>et al</i> ^[8]	133	RME	2.42 (1-6)	-	4.92 (3-11)	5.86 (4-14)	0 (0)	-	
	84	LME	2.47 (1-6)	-	5.19 (2-11)	6.54 (3-25)	0 (0)	-	
Levic <i>et al</i> ^[9]	56	RME	-	-	-	8 (4-100)	0 (0)	-	
	36	LME	-	-	-	7 (3-51)	2 (5.6)	-	
Yoo <i>et al</i> ^[10]	44	RME	-	-	2.58 (+ 1.62)	11.41 (+ 5.56)	0 (0)	-	
	26	LME	-	-	2.48 (+ 1.53)	11.04 (+ 6.33)	0 (0)	-	
Koh <i>et al</i> ^[11]	19	RME	-	-	-	7 (4-21) ¹	0 (0)	1 (5.2)	Bleeding
	19	LME	-	-	-	6 (4-28) ¹	0 (0)	3 (15.7)	Adhesive SBO, colonic infarction, anastomotic leak
Melich <i>et al</i> ^[12]	92	RME	-	-	-	9.6 (8.3-11)	-	6 (6.5)	6 leak/abscess
	106	LME	-	-	-	9.9 (8.5-11.3)	-	5 (4.7)	4 leak/abscess, 1 obstruction due to adhesions
Barnajian <i>et al</i> ^[13]	20	RME	3 (1-8) ¹	-	4 (2-9) ¹	6 (4-31) ¹	0 (0)	2 (10)	Presacral bleeding, pelvic abscess
	20	LME	4 (3-13) ¹	-	4 (4-14) ¹	7 (5-36) ¹	0 (0)	1 (5)	Pancreatic tail injury
	20	OME	4 (2-8) ¹	-	4.5 (2-9) ¹	7 (3-16) ¹	0 (0)	2 (10)	Presacral bleeding, enterotomy
Ielpo <i>et al</i> ^[14]	56	RME	-	-	-	13 (5-60)	0 (0)	3 (5.3)	NS
	87	LME	-	-	-	10 (5-16)	0 (0)	3 (3.4)	NS
Tam <i>et al</i> ^[15]	21	RME	-	-	-	8.7 (4-23)	-	0 (0)	
	21	LME	-	-	-	6 (3-14)	-	1 (5)	Bleeding
Ghezzi <i>et al</i> ^[16]	65	RME	2 (1-2)	1 (1-2)	-	6 (5-8) ¹	0 (0)	3 (4.6)	NS
	109	OME	3 (2-5)	5 (4-6)	-	9 (8-10) ¹	0 (0)	2 (1.8)	NS
Kuo <i>et al</i> ^[17]	36	RME	2.9 (1-6)	-	6.4 (4-12)	14.2 (9-27)	-	-	
	28	LME	3.4 (1-11)	-	5.8 (3-16)	15.1 (7-57)	-	-	
Park <i>et al</i> ^[18]	32	RME	-	-	-	-	-	-	
	32	LME	-	-	-	-	-	-	
Saklani <i>et al</i> ^[19]	74	RME	2.45 (1-10)	-	4.6 (2-13)	8 (4-21)	0 (0)	-	
	64	LME	2.48 (1-6)	-	5.1 (2-14)	9.2 (5-29)	0 (0)	-	
Fernandez <i>et al</i> ^[20]	13	RME	-	-	-	13 ¹	0 (0)	2 (15)	SBO
	59	LME	-	-	-	8 ¹	1 (2)	7 (12)	NS
Erguner <i>et al</i> ^[21]	27	RME	-	-	-	-	1 (3.7)	1 (3.7)	Colonic necrosis
	37	LME	-	-	-	-	1 (2.7)	3 (8.1)	1 ileostomy retraction, 2 anastomotic leak
D'Annibale <i>et al</i> ^[22]	50	RME	-	3 (3-5) ¹	-	8 (7-11) ¹	0 (0)	0 (0)	
	50	LME	-	5 (4-6) ¹	-	10 (8-14) ¹	0 (0)	3 (6)	Anastomotic leak
Kang <i>et al</i> ^[23]	165	RME	2.2 (+ 1.1)	-	4.5 (+ 1.9)	10.8 (+ 5.5)	0 (0)	15 (9)	NS
	165	LME	2.4 (+ 1.2)	-	5.2 (+ 2.4)	13.5 (+ 9.2)	0 (0)	5 (15)	NS
	165	OME	3 (+ 1.4)	-	6.4 (+ 2.5)	16 (+ 8.6)	0 (0)	9 (5.4)	NS
Kim <i>et al</i> ^[25]	62	RME	-	-	6 (+ 5)	12 (+ 6)	-	-	
	147	LME	-	-	7 (+ 5)	14 (+ 9)	-	-	

Park <i>et al</i> ^[24]	40	RME	2.4 (+ 1.6)	-	7.5 (+ 3.5)	10.6 (+ 4.2)	0 (0)	2 (5)	Anastomotic leak
	40	LME	2.5 (+ 1.3)	-	7.7 (+ 2.3)	11.3 (+ 3.6)	0 (0)	1 (2.5)	Anastomotic leak
Kim <i>et al</i> ^[26]	30	RME	-	-	-	-	-	-	
	39	LME	-	-	-	-	-	-	
Bertani <i>et al</i> ^[27]	52	RME	2 (1-5)	2 (1-13)	-	6 (4-51) ¹	-	2 (4)	
	34	OME	3 (1-9)	3 (2-12)	-	7 (4-24) ¹	-	0 (0)	
Kwak <i>et al</i> ^[28]	59	RME	-	-	-	-	-	-	
	59	LME	-	-	-	-	-	-	
Baek <i>et al</i> ^[29]	41	RME	-	2.3 (1-13)	-	6.5 (2-33)	0 (0)	-	
	41	LME	-	2.4 (1-9)	-	6.6 (3-20)	0 (0)	-	
Park <i>et al</i> ^[30]	52	RME	3.2 (+ 1.8)	-	6.7 (+ 3.8)	10.4 (+ 4.7)	0 (0)	-	
	123	LME	3 (+ 1.1)	-	6.1 (+ 2.7)	9.8 (+ 3.8)	0 (0)	-	
	88	OME	4.4 (+ 3)	-	7.6 (+ 3.3)	12.8 (+ 7.1)	1 (1.1)	-	
Patriti <i>et al</i> ^[31]	29	RME	-	-	-	11.9 (6-29)	0 (0)	-	-
	37	LME	-	-	-	9.6 (5-37)	0 (0)	-	-
Baik <i>et al</i> ^[32]	18	RME	1.8 (1-2) ¹	-	-	6.9 (5-10) ¹	-	0 (0)	
	18	LME	2.4 (1-6) ¹	-	-	8.7 (6-12) ¹	-	0 (0)	
Pigazzi <i>et al</i> ^[33]	6	RME	-	-	-	4.5 (3-11)	-	0 (0)	
	6	LME	-	-	-	3.6 (3-6)	-	0 (0)	
Parisi <i>et al</i> ^[34]	40	RME	1 (1-3) ¹	1 (1-5) ¹	2 (2-6) ¹	5 (3-18) ¹	0 (0)	1 (2.5)	Anastomotic leak
Baek <i>et al</i> ^[35]	182	RME	-	-	-	-	-	-	-
Shiomi <i>et al</i> ^[36]	113	RME	2 (1-3) ¹	3 (3-7) ¹	-	7 (6-24) ¹	0 (0)	2 (1.8)	Anastomotic leak
Kim <i>et al</i> ^[37]	200	RME	2.4	-	5	10.7	-	16 (8)	ns
Stănciulea <i>et al</i> ^[38]	100	RME	-	-	-	10 (6-38) ¹	-	6 (6)	3 anastomotic leak, 1 bowel obstruction, 1 bleeding, 1 bowel injury
Zawadzki <i>et al</i> ^[39]	77	RME	-	-	-	6.4 (3-26)	0 (0)	3 (3.9)	Anastomotic leak
Sng <i>et al</i> ^[40]	197	RME	-	-	-	9 (5-122) ¹	-	-	
Du <i>et al</i> ^[41]	22	RME	2.6 (1.41-4.37) ¹	-	-	7.8 (7-13) ¹	-	-	
Alimoglu <i>et al</i> ^[42]	7	RME	-	-	-	8.1 (5-10) ¹	0 (0)	0 (0)	
Akmal <i>et al</i> ^[43]	80	RME	-	2.75 (1-19)	-	7.55 (2-33)	0 (0)	-	
Park <i>et al</i> ^[44]	30	RME	-	-	-	4 (3-6) ¹	0 (0)	-	
Kang <i>et al</i> ^[45]	389	RME	2.3	3.9	-	13.5	0 (0)	36 (9.2)	ns
deSouza <i>et al</i> ^[46]	44	RME	-	-	-	5 (3-36) ¹	1 (0.46)	2 (0.92)	1 anastomotic leak
Pigazzi <i>et al</i> ^[47]	143	RME	-	2.7 (1-19)	-	8.3 (2-33)	0 (0)	-	
Choi <i>et al</i> ^[48]	50	RME	1.9 (1-3)	2.6 (2-12)	-	9.2 (5-24)	-	0 (0)	
Ng <i>et al</i> ^[49]	8	RME	-	-	-	5 (4-30) ¹	0 (0)	-	-
Hellan <i>et al</i> ^[50]	39	RME	-	2 (1-11) ¹	-	4 (2-22) ¹	0 (0)	4 (10.3)	Anastomotic leak

¹Values are expressed as mean, solid diet includes soft diet. SBO: Small bowel obstruction; RME: Robotic mesorectal excision; LME: Laparoscopic mesorectal excision; OME: Open mesorectal excision; POD: Post operative day.

patients. Among the 19 comparative^[8-10,13,15,17,19,21-26,28-33] studies only Park *et al*^[24] reported a longer distal margin in the robotic than in the laparoscopic group ($P = 0.04$). No significant difference in distal margins tumor involvement was reported when the robotic and laparoscopic approaches were compared.

Mean circumferential resection margins (CRM) after robotic rectal surgery were reported in 9 studies^[9,13,17,21,25,30,43,44,47] ranging from 1.8 mm^[43] to 11 mm^[44]. CRM tumor involvement was available in 32 studies^[8,10-12,14-17,19,20,22-30,35-37,39,40,42,44-50] and ranged from 0%^[15,16,20,22,36,42,44,46,49,50] to 11.1%^[17] of patients with a 2.94 overall rate (76 out of 2583 patients).

Mean CRM after laparoscopic rectal surgery were reported in 6^[9,13,17,21,25,30] comparative studies. It ranged

from 4 mm^[21] to 8.2 mm^[30]. CRM involvement was reported in 17 studies^[8,10-12,14,15,17,19,20,22-26,28-30] and occurred in 51 out of 1158 patients (4.4%) Where the 2 procedures were compared only D'Annibale *et al*^[22] observed a significantly greater number of patients with positive CRM in the laparoscopic series when compared with the robotic one.

Only in 11 papers^[9,11,13,20,21,26,32,34,36,41,44] reported the quality of mesorectum. Complete mesorectum excision ranged from 100%^[11,36] to 60%^[9] in the robotic series and from 100%^[11] to 40.6%^[9] after laparoscopy. Total mesorectal excision was achieved in 83.62% of robotic cases vs 77.22% of laparoscopic ones. None of the 7 comparative studies showed a significant difference in the quality of mesorectum between the 2 procedures.

Table 5 Complications according to Clavien Dindo classification

Ref.	Pts	Mesorectum	Complicated pts (%)	1 (%)	2 (%)	3 (%)		4 (%)	5 (%)
						3a	3b		
Park <i>et al</i> ^[8]	133	RME	26 (19.5)	11 (42.3)	5 (19.2)		9 (34.6)	1 (3.8)	
	84	LME	19 (22.6)	7 (36.8)	4 (21)		6 (31.6)	2 (10.5)	
Yoo <i>et al</i> ^[10]	44	RME	17 (38.6)	13 (76.5)			4 (23.5)		
	26	LME	7 (26.9)	5 (71.4)			2 (28.5)		
Koh <i>et al</i> ^[11]	19	RME	3 (15.7)	2 (66.7)			1 (33.3)		
	19	LME	7 (36.8)	4 (57)			3 (43)		
Melich <i>et al</i> ^[12]	92	RME	17 (18.4)	11 (64.7)			6 (35.3)		
	106	LME	18 (17)	13 (72.2)			5 (27.8)		
Barnajian <i>et al</i> ^[13]	20	RME	8 (40)		3	3 (37.5)	2 (25)		
	20	LME	4 (10)	2		1	1		
	20	OME	8 (40)		5		2	1 (33.3)	
Ielpo <i>et al</i> ^[14]	56	RME	15 (26.8)	11 (73.3)			4 (26.7)		
	87	LME	20 (23)	15 (75)			5 (25)		
Ghezzi <i>et al</i> ^[16]	65	RME	27 (41.5)	22 (81.5)			5 (18.5)		
	109	OME	45 (41.3)	38 (84.5)			7 (15.5)		
Kuo <i>et al</i> ^[17]	36	RME	11 (30.5)	4 (36.3)		3 (27.2)	4 (36.3)		
	28	LME	14 (50)	11 (78.6)		1 (7)	2 (14.2)		
Fernandez <i>et al</i> ^[20]	13	RME					2		
	59	LME							
Erguner <i>et al</i> ^[21]	27	RME	3 (11.1)	2 (66.7)			1 (33.3)		
	37	LME	8 (21.6)	5 (62.5)			3 (37.5)		
D'Annibale <i>et al</i> ^[22]	50	RME	5 (10)	5 (100)					
	50	LME	10 (20)	7 (70)			3 (30)		
Kang <i>et al</i> ^[23]	165	RME	34 (20.6)	16 (47.1)			3 (8.8)		
	165	LME	46 (27.9)	20 (43.5)			1 (2.2)		
	165	OME	41 (24.8)	30 (73.2)			2 (4.9)		
Park <i>et al</i> ^[24]	40	RME	6 (15)	4 (66.7)			2 (33.3)		
	40	LME	5 (12.5)	4 (80)			1 (20)		
Park <i>et al</i> ^[30]	52	RME	10 (19.2)	6 (60)			4 (40)		
	123	LME	15 (12.2)	9 (60)			6 (40)		
	88	OME	18 (20.5)	9 (50)			9 (50)		
Baik <i>et al</i> ^[32]	18	RME	4 (22.2)	3 (75)	1 (25)				
	18	LME	1 (5.5)		1 (100)				
Pigazzi <i>et al</i> ^[33]	6	RME	1 (16.6)		1 (100)				
	6	LME	1 (16.6)			1 (100)			
Parisi <i>et al</i> ^[34]	40	RME	4 (10)	1 (25)	1 (25)		2 (50)		
Shiomi <i>et al</i> ^[36]	113	RME	23 (20.3)	10 (43.5)	10 (43.5)	1 (4.3)	2 (8.7)		
Kim <i>et al</i> ^[37]	200	RME				16 (59.2)			
Stănculea <i>et al</i> ^[38]	100	RME	18 (18)		10 (55.5)	2 (5.5)	6 (38.9)		
Zawadzki <i>et al</i> ^[39]	77	RME			2	3			
Sng <i>et al</i> ^[40]	197	RME	74 (37)	58 (78.3)	5 (6.8)	9 (12.1)	1 (1.3)	1 (1.3)	
Du <i>et al</i> ^[41]	22 (4.5)	RME	1 (4.5)	1 (100)	0				
Alimoglu <i>et al</i> ^[42]	7	RME	2 (28.5)	2 (100)					
Kang <i>et al</i> ^[45]	389	RME	74 (19)	34 (45.9)	4 (5.4)	36 (48.6)			
deSouza <i>et al</i> ^[46]	44	RME	19 (43)	15 (79)	1 (5.2)	1 (5.2)	1 (5.2)	1 (5.2)	
Choi <i>et al</i> ^[48]	50	RME	9 (18)		4 (44.4)	5 (55.5)			
Hellan <i>et al</i> ^[50]	39	RME	15 (38.4)	11 (73.3)	4 (26.7)				

RME: Robotic mesorectal excision; LME: Laparoscopic mesorectal excision; OME: Open mesorectal excision.

Short-term oncologic outcomes

Only 11 authors^[8-10,16,19,25,28,31,38,42,47] reported short term oncologic outcomes (Table 8). The main drawback is the heterogeneity of the length of follow up ranging from 1 mo^[9,42] to 80 mo^[8] making results difficult to compare. The disease free survival in the laparoscopic group ranged from 75%^[10] to 89.2%^[31] with local recurrence ranging from 0%^[9,42] to 16.6%^[8] and an overall survival ranging from 88.5%^[10] to 98%^[24]. The disease free survival in the robotic group ranged from 70.4%^[16] to 100%^[31,42] with local recurrence ranging from 0%^[9,31,42] to 12.8%^[10] and an overall survival ranging from 85%^[16] to 100%^[42].

CONCLUSION AND DISCUSSION

Robotic rectal surgery is constantly increasing over the years. Previous reviews have already demonstrated its safety and feasibility^[51-53], although there are not published studies demonstrating its superiority over the laparoscopic approach mainly due to the lack of randomized control trials. This lack of evidence about the effectiveness of robotic rectal surgery is in contrast with the overall opinion of surgeons that report an easier surgical approach especially to narrow and difficult anatomic spaces such as the pelvis. Several authors^[52-54] reported 3D high definition vision, wrist-like movement

Table 6 Short term oncologic outcomes

Ref.	Pts	Mesorectum	DSF% (yr)	LR (%)	Distant metastases (%)	OS % (yr)	F-u mo (median)
Park <i>et al</i> ^[8]	133	RME	81.9 (5)	3 (2.3)	16 (12)	92.8 (5)	58 (4-80)
	84	LME	78.7 (5)	1 (1.2)	14 (16.6)	93.5 (5)	58 (4-80)
Levic <i>et al</i> ^[9]	56	RME		0 (0)	8 (14.3)		12 (1-31)
	36	LME		0 (0)	2 (5.6)		10 (1-33)
Yoo <i>et al</i> ^[10]	43 ¹	RME	76.7 (3)	6 (12.8)		95.2 (3)	33.9 (4.4-61.3)
	26	LME	75 (3)	2 (8.3)		88.5 (3)	36.5 (3.7-69.9)
Ghezzi <i>et al</i> ^[16]	65	RME	73.2 (5)	2 (3.2)	19 (29.6)	85 (5)	60
	109	OME	69.5 (5)	17.5 (16.1)	26 (24.2)	76.1 (5)	60
Saklani <i>et al</i> ^[19]	74	RME	77.7 (3)	2 (2.7)		90 (3)	30.1 (11-61) ²
	64	LME	78.8 (3)	4 (6.3)		92.1 (3)	30.1 (11-61) ²
Kim <i>et al</i> ^[25]	62	RME		0 (0)	3 (4.2)	98 (1.5)	17.4
	147	LME		1 (0.7)	8 (5.4)	98 (1.7)	20.6
Kwak <i>et al</i> ^[28]	59	RME		1 (1.8)	2 (3.6)		17 (11-25)
	59	LME		1 (1.9)	2 (3.7)		13 (9-22)
Patriti <i>et al</i> ^[31]	29	RME	100 (3)	0 (0)	0 (0)	96.6 (2.4)	29.2 ²
	37	LME	83.7 (3)	2 (5.4)	4 (6)	97.2 (1.5)	18.7 ²
Stănciulea <i>et al</i> ^[38]	100	RME		2 (2)		90 (3)	24 (9-63)
Alimoglu <i>et al</i> ^[42]	7	RME	100 (1)	0 (0)	0 (0)	100 (1)	12 (6-21) ²
Pigazzi <i>et al</i> ^[47]	143	RME	77.6 (3)	2 (1.4)	13 (9)	97 (3)	17.4 (0.1-52.5) ²

¹1 patient excluded (palliative ISR); ²Mean. DSF: Disease free survival rate; LR: Local recurrence; OS: Overall survival; RME: Robotic mesorectal excision; LME: Laparoscopic mesorectal excision; OME: Open mesorectal excision.

Table 7 Histopathological data

Ref.	Pts	Mesorectum	Harvested nodes	Quality of mesorectum (complete)	Proximal margin (mm)	Distal margin (mm)	Distal margin + (%)	CRM (mm)	CRM + (%)	pTpn stage (%)				
										0	1	2	3	4
Park <i>et al</i> ^[8]	133	RME	16.34 (2-43)	-	111.7 (40-350)	27.5 (10-140)	0 (0)	-	9 (6.8)	0 (0)	49 (36.8)	36 (27.1)	48 (36.1)	0 (0)
	84	LME	16.63 (2-49)	-	105.1 (40-340)	28.7 (10-90)	0 (0)	-	6 (7.1)	0 (0)	22 (26.2)	28 (33.3)	34 (40.5)	0 (0)
Levic <i>et al</i> ^[9]	56	RME	21 (7-83) ¹	34	-	30 (5-80)	1 (0.56)	9 (0-60) ¹	-	3 (5.4)	12 (21.4)	20 (35.7)	21 (37.5)	0 (0)
	36	LME	13 (3-33) ¹	26	-	30 (5-75)	0 (0)	10 (1-43) ¹	-	1 (2.8)	6 (16.7)	15 (41.7)	14 (38.8)	0 (0)
Yoo <i>et al</i> ^[10]	44	RME	13.93 (+ 9.27)	-	225.2 (+ 102.5)	13.3 (+ 9.7)	-	-	4 (9.1)	5 (11.4)	14 (31.8)	11 (25)	9 (20.5)	5 (11.4)
	26	LME	21.42 (+ 15.71)	-	208.4 (+ 89.5)	16.7 (+ 30)	-	-	5 (19.2)	1 (3.8)	7 (26.9)	8 (30.8)	8 (30.8)	2 (7.7)
Koh <i>et al</i> ^[11]	19	RME	16 (4-24) ¹	19	-	-	1 (5.2)	-	1 (5.2)	2 (10.5)	3 (15.7)	4 (21)	9 (47.3)	1 (5.2)
	19	LME	14 (5-27) ¹	19	-	-	0 (0)	-	0 (0)	0 (0)	5 (26.3)	4 (21)	9 (47.3)	1 (5.2)
Melich <i>et al</i> ^[12]	92	RME	17.2 (15-19.5)	-	-	-	1 (1.1)	-	3 (3.3)	-	-	-	-	-
	106	LME	16.3 (14.4-18.1)	-	-	-	0 (0)	-	3 (2.8)	-	-	-	-	-
Barnajian <i>et al</i> ^[13]	20	RME	14 (3-22) ¹	16	-	20.5 (5-50) ¹	-	10.5 (1-30) ¹	-	0 (0)	6 (40)	4 (25)	10 (35)	0 (0)
	20	LME	11 (4-18) ¹	19	-	21.5 (1-55) ¹	-	4 (0-30) ¹	-	0 (0)	7 (35)	3 (15)	10 (50)	0 (0)
	20	OME	12 (4-20) ¹	19	-	20.5 (1-45) ¹	-	8 (0-30) ¹	-	0 (0)	8 (40)	3 (15)	9 (45)	0 (0)
Ielpo <i>et al</i> ^[14]	56	RME	10 (0-29)	-	-	-	-	-	2 (3.6)	0 (0)	14 (25)	21 (37.5)	21 (37.5)	0 (0)
	87	LME	9 (0-17)	-	-	-	-	-	2 (2.3)	0 (0)	19 (21.8)	38 (43.6)	30 (34.5)	0 (0)
Tam <i>et al</i> ^[15]	21	RME	19.7 (8-40)	-	-	460 (10-180)	0 (0)	-	0 (0)	2 (10)	5 (24)	4 (19)	9 (43)	1 (5)
	21	LME	14.8 (8-21)	-	-	510 (5-80)	1 (5)	-	1 (5%)	3 (14)	7 (33)	4 (19)	7 (33)	0 (0)
Ghezzi <i>et al</i> ^[16]	65	RME	20.1	-	-	27 (16-44)	-	-	0 (0)	10 (15.4)	5 (7.7)	17 (26.2)	27 (41.5)	6 (9.2)
	109	OME	14.1	-	-	22 (15-30)	-	-	2 (1.8)	15 (13.8)	10 (9.2)	38 (34.9)	42 (38.5)	4 (3.7)
Kuo <i>et al</i> ^[17]	36	RME	14 (2-33)	-	-	22 (4-42)	0 (0)	6.7 (0-18)	4 (11.1)	7 (19.4)	4 (11.1)	11 (30.5)	14 (38.8)	0 (0)
	28	LME	13.9 (3-31)	-	-	17.9 (1-60)	1 (3.6)	7 (0-16)	4 (14.2)	6 (21.4)	2 (7.1)	8 (28.6)	12 (42.8)	0 (0)
Park <i>et al</i> ^[18]	32	RME	-	-	-	-	-	-	-	-	-	-	-	-
	32	LME	-	-	-	-	-	-	-	-	-	-	-	-

Saklani <i>et al</i> ^[19]	74	RME	11.6 (1-36)	-	128 (50-240)	17 (1-60)	-	-	3 (4)	18 (24.3)	16 (21.6)	22 (29.7)	18 (24.3)	0 (0)
	64	LME	14.7 (1-27)	-	140 (55-280)	22 (2-70)	-	-	1 (1.6)	8 (12.5)	13 (20.3)	23 (35.9)	20 (31.3)	0 (0)
Fernandez <i>et al</i> ^[20]	13	RME	16	9	-	-	0 (0)	-	0 (0)	-	-	-	-	-
	59	LME	20	24	-	-	1 (2)	-	1 (2)	-	-	-	-	-
Erguner <i>et al</i> ^[21]	27	RME	16 (3-38)	19	120 (40-180)	40 (30-80)	0 (0)	4 (2-8)	-	0 (0)	15 (55.5)	11 (40.7)	1 (3.7)	0 (0)
	37	LME	16 (3-31)	17	140 (45-230)	25 (5-50)	0 (0)	4 (1-10)	-	0 (0)	17 (46)	16 (43.2)	4 (10.8)	0 (0)
D'Annibale <i>et al</i> ^[22]	50	RME	16.5 (11-44)	-	-	30 (20-70)	-	-	0 (0)	-	-	-	-	-
	50	LME	13.8 (4-29)	-	-	30 (10-60)	-	-	6 (12)	-	-	-	-	-
Kang <i>et al</i> ^[23]	165	RME	15 (+ 9.4)	-	120 (+ 49)	19 (+ 14)	0 (0)	-	7 (4.2)	4 (2.4)	56 (33.9)	51 (30.9)	54 (32.7)	0 (0)
	165	LME	15.6 (+ 9.1)	-	113 (+ 51)	20 (+ 17)	0 (0)	-	11 (6.7)	9 (5.4)	55 (33.1)	47 (28.5)	54 (32.7)	0 (0)
	165	OME	17.4 (+ 10.9)	-	114 (+ 55)	22 (+ 17)	0 (0)	-	17 (10.3)	14 (8.5)	55 (33.3)	41 (24.8)	55 (33.3)	0 (0)
Kim <i>et al</i> ^[25]	62	RME	16 (+ 10)	-	-	30 (+ 14)	-	-	2 (3.2)	4 (6.5)	17 (27.4)	16 (25.8)	24 (38.7)	0 (0)
	147	LME	16 (+ 9)	-	-	25 (+ 16)	-	-	4 (2.7)	6 (4.1)	55 (37.7)	35 (24)	46 (31.5)	4 (2.7)
Park <i>et al</i> ^[24]	40	RME	12.9 (+7.5)	-	198 (+ 69)	14 (+ 9)	0 (0)	6.2 (4.7)	3 (7.5)	0 (0)	19 (47.5)	9 (22.5)	11 (27.7)	1 (2.5)
	40	LME	13.3 (+8.6)	-	213 (+ 139)	13 (+ 9)	0 (0)	6.9 (5.1)	2 (5)	0 (0)	13 (32.5)	15 (37.5)	11 (27.5)	1 (2.5)
Kim <i>et al</i> ^[26]	30	RME	-	29	-	27.9 (+ 10.2)	0 (0)	-	2 (6)	-	-	-	-	-
	39	LME	-	37	-	28.6 (+ 13.6)	0 (0)	-	1 (2.5)	-	-	-	-	-
Bertani <i>et al</i> ^[27]	52	RME	20.5 (5-43) ¹	-	-	26 (1-70)	-	-	2 (4)	-	-	-	-	-
	34	OME	16 (6-46) ¹	-	-	26 (1-80)	-	-	2 (6)	-	-	-	-	-
Kwak <i>et al</i> ^[28]	59	RME	20 (12-27) ¹	-	-	22 (15-30)	0 (0)	-	1 (1.7)	3 (5.1)	16 (27.1)	23 (39)	13 (22)	4 (6.8)
	59	LME	21 (14-28) ¹	-	-	20 (12-35)	0 (0)	-	0 (0)	3 (5.1)	16 (27.1)	23 (39)	12 (20.3)	5 (8.5)
Baek <i>et al</i> ^[29]	41	RME	13.1 (3.33)	-	-	36 (4-100)	0 (0)	-	1 (2.4)	7 (17.1)	12 (29.3)	4 (9.8)	15 (36.6)	3 (7.3)
	41	LME	16.2 (5-39)	-	-	38 (4-110)	0 (0)	-	2 (4.9)	3 (7.3)	15 (36.6)	3 (7.3)	19 (46.3)	1 (2.4)
Park <i>et al</i> ^[30]	52	RME	19.4 (+ 10.2)	-	165 (+ 60)	28 (+ 19)	0 (0)	7.9 (+ 4.5)	1 (1.9)	0 (0)	15 (28.8)	15 (28.8)	22 (42.3)	0 (0)
	123	LME	15.9 (+ 10.1)	-	169 (+ 84)	32 (+ 21)	0 (0)	8.2 (+ 5.8)	3 (2.4)	0 (0)	34 (27.6)	52 (42.3)	37 (30.1)	0 (0)
	88	OME	18.5 (+ 10.9)	-	124 (+ 66)	23 (+ 15)	0 (0)	8.5 (+ 5.7)	2 (2.3)	0 (0)	27 (30.7)	32 (36.4)	29 (33)	0 (0)
Patriti <i>et al</i> ^[31]	29	RME	10.3 (+ 4)	-	-	21 (+ 9)	-	-	-	0 (0)	11 (38)	9 (31)	7 (24.1)	2 (6.9)
	37	LME	11.2 (+ 5)	-	-	45 (+ 72)	-	-	-	0 (0)	17 (46)	8 (21.6)	10 (27.2)	2 (5.4)
Baik <i>et al</i> ^[32]	18	RME	20 (6-49)	17	109 (75-200)	40 (10-55)	-	-	-	0 (0)	5 (27.8)	4 (22.2)	9 (50)	0 (0)
	18	LME	17.4 (9-42)	13	103 (55-85)	37 (15-60)	-	-	-	0 (0)	5 (27.8)	4 (22.2)	9 (50)	0 (0)
Pigazzi <i>et al</i> ^[33]	6	RME	14 (9-28)	-	-	38 (18-90)	-	-	-	-	-	-	-	-
	6	LME	17 (9-39)	-	-	35 (22-50)	-	-	-	-	-	-	-	-
Parisi <i>et al</i> ^[34]	40	RME	19 (6-35) ¹	32	118.5 (65-390) ¹	40 (20-80) ¹	0 (0)	-	-	2 (5)	10 (25)	9 (22.5)	19 (47.5)	0 (0)
Baek <i>et al</i> ^[35]	182	RME	14.8 (2-47)	-	-	22 (+ 14.3)	-	-	10 (5.5)	5 (2.7)	57 (31.3)	52 (28.5)	62 (34)	6 (3.3)
Shiomi <i>et al</i> ^[36]	113	RME	32 (11-112) ¹	113	180 (65-376)	26 (5-100)	0 (0)	-	0 (0)	5 (4.4)	35 (31)	28 (24.7)	38 (33.6)	7 (6.2)
Kim <i>et al</i> ^[37]	200	RME	16.1	-	132.5	22	0 (0)	-	2 (1)	-	-	-	-	-
Stănciulea <i>et al</i> ^[38]	100	RME	14 (4-32) ¹	-	-	30 (2-70) ¹	-	-	-	5 (5)	24 (24)	43 (43)	21 (21)	7 (7)
Stănciulea <i>et al</i> ^[38]	77	RME	12.9 (3-45)	-	-	-	2 (2.6)	-	1 (1.2)	26 (34)	8 (10)	15 (19)	26 (34)	2 (3)
Sng <i>et al</i> ^[40]	197	RME	16 (1-80) ¹	-	-	17 (0-8.3) ¹	-	-	2 (2.5)	-	-	-	-	-
Du <i>et al</i> ^[41]	22	RME	14.3 (8-27) ¹	19	-	26 (10-55)	-	-	-	0 (0)	1 (4.5)	9 (40.9)	12 (54.5)	0 (0)
Alimoglu <i>et al</i> ^[42]	7	RME	16 (14-21)	-	-	-	-	-	0 (0)	0 (0)	3 (42.8)	1 (14.2)	3 (42.8)	0 (0)
Akmal <i>et al</i> ^[43]	80	RME	14.2 (2-33)	-	-	32.5 (2-100)	-	1.8 (0-45)	-	15 (18.8)	20 (25)	12 (15)	27 (33.8)	5 (6.3)
Park <i>et al</i> ^[44]	30	RME	20 (14-25) ¹	25	-	-	-	11 (5-20)	0 (0)	6 (20)	7 (23.3)	4 (13.3)	10 (33.3)	3 (10)
Kang <i>et al</i> ^[45]	389	RME	15.7 (+ 10)	-	11.7	2.15	-	-	14 (3.6)	24 (6.2)	122 (31.4)	103 (26.5)	140 (36)	0 (0)
deSouza <i>et al</i> ^[46]	44	RME	14 (5-45)	-	-	-	1 (2.7)	-	0 (0)	4 (9.1)	14 (31.8)	15 (34.1)	8 (18.2)	3 (6.8)
Pigazzi <i>et al</i> ^[47]	143	RME	14.1 (1-39)	-	-	29 (0-100)	-	19 (1-45)	1 (0.7)	18 (12.6)	36 (25.2)	36 (25.2)	53 (37)	0 (0)
Choi <i>et al</i> ^[48]	50	RME	20.6 (6-48)	-	-	19 (5-45)	0 (0)	-	1 (2)	0 (0)	10 (20)	19 (38)	19 (38)	2 (4)
Ng <i>et al</i> ^[49]	8	RME	15 (2-26) ¹	-	-	-	-	-	0 (0)	0 (0)	3 (37.5)	2 (25)	2 (25)	0
Hellan <i>et al</i> ^[50]	39	RME	13 (7-28) ¹	-	-	26.5 (4-75) ¹	0 (0)	-	0 (0)	8 (20.5)	13 (33.3)	4 (10.3)	13 (33.3)	1 (2.6)

¹Median. RME: Robotic mesorectal excision; LME: Laparoscopic mesorectal excision; OME: Open mesorectal excision.

Table 8 Short term oncologic outcomes

Ref.	Pts	Mesorectum	DSF% (yr)	LR (%)	Distant mtx (%)	OS % (yr)	F-u mo (median)
Park <i>et al</i> ^[8]	133	RME	81.9 (5)	3 (2.3)	16 (12)	92.8 (5)	58 (4-80)
Levic <i>et al</i> ^[9]	84	LME	78.7 (5)	1 (1.2)	14 (16.6)	93.5 (5)	58 (4-80)
	56	RME		0 (0)	8 (14.3)		12 (1-31)
Yoo <i>et al</i> ^[10]	36	LME		0 (0)	2 (5.6)		10 (1-33)
	43 ¹	RME	76.7 (3)	6 (12.8)		95.2 (3)	33.9 (4.4-61.3)
Ghezzi <i>et al</i> ^[16]	26	LME	75 (3)	2 (8.3)		88.5 (3)	36.5 (3.7-69.9)
	65	RME	73.2 (5)	2 (3.2)	19 (29.6)	85 (5)	60
Saklani <i>et al</i> ^[19]	109	OME	69.5 (5)	17.5 (16.1)	26 (24.2)	76.1 (5)	60
	74	RME	77.7 (3)	2 (2.7)		90 (3)	30.1 (11-61) ²
Kim <i>et al</i> ^[25]	64	LME	78.8 (3)	4 (6.3)		92.1 (3)	30.1 (11-61) ²
	62	RME		0 (0)	3 (4.2)	98 (1.5)	17.4
Kwak <i>et al</i> ^[28]	147	LME		1 (0.7)	8 (5.4)	98 (1.7)	20.6
	59	RME		1 (1.8)	2 (3.6)		17 (11-25)
Patrioti <i>et al</i> ^[31]	59	LME		1 (1.9)	2 (3.7)		13 (9-22)
	29	RME	100 (3)	0 (0)	0 (0)	96.6 (2.4)	29.22
Stănciulea <i>et al</i> ^[38]	37	LME	83.7 (3)	2 (5.4)	4 (6)	97.2 (1.5)	18.72
	100	RME		2 (2)		90 (3)	24 (9-63)
Alimoglu <i>et al</i> ^[42]	7	RME	100 (1)	0 (0)	0 (0)	100 (1)	12 (6-21) ²
Pigazzi <i>et al</i> ^[47]	143	RME	77.6 (3)	2 (1.4)	13 (9)	97 (3)	17.4 (0.1-52.5) ²

¹1 patient excluded (palliative ISR); ²Mean. DSF: Disease free survival rate; RME: Robotic mesorectal excision; LME: Laparoscopic mesorectal excision; OME: Open mesorectal excision.

of instruments (endowrist™), stable camera holding, motion filter for tremor-free surgery and improved ergonomics as major improvements in rectal surgery but it seems that these technical benefits have not reflected better clinical outcomes yet. This review aimed to analyze robotic rectal surgery from the first report to nowadays in order to focus on the current state and assess any benefits of robotic rectal surgery and its evolution through the years.

A well-established finding of this review is the longer operative time of robotic surgery when compared to the laparoscopic one. This is most likely due not to longer dissection but to non-surgical technical time. In fact in the totally robotic approach the docking and undocking has to be performed twice and in the hybrid approach there is the need to switch from laparoscopy to robot. A totally robotic technique without undocking is feasible, but this approach is technically much more difficult and as a consequence, a longer operative time is needed^[10,12,14,16,17,22-24,27,28,37,38,40-42,48]. Traditionally, longer operative time is related with increased morbidity, most likely related to the difficulty of the operation^[53]. However prolonged times in robotic surgery are not associated with an increased complication rate as demonstrated by this review and previously published review and meta-analysis^[55].

In our review 2^[21,23], out of 16 comparative studies reported a significantly lower estimated blood loss after robotic rectal surgery confirming that there is still no evidence that robotic rectal surgery for cancer may be associated with a lower intraoperative blood loss.

As regards conversion rates to open surgery, 3^[8,22,31] out of 22 comparative studies reported significant lower complication rates in robotic patients. Many authors associated these results to better visualization, 3D view, endowrist™ technology and stable camera holding

resulting in an easier dissection in narrow anatomical fields such as the pelvis^[56]. Even the results reported by Ielpo *et al*^[14] suggest that the robotic approach has lower conversion rates when the tumor location requests a low anterior resection and as a consequence, when the operations is technically more challenging. Since converted cases are associated to greater morbidity and tumor recurrence^[57], robotic surgery could provide better oncologic long term results as well as a decreased perioperative morbidity.

The difference in protective ileostomy creation observed in this review can be related to several factors: The surgeon's habit, the tumor location, the surgeon's learning curve. Moreover, a trend toward an increasing stoma creation after robotic surgery could have been verified because of the initial worries about the new technique. On the bases of our findings the robotic approach seems associated with a higher rate of protective stoma creation.

One of the main benefits of minimally invasive surgery is the early recover. In this review we were unable to draw definitive results about any benefit of the robotic technique over conventional laparoscopy. Length of hospital stay, day of 1st flatus, 1st solid diet and 1st liquid diet were substantially similar in both the robotic and laparoscopic series even if some authors reported some advantages for either the robotic or the laparoscopic technique^[8,9,15,22-24,30,32].

Anastomotic leak is the most severe surgical complication in rectal surgery. Well known risk factors for anastomotic leak are represented by cancers located less than 6 cm from anal verge, neoadjuvant radio-chemotherapy, obesity and intraoperative blood transfusions^[58-63]. In this review the overall anastomotic leak rates in the robotic and laparoscopic series were similar (7.3% vs 7.6%) with no comparative study

reporting any significant difference between the 2 types of procedure. All together these results demonstrate that robotic surgery does not reduce the anastomotic leak rate. Nevertheless results of comparative studies are contradictory since 9^[11,15,19,20-22,23,25,30] of these reported less anastomotic leaks in the robotic group and 9^[8-10,14,17,24,28,29,31] in the laparoscopic one, but none of these results was significant. Looking at intraoperative complications, only Levic *et al.*^[9] reported a significant, higher rate in the robotic patients (4.48% vs 0%). However it must be considered that in this study there were more obese patients in the robotic group and all robotic and laparoscopic operations were performed in 2 different hospitals.

The number of harvested and examined lymph nodes is pivotal in the postoperative tumor staging whose accuracy increases with the number of nodes retrieved within the surgical specimen. The robotic platform with its 3D high definition vision and wrist-like movement of instruments should improve the lymph nodes retrieving. Nevertheless, the difference between the mean harvested lymph nodes in the robotic and laparoscopic series was not substantial in our review (15.1 vs 15.7 respectively) and only 2 authors^[9,10] reported a significant higher number of retrieved lymph nodes in the robotic group.

The length of tumor involvement of both the distal and circumferential resection margins is considered an important parameter in evaluating the treatment of rectal cancer. Findings from the present review seems to determinate the lack of any advantages of robotic surgery over the laparoscopic approach. This issue might be explained by the likely surgeon's trend to prefer robotic approach in more advanced and distal tumors because of the theoretical superiority of this technique in pelvic dissection. In this review indeed 7 authors^[10,11,15,20,22,25,31] reported a significant lower distance of the tumor from anal verge when the robotic approach was compared with the laparoscopic one. Two comparative studies^[13,22] reported even a significant wider CRM in their robotic series when compared to the laparoscopic ones. However a possible bias in the evaluation of this parameter is the non-uniform recording of data: some authors report median values, others the mean values making data not comparable. Even definition of circumferential resection margin is still not clear as it is currently considered as positive as positive if < 1 mm^[8,11,14,19,24,25,30,35,64] by some authors and < 2 mm^[10,12,15-17,20,22,23,26-29,36,37,39,40,42,44-50] by others.

Thanks to its technical characteristics the robot platform should help in performing total and complete mesorectal excision that is an important target in rectal surgery since it potentially reflects the radicality of the operation. Unfortunately even if this is a major parameter in evaluating the radicality of the intervention, only 11 out of 43 studies in this review have addressed this important parameter. On the basis of our results any superiority of robotic mesorectal excision over the laparoscopic one cannot be demonstrated.

Robotic surgery may help in the identification and preservation of autonomic nerves due to high definition 3D image. Common sites of potential nerve damage are the superior hypogastric plexus, leading to ejaculation dysfunction in males and impaired lubrication in females, and the pelvic splanchnic nerve/pelvic plexus leading to erectile dysfunction in men. According to results of the CLASSIC trial^[59] the risk of an autonomic injury with sexual dysfunction in males is significantly higher in laparoscopic surgery when compared to the open approach. The perceived advantages of robotic surgery may translate to decreased incidence of urinary dysfunction and erectile dysfunction in males. Although some preliminary results suggested that robotic assisted rectal surgery is superior to conventional laparoscopic surgery in preventing sexual or urinary dysfunction^[63,64], we cannot provide definitive results since only few studies addressed this issue with high heterogeneity in the scores systems used for the analysis. Furthermore not all the patients in the studies agreed in answering questionnaires and this could lead to a possible type II error. Some authors^[26,18] reported an earlier recovery of erectile, sexual desire and urinary function when the robotic group was compared with the laparoscopic one but they did not report any difference in long-term follow-up.

In conclusion, results from the present review show that robotic surgery is as feasible and safe as conventional laparoscopy in the treatment of rectal cancer, with the only drawback of longer operative time. The magnified view, the improved ergonomics and dexterity might improve the diffusion of minimally invasive approach in the treatment of rectal cancer. Potential clinical benefits of the robotic technique must be demonstrated, if any, only by RCTs.

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Molecular predictive markers in tumors of the gastrointestinal tract

Eirini Papadopoulou, Vasiliki Metaxa-Mariatou, Georgios Tsaousis, Nikolaos Tsoulos, Angeliki Tsigirigoti, Chrisoula Efstathiadou, Angela Apeessos, Konstantinos Agiannitopoulos, Georgia Pepe, Eugenia Bourkoula, George Nasioulas

Eirini Papadopoulou, Vasiliki Metaxa-Mariatou, Georgios Tsaousis, Nikolaos Tsoulos, Angeliki Tsigirigoti, Chrisoula Efstathiadou, Angela Apeessos, Konstantinos Agiannitopoulos, Georgia Pepe, Eugenia Bourkoula, George Nasioulas, Department of Molecular Biology, GeneKor Medical S.A., 15344 Gerakas, Greece

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Correspondence to: George Nasioulas, PhD, Department of Molecular Biology, GeneKor Medical S.A., 52 Spaton Ave, 15344 Gerakas, Greece. gnasioulas@genekor.com
Telephone: +30-210-6032138
Fax: +30-210-6032148

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Abstract

Gastrointestinal malignancies are among the leading causes of cancer-related deaths worldwide. Like all human malignancies they are characterized by accumulation of mutations which lead to inactivation of tumor suppressor genes or activation of oncogenes. Advances in Molecular Biology techniques have allowed for more accurate analysis of tumors' genetic profiling using new breakthrough technologies such as next generation sequencing (NGS), leading to the development of targeted therapeutical approaches based upon biomarker-selection. During the last 10 years tremendous advances in the development of targeted therapies for patients with advanced cancer have been made, thus various targeted agents, associated with predictive biomarkers, have been developed or are in development for the treatment of patients with gastrointestinal cancer patients. This review summarizes the advances in the field of molecular biomarkers in tumors of the gastrointestinal tract, with focus on the available NGS platforms that enable comprehensive tumor molecular profile analysis.

Key words: Predictive biomarkers; Targeted therapy; Next generation sequencing; Gastrointestinal tract; Somatic mutations; Liquid biopsy

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Core tip: Gastrointestinal cancers are among the leading causes of cancer morbidity and mortality worldwide. So far, various targeted agents associated with predictive biomarkers are available or are under development for the selection of treatment in patients with gastrointestinal cancer. Advances in high-throughput technologies such as next generation sequencing and the use of noninvasive

materials for tumor characterization, such as liquid biopsies, will facilitate tumor molecular profiling and lead to the establishment of further targeted treatment therapies.

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INTRODUCTION

The comprehension of the importance of tumor biology has led to the development of new drugs that target specific molecules involved in carcinogenesis. The efficacy of such targeted therapies often depends on the presence or absence of gene alterations that encode for the protein-target or for proteins involved in the molecular pathway targeted by the specific medication. This targeted therapeutical approach is based on the tumor's molecular analysis in order to select patients with increased probability to respond to the treatment given. Advances in Molecular Biology techniques have permitted comprehensive tumor genomic profiling using new breakthrough technologies such as next generation sequencing (NGS)^[1-3].

Nowadays, biomarkers are used in the management of patients with cancer and can be divided into predictive and prognostic. Prognostic biomarkers are defined as those that provide information on the possible outcome of cancer in a particular patient regardless of treatment. Predictive biomarkers provide information on the potential benefit of the administrated treatment (whether this relates to the tumor's volume shrinkage or survival). Predictive biomarkers can be used to identify subpopulations of patients that are likely to respond to a particular treatment^[1]. They can be subdivided in positive and negative predictive biomarkers. The first are used for positive selection of patients who are likely to benefit from targeted therapy, whereas the latter for resistance prediction^[1].

The number of genes involved in targeted therapy (predictive biomarkers), is increasing continuously. The simultaneous analysis of these biomarkers is feasible using molecular biology technologies that allow accurate, fast and cost effective genomic analysis with limited requirements concerning the quantity of the biological material used^[1,4]. NGS has all the features required to carry out such analysis and provides simultaneous information on a large number of actionable alterations in tumor tissues and thus a more precise molecular characterization of the tumor. The massive amount of genetic information produced is the main advantage of this technology. However, it also constitutes its main

challenge, requiring usage of appropriate software and bioinformatics tools, along with web-based tools for data analysis, management and interpretation^[5].

The human gastrointestinal (GI) tract is an organ system which includes all structures between mouth and anus and is divided into upper (buccal cavity, pharynx, esophagus, stomach and duodenum) and lower (small and large intestine) GI tracts. GI cancers are complex diseases and refer to malignant conditions that affect the digestive system. The current review will focus on the advances in the field of molecular biomarkers and the application of high throughput technologies, in the most common tumors of the gastrointestinal tract.

Esophageal cancer

Esophageal cancer is one of the most aggressive malignancies with a rapidly increasing incidence rate in the recent decades. There are two predominant histological types: Esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC) of the distal esophagus and the gastroesophageal junction. Smoking and heavy alcohol consumption are associated with increased risk of ESCC, while gastroesophageal reflux disease and Barrett esophagus may increase the risk of EAC^[6].

TP53 mutations are identified in about 50% of esophageal cancers and are associated with poorer survival^[7]. Apart from mutations in *TP53* ESCC and EAC seem to differ significantly in the genetic alterations pattern. Agrawal *et al.*^[8] using NGS reported a substantial disparity in the spectrum of mutations, with more insertions/deletions in ESCCs, A:T>C:G transversions in EACs, and C:G>G:C transversions in ESCCs. Inactivating mutations of *NOTCH1* are identified in about 20% of ESCCs but not in EACs. Somatic aberrations in EACs are mainly identified in the Wnt, cell cycle and Notch pathways^[9,10]. A number of genes that can be used as predictive markers for targeted therapy have been explored for somatic mutations in esophageal adenocarcinoma, including genes of the RAF/MEK/ERK (MAPK) kinase pathway such as *EGFR*, *BRAF*, *KRAS*, *PIK3CA*^[11]. However the reported frequency of somatic mutations identified appears to be low and this is obvious when accessing data from the Catalogue of Somatic Mutations in Cancer database (COSMIC, cancer.sanger.ac.uk) (Figure 1), which is currently the most comprehensive global resource accessing the world literature on somatic mutations in human cancer^[12]. In a recent study, NGS-based comprehensive genomic profiling was used to analyze ESCC and EAC tumors^[13]. The analysis showed that the esophageal histotypes differ significantly in genomic alterations profile, with *KRAS* and *ERBB2* far more frequently altered in EAC compared to ESCC. In contrast, genes of the mechanistic target of rapamycin (MTOR) pathway (*PIK3CA* and *PTEN*) and *NOTCH1* are more frequently altered in ESCC compared to EAC. They also have different amplification patterns (Figure 2).

ESCC and EAC also differ in the gene amplification

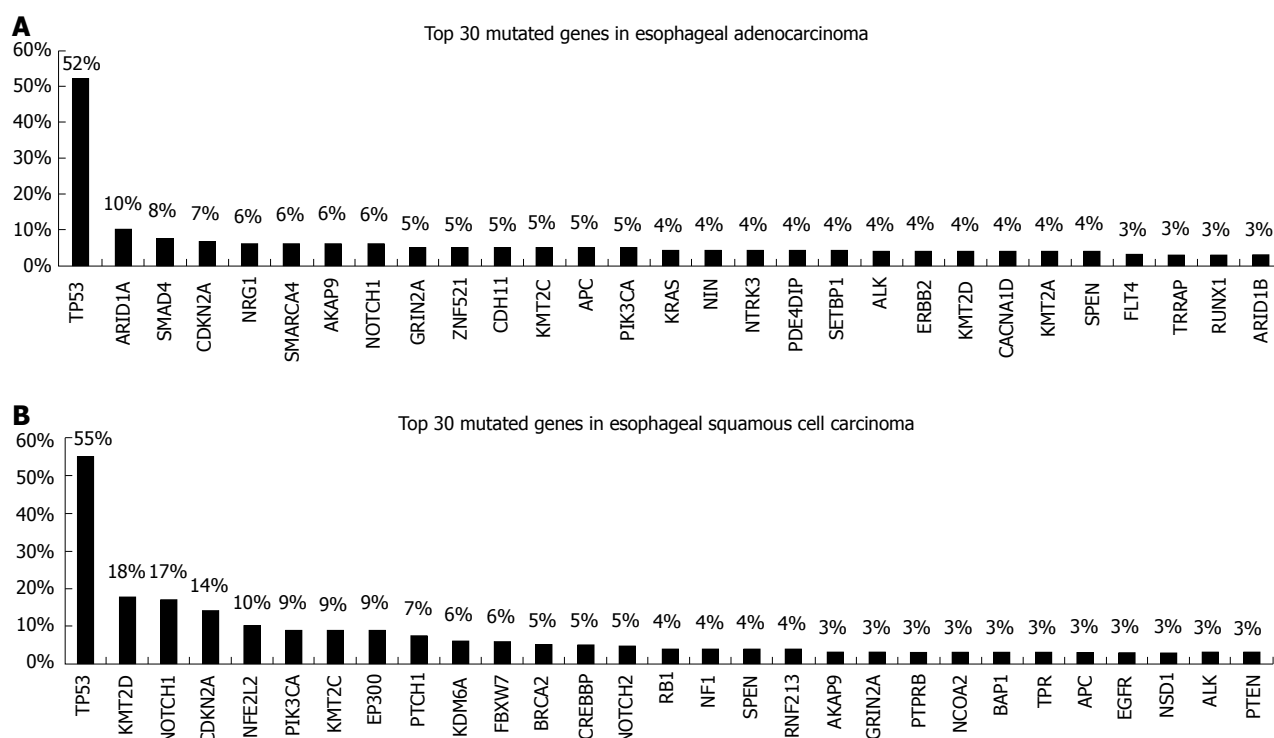


Figure 1 Bar chart showing the most frequently mutated genes in esophageal cancer according to catalogue of somatic mutations in cancer database. In the Y-axis the percentage of observed mutation frequency is represented. In the X-axis the most frequently mutated genes are listed. A: Top 30 mutated genes in esophageal adenocarcinoma; B: Top 30 mutated genes in esophageal squamous cell carcinoma.

and/or protein (over)expression of the receptor tyrosin kinases (RTKs) EGFR and HER2 making them possible prognostic markers and as therapeutic targets^[7,14]. EGFR is frequently overexpressed in ESCCs, while HER2 overexpression occurs mainly in EACs. Thus the trastuzumab-platinum regimen is currently used for the 15% of the EACs patients that test positive for HER2 (ERBB2) amplification or overexpression^[13,14].

Numerous preclinical studies addressed EGFR and HER2 inhibition in esophageal cancer cell lines and there are various phase II/III clinical trials testing EGFR, HER2, and VEGF targeting therapies for esophageal cancer^[7,15]. However, the results obtained to date do not allow the use of these agents in clinical practice. Upon trial completion several clinical studies have concluded, that in order to select patients who will respond to RTK-targeted therapy, there is a need for molecular patient stratification before treatment.

In a disease with historically poor outcomes and limited options, comprehensive genomic profiling of relapsed and refractory cancers, including distinct evaluation for EAC and ESCC has led to promising information suggesting targeted therapies for future consideration.

Gastric cancer

Gastric cancer (GC) develops from the inner lining of the stomach and is a very aggressive malignancy, with poor prognosis and very high cancer related mortality. The high mortality rate is largely due to the late stages of cancer diagnosis and to the lack of effective medical

treatment for advanced stages of this disease^[16,17]. The majority of these cancers are adenocarcinomas and can be further classified as diffuse (poorly differentiated) or intestinal (well-differentiated) types that have distinct molecular profiles^[16,17].

Concerning the causes of GC, it can be viewed as a multifactorial disease since many inherited and environmental factors play a role in its development. Infectious agents such as *Helicobacter pylori* and EBV, dietary habits and the genetic background are considered as causative agents^[17].

Given the variety of causes of the disease, it is not surprising that these tumors present a high level of biological heterogeneity, with distinct molecular profile for each patient. Genetic and epigenetic alterations play important role in GCs therefore, targeted therapy based on the biology of the individual patient could improve treatment outcome^[17-19].

ERBB2 amplifications occur frequently in gastric tumors (2%-27%)^[12,20]. Trastuzumab, a monoclonal antibody against HER2/neu receptor, was the first targeted agent to be used in the treatment of ERBB2-positive advanced gastric and gastroesophageal junction (GEJ) adenocarcinoma^[20]. Several molecular targeted agents associated with a survival benefit in other cancer types are now under clinical investigation for the treatment of gastric cancer, including inhibitors of EGFR, MET, FGFR, VEGF, and PI3K^[18,20]. Additionally, *CDH1* gene mutations at the somatic level are considered of prognostic significance^[19].

Several studies have investigated gastric cancer's

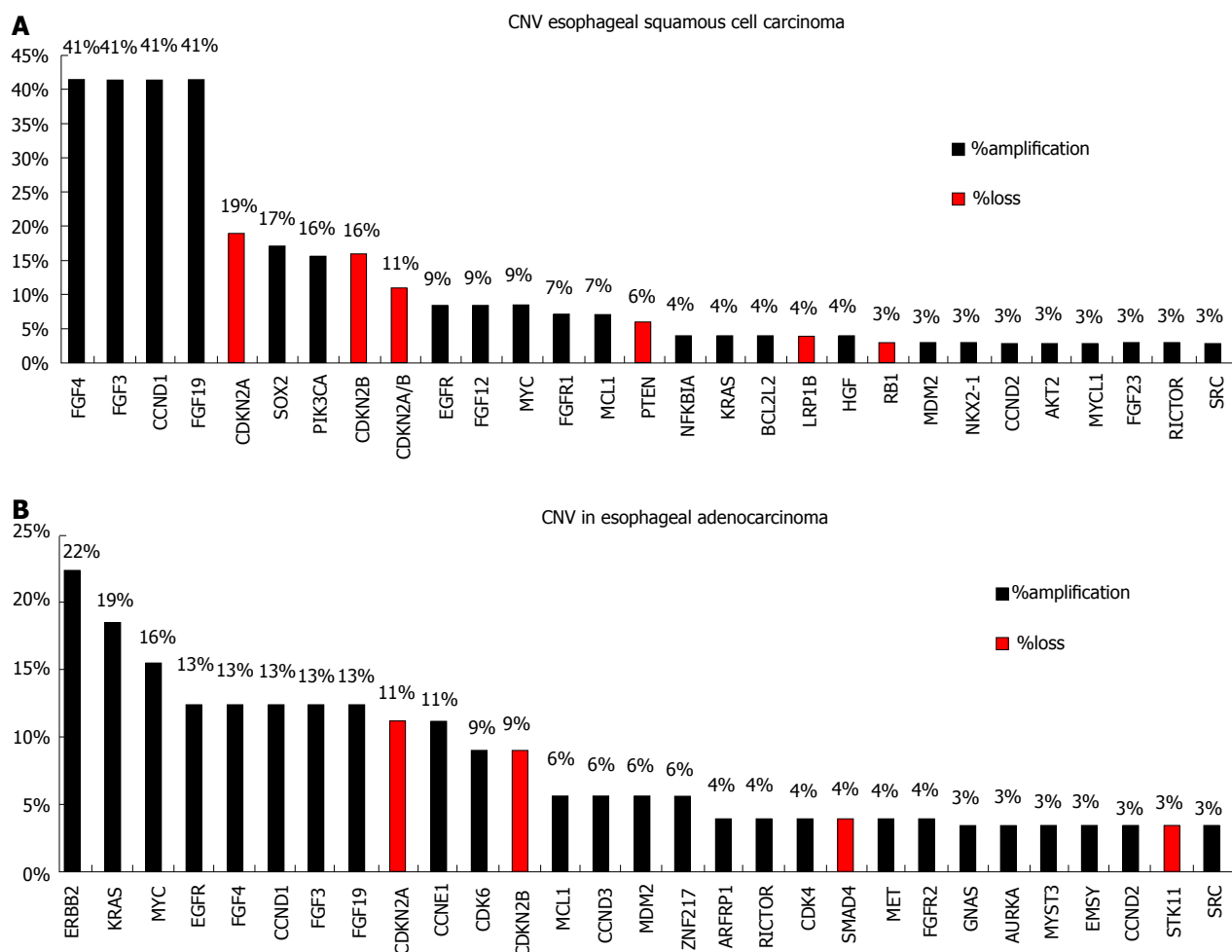


Figure 2 Bar chart showing the copy number variations in esophageal cancer according to the study performed^[13]. In the Y-axis the percentage of CNV frequency is represented. In the X-axis the most frequently altered genes are listed. A: Genes most commonly affected by CNV (amplification or loss) in esophageal adenocarcinoma; B: Genes most commonly affected by CNV (amplification or loss) in esophageal squamous cell carcinoma. CNV: Copy number variation.

molecular profile using whole genome as well as targeted NGS approaches^[19,21-23]. The presence of somatic mutations and copy number variations (CNV) in many cancer driver genes has been revealed. Among the cancer genes frequently mutated in gastric cancer *P53*, *ARD1A*, *CDH1*, *PIK3CA*, *APC*, *CTNNB1*, *ERBB3*, *ATM*, *KRAS* are the most important prognostic and/or predictive markers (Figure 3)^[12]. Consequently, molecular profile-directed therapy seems to be a promising strategy for the improvement of standard chemotherapy effectiveness.

CNVs have been observed for *HER2*, *FGFR2*, and *MET* that represent viable treatment targets for which therapeutics are already approved or are currently under investigation^[24] (Figure 4).

The high heterogeneity of these tumors triggered scientists to attempt their molecular characterization. In a study conducted by The Cancer Genome Atlas, molecular classification four major genomic subtypes of gastric cancer were defined: EBV-infected tumors; MSI tumors; genomically stable tumors; and chromosomally unstable tumors^[21].

In a recent study, Li *et al.*^[19], using whole genome

NGS data were able to classify gastric cancers into regular (86.8%) and hyper-mutated (13.2%) subtypes based on mutation burden. Additionally, in the "regular" mutated cohort a further classification, using 40 significantly mutated genes, could be obtained, separating the patients to S1 and S2 subtypes with distinct prognostic outcomes.

Gastrointestinal stromal tumors

Gastrointestinal stromal tumors (GISTs) are rare tumors of the gastrointestinal tract. They are mesenchymal in origin and are characterized by overexpression of the KIT protein^[25]. Morphological diagnosis based on microscopic examination is the standard for GIST diagnosis. They occur anywhere within the GI tract, but they are most common in the stomach (60%) or small intestine (30%)^[26]. Their diagnosis is based on the expression of the transmembrane tyrosine kinase (TK) receptor, KIT, since 95% of GISTs express CD 117 antibody. In 80% of the cases, somatic mutations in the *ckIT* gene are observed, resulting in constitutive receptor activation. Additionally, in 5%-10% of the cases without *ckIT* mutations, the TK receptor *PDGFRA* is

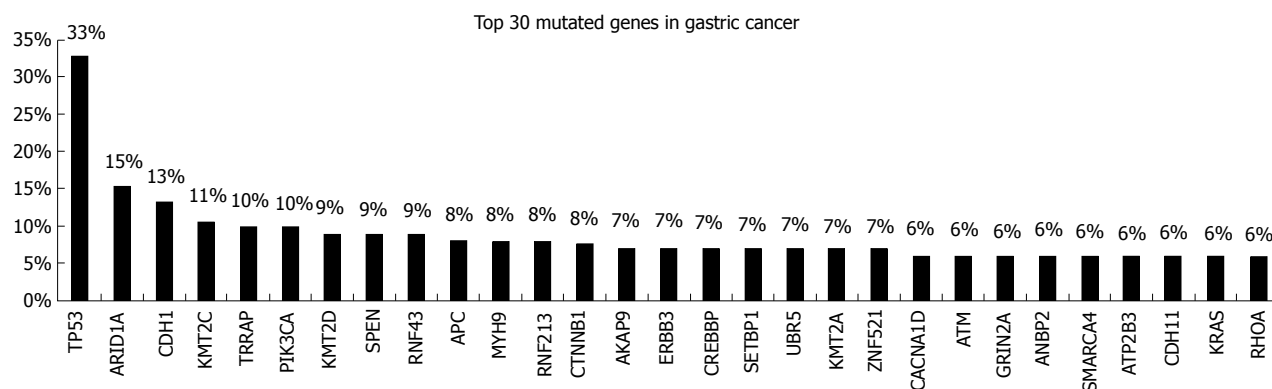


Figure 3 Bar chart showing the most frequently mutated genes in gastric cancer according to catalogue of somatic mutations in cancer database. In the Y-axis the percentage of observed mutation frequency is represented. In the X-axis the genes are listed.

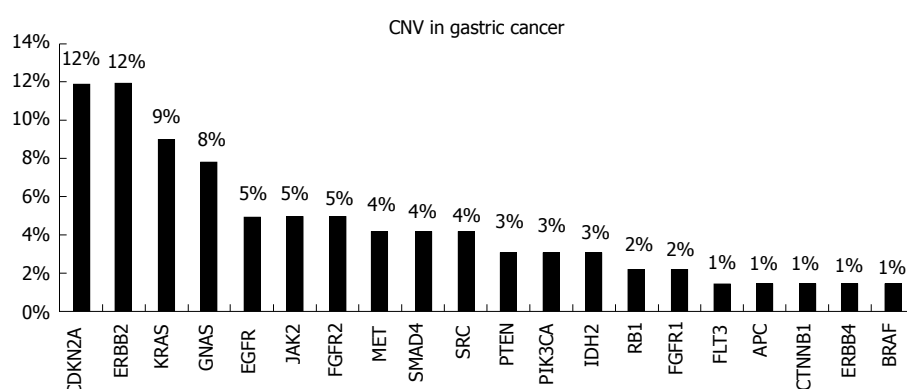


Figure 4 Copy number variation in the most important treatment targetable genes in gastric cancer. In the Y-axis the percentage of observed CNV is represented. In the X-axis the genes are listed. CNV: Copy number variation.

mutated^[27]. The mutation spectrum of these tumors is very limited as we observe in COSMIC database (Figure 5)^[12]. Tyrosine kinase inhibitors (TKIs) like imatinib, sunitinib, and more recently regorafenib, have proven effectiveness in suppressing the growth of metastatic GISTs, allowing patients to live far longer than during the previous era of ineffective chemotherapy^[28-30]. The response to targeted therapy with TKIs is mainly dependent on the presence and type of mutation. Patients with mutations in exon 11 of the *ckit* are highly responsive to imatinib, while the presence of a mutation in exon 9 of this gene implies intermediate response rates and necessitates a double dose of drug administration. Furthermore, resistance mutations to imatinib are also observed in the *ckit*/*PDGFRA* genes. These mutations can be present in the primary tumor or arise as a result of the drug administration (secondary mutations)^[29].

Colorectal cancer

The cancer of colon and rectum (colorectal, CRC) is the third most common cancer worldwide with 95% of these tumors being classified as adenocarcinomas. It's a leading cause of cancer related deaths; however, colorectal cancer mortality is declining in the last decades, mainly due to early diagnosis and the presence of new therapy strategies^[31,32]. This malignancy is one of the

first paradigms of the benefits that can be derived from the application of personalized treatment in cancer therapy^[33-35].

Genetic alterations in colorectal cancer include mainly single-base substitutions (SBS). Nevertheless, small insertions and deletions (indels), amplifications, homozygous deletions and translocations can also be observed^[36].

Five hundred and seventy-two cancer relevant genes are included in the Catalogue of Somatic Mutations in Cancer (COSMIC, cancer.sanger.ac.uk)^[12]. Somatic mutations in CRC cancer are observed in the majority of these genes. The pattern of genomic alterations was identified through Massively Parallel Sequencing studies, revealing the inter- and intra-tumor genetic heterogeneity of these tumors^[37-39]. Apart from single base substitutions, gene amplifications are also observed (Figure 6). Mutations in many important biologic pathways occur. In Table 1 the frequency of mutations in important molecular signaling pathways and the related therapies are represented. The gene mutation frequency is calculated using data from samples analysed by whole genome screening in the COSMIC database. The information concerning the therapies targeting each pathway was retrieved from MyCancer Genome knowledge database (www.mycancergenome.org/), that provides reliable information concerning

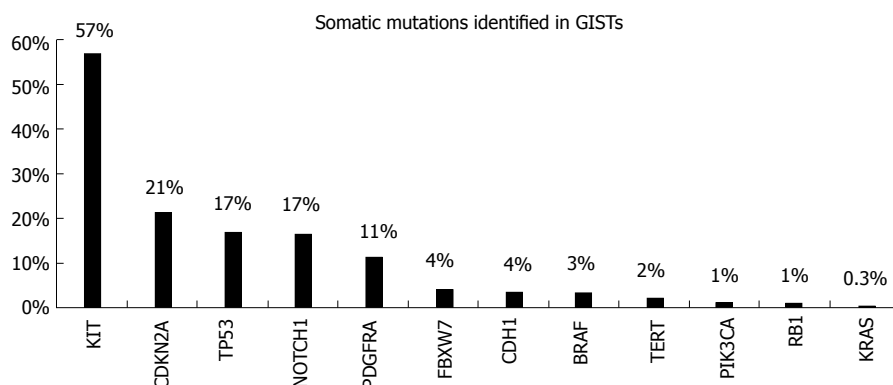


Figure 5 Bar chart showing the most frequently mutated genes in gastrointestinal stromal tumors according to catalogue of somatic mutations in cancer database. In the Y-axis the percentage of observed mutation frequency is represented. In the X-axis the genes are listed. GIST: Gastrointestinal stromal tumors.

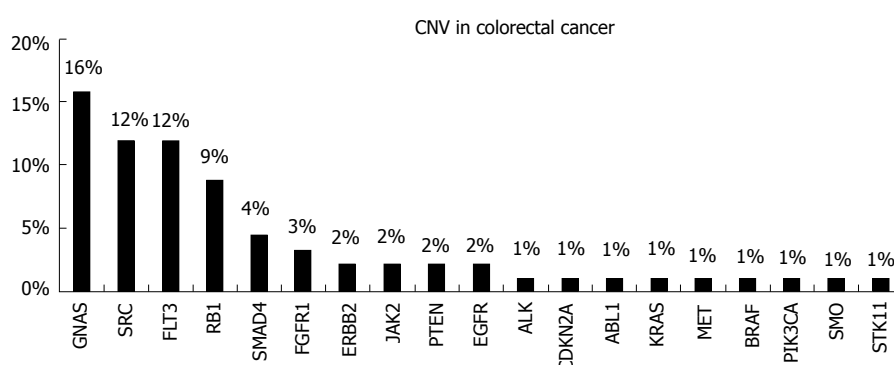


Figure 6 Copy number variation in the most important treatment targetable genes in colorectal cancer. CNV: Copy number variation.

important cancer related genes and their correlation with treatment options (Table 1)^[40].

The *RAS* proto-oncogenes (*HRAS*, *KRAS* and *NRAS*) encode a family of highly homologous proteins. They participate in a signal transduction cascade, namely the *RAS*/*RAF*/*MEK*/*ERK* pathway, which regulates the growth and survival properties of the cells. They are controlled by extracellular signals transmitted by the transmembrane receptor tyrosine kinase (TK), *EGFR*^[34]. This TK and the *RAS*/*RAF*/*MEK*/*ERK* pathways it controls, play an important role in colorectal carcinogenesis, making it a good target for biological therapy of this disease.

Two monoclonal antibodies were designed as effective inhibitors of *EGFR*. Cetuximab (Erbix, Merck KGaA, Darmstadt, Germany) is a chimeric mouse/human antibody, and Panitumumab (Vectibix, Amgen Thousand Oaks, CA, United States), is a fully human antibody^[33-35]. They both target the extracellular domain of the *EGFR* protein and compete with ligands, blocking ligand induced intracellular signal transmission. However, anti-*EGFR* treatment is not effective in patients harboring activating mutations in genes that participate in the intracellular transduction *RAS*/*RAF*/*MEK*/*ERK* pathway. This is due to the constitutive, independent of ligand, activation of the mutated proteins^[33].

In total, activating mutations in the *RAS* genes, mainly in codons 12, 13 or 61, occur in approximately

20% of all human cancers. Mutations in *KRAS* account for about 85% of all *RAS* mutations in human tumors, *NRAS* for about 15%, and *HRAS* for less than 1%^[41,42]. Which particular *RAS* gene is mutated seems to be tumor specific. Colonic, pancreatic and lung cancers have high frequencies of *KRAS* mutations^[41,42].

Acquired mutations in *KRAS* and *NRAS* are commonly used to identify colorectal cancer patients who are unlikely to benefit from anti-*EGFR* therapy. Approximately 40% of colorectal cancer tumors harbor mutations in the *KRAS* gene, with the majority of the mutations occurring in codons 12, 13 and 61. In 5% of the colorectal cancer cases a mutation occurs in the *NRAS* gene^[35,41].

Another important gene of the *RAS*/*RAF*/*MEK*/*ERK* pathway is *BRAF*. Mutations in the *BRAF* gene (exons 11 and 15) have been detected in about 12% of colorectal cancers and are mutually exclusive with *RAS* mutations^[12,43,44]. The *BRAF* activating aberrations, result in constitutive *BRAF* kinase activity, *ERK* signaling, proliferation and transformation^[44]. The majority of *BRAF* mutations are observed in exon 15 (codon 600) and a minority of mutations are observed in exon 11^[44,45].

Several studies have reported that patients with metastatic CRC (mCRC) that harbor *BRAF* mutations do not respond to the anti-*EGFR* antibody agents cetuximab or panitumumab^[43,46,47]. However it is unclear if the presence of *BRAF* mutations in CRC cancer can be

Table 1 Overall gene mutation frequency in each molecular signaling pathway

Biologic pathway	Frequency of mutation in genes involved in each pathway	Therapies that target the pathway
Beta-catenin/WNT signaling	75%	FZD, GSK inhibitors
Cell cycle control	68%	CDK, CDK1, CDK2, CDK4/6 inhibitors
Receptor tyrosine kinase/growth factor signaling	67%	Therapeutic antibodies/tyrosine kinase inhibitors
MAP kinase signaling	61%	BRAF, ERK, MEK AND SRC inhibitors
PI3K/AKT1/MTOR	52%	Allosteric mTORC1 inhibitors/mTORC1/2 catalytic inhibitors
DNA damage/repair	48%	PARP INHIBITORS
TGFbeta signaling	37%	TGFBRI inhibitors
Chromatin remodeling/DNA methylation	32%	DNMT inhibitors, Histone deacetylase
Immune checkpoints	26%	Anti-CTLA4 antibodies, anti-PD-1 antibodies, Anti-PD-L1 antibodies, Immunotherapies
JAK/STAT signaling	23%	JAK inhibitors
Hedgehog signaling	12%	SMO inhibitors

The information concerning therapies that target each molecular pathway was retrieved for MyCancer Genome Site (Available from: URL: <http://www.mycancergenome.org/>).

used as a predictive marker or if it has only a prognostic value, independent of treatment, since different studies arrive at controversial conclusions concerning its clinical significance^[45,48].

The *PIK3CA* gene encodes the catalytic subunit of phosphatidylinositol 3-kinase while belongs to a family of lipid kinases. These kinases regulate a diverse range of cellular processes including cell proliferation, adhesion, survival, and migration^[49]. Mutations in *PIK3CA* stimulate downstream AKT-mTOR signaling pathways, thereby promoting growth-factor independent growth, cell invasion and metastasis. *PIK3CA* mutations have been reported in multiple malignancies, including approximately 25% of gastric, 4% of lung, 25% of breast, and 20% of colorectal cancers^[50]. The majority (80%) of *PIK3CA* mutations cluster in 2 "hotspot" regions, the helical domain (exon 9) and the kinase domain (exon 20). Concomitant *PIK3CA* mutations in exons 9 and 20 seem to be linked to significantly worse cancer-specific survival^[51]. *PIK3CA* mutations may also be associated with clinical resistance to EGFR-targeted monoclonal antibodies, but there have been conflicting results^[52-55]. A meta-analysis comprising 864 patients, from 11 studies, with colorectal cancer treated with cetuximab or panitumumab-based therapy showed that *PIK3CA* mutations, particularly in exon 20, are significantly associated with worse response and shorter progression-free and overall survival^[51]. Somatic *PIK3CA* mutations have also been associated with superior colorectal cancer-specific survival in patients who regularly intake aspirin after diagnosis^[56]. *PIK3CA* activating mutations may also predict sensitivity to inhibitors of the PI3K-AKT-mTOR pathway^[57]. Inhibitors of mTOR, PI3K, and AKT, alone or in combination with other therapies are in clinical trials in solid tumors^[58,59].

A number of rare gene mutations occurring in the PI3K/AKT/mTOR pathway are potentially actionable in colorectal cancer. *PTEN* is a key negative regulator of the PI3K pathway. *PTEN* gene mutations occur in about 5% of colorectal cancers^[12,60,61]. *PTEN* inactivating mutations and *PTEN* loss have as a consequence the

upregulation of the PI3K/ AKT pathway^[54,61].

Currently, the prognostic and predictive significance of *PTEN* mutations or *PTEN* loss of expression is under investigation. In retrospective studies, *PTEN* loss was associated with decreased sensitivity of colorectal cancer tumors to anti-EGFR antibodies^[60-62]. Preclinical data and *in vitro* studies suggest that it may be associated with sensitivity to PI3K and mTOR inhibitors. Based on these data, several PI3K and mTOR inhibitors are currently in clinical trials for the treatment of patients with *PTEN*-deficient cancers^[61,63].

AKT (Protein kinase B, PKB) is a serine/threonine kinase that is encoded by three genes *AKT1*, 2 and 3. Somatic mutations in the *AKT1* gene occur in colorectal cancer in about 1% of the cases according to the COSMIC database^[12]. The only mutation observed is the activating mutation *E17K*, which is also observed in other types of cancer^[64]. *AKT1* is a critical component of the PI3K/AKT/mTOR pathway, thus it has become an attractive target for therapeutic intervention^[49,65]. *AKT1 E17K* mutations have also been associated with primary resistance to cetuximab^[66].

In colorectal cancer, DNA mismatch repair (MMR) system deficiency occurs frequently and leading to microsatellite instability (MSI). These are small changes in the DNA sequence that occur during DNA replication and are usually additions or deletions of one or two nucleotide bases^[67,68]. This phenomenon is most common in areas of the genome that contain repetitive DNA sequences with a repeat unit, from one to four bases, and are known as Microsatellite regions^[67,69]. The presence of microsatellite instability (MSI High) is a good prognostic marker^[67,70]. It is found in 90% of cases of tumors arising patients with hereditary Lynch syndrome and in 10%-15% of the sporadic cancers^[71]. Sporadic MSI-H tumors can be distinguished from the hereditary ones through somatic mutation analysis of the *BRAF* gene or loss of *MLH1* expression^[72,73]. Somatic mutations in the *BRAF* gene occurs only in sporadic MSI-H tumors but not in Lynch-associated CRC cancers. Similarly, *hMLH1* promoter methylation rarely occurs in

Lynch syndrome-associated cancers, while is common in sporadic MSI-high cancers^[73].

Recent studies have indicated that MSI high tumors, both sporadic and hereditary, are less aggressive and are related with low probability of lymph node and distant recurrences^[70]. In addition they respond differently to chemotherapy, since they are less sensitive to Topoisomerase inhibitors and to the treatment with 5-fluorouracil^[74-76]. Additionally, it has been proposed that MMR-deficient tumors are more responsive to PD-1 blockade than the mismatch repair-proficient tumors^[77].

Gene expression profiling (GEP) is an emerging tool which aims to identify differentially expressed subsets of genes (gene signatures) in groups of patients with distinct clinical outcomes. Several commercial GEP tests are currently available for stage II/IIIa colorectal cancer patients. Oncotype DX[®] Colon Cancer Assay (Genomic Health, Inc., Redwood City, CA) and ColoPrint (Agendia, BV, Amsterdam, Holland) are the most promising gene signature tests^[78]. Both tests provide a risk of recurrence, but OncotypeDx has the advantage of being applicable to Formalin Fixed Paraffin Embedded (FFPE) tissue for analysis, while ColoPrint requires fresh tissue which is not easily available. Oncotype DX[®] Colon Cancer Assay is a quantitative reverse transcription polymerase chain reaction (RT-qPCR) assay on RNA extracted from FFPE tumor tissue, used to assess risk of recurrence in stage II colon cancer patients at three years after surgery^[79-81]. The test uses gene expression profiling of 12 genes that include seven prognostic genes and five reference genes, in order to provide a Recurrence Score (RS). The RS allows patients and physicians to determine the risk of developing a distant metastasis. In a retrospectively performed study by Yothers *et al.*^[82], RS was the strongest predictor of disease recurrence independent of other factors, such as T-stage, mismatch repair status, number of nodes examined, tumor grade, and lymphovascular invasion. The greatest utility of this test seems to be in the prediction of recurrence risk in T3, mismatch repair-proficient (MMR-P) stage II colon cancer patients^[82]. However, it has also been validated in stage III patients with very promising results^[83]. The continuous RS predicted recurrence as well as disease free survival (DFS) and overall survival (OS) in all three patient subgroups (stage II, IIIA/B, and IIIC). The use of this assay could lead to overall reduction in adjuvant chemotherapy use in this subgroup of stage II/III colon cancer patients^[83].

LIQUID BIOPSY

Until recently, the best material for somatic mutation analysis was considered formalin fixed paraffin embedded (FFPE) tumor tissue. FFPE tissue is a widely available material, easy to use and maintain. In addition, the cancer tissue can be selected and mutation analysis

can be performed without contamination by normal tissues^[42]. This increases the sensitivity of mutation detection assays which is very important because, due to tumor heterogeneity, somatic mutations can sometimes be present at a very low percentage. However, FFPE tissue material also has several disadvantages^[84,85]. First of all in some cases it is not available. This is the case of non-operable tumors. Furthermore, the examination of a limited tumor area present in a paraffin block doesn't take into account tumor molecular heterogeneity and does not necessarily reflect the molecular profile of other tumors or metastasis that are eventually present in the patient's body^[85]. Additionally, the genetic material obtained, due to the paraffinization process, is sometimes of very bad quality and not suitable for molecular analysis^[86]. Most importantly, tumor molecular profile is altered mainly following therapy and those alterations cannot be detected by analyzing the primary tumor material^[87].

Nowadays, the presence of cell-free tumor derived nucleic acids (ctDNA/ctRNA) in cancer patients body fluids (plasma, serum, Broncho-alveolar, urine, stool, etc.) is well documented^[84]. The term Liquid Biopsy has emerged indicating the use of these noninvasive materials for tumor characterization. The mutation status detected in a liquid biopsy reflects the status present in the patient's tumor. Furthermore Liquid Biopsy analyses take into account intra-tumor or inter metastatic heterogeneity and could eventually detect more tumor alterations compared with the analysis of a specific area in a FFPE tissue^[83,84]. A variety of sensitive methods can be used for the detection of ctDNA in plasma samples, including digital PCR, Real time PCR, Arms PCR and NGS^[84].

The utility of liquid biopsy analysis has been proven in many studies that used ctDNA for the detection of tumor specific alterations in plasma with prognostic and/or predictive significance^[87-98]. A liquid biopsy analysis can be performed before treatment as well as for patients monitoring during therapy. It is also very helpful in the detection of secondary mutations that arise due to targeted therapy. The detection of secondary mutations in plasma can modify the treatment strategy for those patients (Table 2).

NGS METHODOLOGIES

NGS is a general term referring to all post-Sanger sequencing technologies that are able to massively sequence millions of DNA segments^[99,100]. The goal of these technologies is to increase sequencing capacity and speed at a lower cost. Furthermore, the sensitivity obtained is superior to that of the conventional sequencing technology, making possible the detection of mutations that are present at very low percentages in a background of normal DNA, which is very important for somatic mutation detection. Currently the most widely used platforms are those offered by Illumina, Inc. (United States); Thermo Fisher Scientific, Inc. (United

Table 2 Gene mutations identified in ctDNA of patients with tumors of the gastrointestinal tract and their correlation with possible clinical implications

Gene	Mutation type	Tumor type	Possible clinical implications	Ref.
KRAS/NRAS	Point mutation/amplification	Colorectal/pancreatic cancer	Resistance to anti-EGFR therapy/sensitivity to MEK inhibitors	[88-90]
BRAF	Point mutation	Colorectal cancer	Resistance to anti-EGFR therapy/sensitivity to MEK inhibitors	[88,91]
MET	Amplification/alteration	Colorectal/esophageal cancer	Resistance to anti-EGFR therapy/sensitivity to MEK inhibitors	[92-94]
HER2	Amplification	Colorectal/gastric cancer	Resistance to anti-EGFR therapy/sensitivity to Anti HER2 inhibitors	[95,96]
EGFR	Point mutation	Colorectal/pancreatic cancer	Panitumumab	[97,98]
PIK3CA	Point mutation	Colorectal/pancreatic cancer	mTOR inhibitor	[89]
Ckit	Point mutations	GISTS	Imatinib or dose escalation or alternative TKIs	[99,100]
PDGFRA	Point mutations	GISTS	Imatinib or dose escalation or alternative TKIs	[99,100]

TKI: Tyrosine kinase inhibitors; GIST: Gastrointestinal stromal tumors; EGFR: Epidermal growth factor receptor; mTOR: Mechanistic target of rapamycin.

Sates) and Roche Holding AG (Switzerland)^[101-103]. The first NGS platform was created by Roche and used emulsion PCR (emPCR) to clonally amplify the fragments that are then sequenced *via* sequencing-by-synthesis (SBS) technology^[101]. The Illumina platform is currently widely used in the NGS market and involves bridge amplification, of a solid surface-bound DNA, to clonally amplify the fragments that are then sequenced using SBS chemistry^[102]. Unlike the previous two technologies, the Life technology platform uses Ion semiconductor sequencing, instead of fluorescence based sequencing, detecting the protons released as nucleotides are incorporated during synthesis^[103,104].

The past years have seen an accelerating outbreak of publications in which NGS is applied for a variety of goals such as full-genome resequencing or more targeted mutation detection. Worldwide collaborative efforts, such as COSMIC database, International Genome Consortium (ICGC) and The Cancer Genome Atlas (TCGA) project, enabled to catalogue NGS data of thousands of cancer genomes across many disease types^[105,106]. Targeted NGS, involving gene panels, is a quicker and cost effective alternative to whole genome sequencing or exome sequencing. Targeted NGS panels for somatic mutation detection include actionable cancer genes and allow the determination of the patient's tumor molecular profile. The goal of their use is to increase the percentage of patients with detected actionable alterations and with copy number data, allowing them to be included in clinical trials^[107-109].

Such panels are currently available or can be custom made. They exhibit high rates of sensitivity, specificity and repeatability; therefore they are optimal for diagnostic use. Benchtop NGS sequencers are now offered by both Illumina (MiSeq) and Thermo Fisher (PGM™ and Ion Proton™). The availability of the equipment required and the cost effectiveness of the analysis allows its implementation in local specialized laboratories^[108,109]. However, the reliability of these tests should be reassured. Thus, NGS performing laboratories should have specialized personnel and equipment which will provide adequate data analysis management and interpretation with the aid of appropriate software and bioinformatics tools. Importantly, these tests should be

operated under the guidelines of a quality assurance system^[110,111].

Concerning the selection of the appropriate sequencing platform, it should be based on the individual laboratory's needs. All NGS platforms have advantages and disadvantages and the choice of the platform used should be based on the application for which it is required. For example, the MiSeq platform (Illumina) has lower error rates especially in the homo-polymer regions compared to both Ion Proton and PGM (Life Technology). However it requires higher DNA concentration and quality, which is not always available when the starting material is FFPE tissue. On the other hand, the NGS platforms offered by Thermo Fisher provide a fast and cost-effective sequencing solution with good analytical performance. Additionally, they more compatible with low DNA concentrations and partially degraded poor quality DNA from FFPE samples^[107-109,112]. Consequently, they provide an attractive option of clinical utility for the detection of cancer hotspot mutation analysis.

CONCLUSION

This review summarizes the use of biomarkers in the most common cancers of the GI tract. They are used for positive selection of patients who are likely to benefit from targeted therapy or for resistance prediction. Biomarker based targeted treatment is established in a subset of patients with gastrointestinal cancer. Meta-analysis studies have shown that biomarker based treatment is a promising approach and is associated with improved treatment outcome^[113-115]. However, ongoing clinical trials, identification of novel biomarkers as well as further advances in high-throughput technologies will hopefully result in further development of therapeutic targets, treatment strategies and improved survival for these patients in the near future.

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Clinical impact of chemotherapy to improve tumor microenvironment of pancreatic cancer

Takahiro Tsuchikawa, Shintaro Takeuchi, Toru Nakamura, Toshiaki Shichinohe, Satoshi Hirano

Takahiro Tsuchikawa, Shintaro Takeuchi, Toru Nakamura, Toshiaki Shichinohe, Satoshi Hirano, Department of Gastroenterological Surgery II, Hokkaido University Graduate School of Medicine, Sapporo 060-8638, Japan

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Correspondence to: Dr. Takahiro Tsuchikawa, Associate Professor, Department of Gastroenterological Surgery II, Hokkaido University Graduate School of Medicine, N-15 W-7, Sapporo 060-8638, Japan. tsuchi-t@med.hokudai.ac.jp
Telephone: +81-11-7067714
Fax: +81-11-7067158

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Abstract

A perioperative multimodal strategy including combina-

tion chemotherapy and radiotherapy, in addition to surgical resection, has been acknowledged to improve patient prognosis. However chemotherapy has not been actively applied as an immunomodulating modality because of concerns about various immunosuppressive effects. It has recently been shown that certain chemotherapeutic agents could modify tumor microenvironment and host immune responses through several underlying mechanisms such as immunogenic cell death, local T-cell infiltration and also the eradication of immune-suppressing regulatory cells such as regulatory T cells (Tregs) and myeloid-derived suppressor cells. With the better understanding of the cell components in the tumor microenvironment and the effect of chemotherapy to improve tumor microenvironment, it has been gradually clear that the chemotherapeutic agents is two-edged sword to have both immune promoting and suppressing effects. The cellular components of the tumor microenvironment include infiltrating T lymphocytes, dendritic cells, regulatory T cells, tumor associated macrophages, myeloid derived suppressor cells and cancer associated fibroblasts. Based on the better understanding of tumor microenvironment following chemotherapy, the treatment protocol could be modified as personalized medicine and the prognosis of pancreas cancer would be more improved utilizing multimodal chemotherapy. Here we review the recent advances of chemotherapy to improve tumor microenvironment of pancreatic cancer, introducing the unique feature of tumor microenvironment of pancreatic cancer, interaction between anti-cancer reagents and these constituting cells and future prospects.

Key words: Pancreas cancer; Microenvironment; Chemotherapy; Immune cells; Immunomodulation

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Core tip: It has been gradually clear that the chemotherapeutic agents are two-edged sword to have both immune promoting and suppressing effects. The cellular

components of the tumor microenvironment including infiltrating T lymphocytes, dendritic cells, regulatory T cells, tumor associated macrophages, myeloid derived suppressor cells and cancer associated fibroblasts could be improved. Based on the better understanding of tumor microenvironment following chemotherapy, the treatment protocol could be modified as personalized medicine and the prognosis of pancreas cancer would be more improved utilizing multimodal treatment strategy.

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INTRODUCTION

Pancreatic carcinoma is an extremely aggressive malignant tumor and the fifth leading cause of death worldwide and is expected to be the second by 2030 in Western countries^[1,2]. The only curative option is surgical resection, but the 5-year overall survival (OS) rate still needs to be improved from the current 10%-15% even after curative resection^[1,3]. A perioperative multimodal strategy including combination chemotherapy and radiotherapy, in addition to surgical resection, has been acknowledged to improve patient prognosis. New cytotoxic agents such as gemcitabine, Tegafur-gimeracil-oteracil potassium (TS-1) and combination chemotherapy with 5-fluorouracil (5-FU), oxaliplatin, and irinotecan along with perioperative chemoradiotherapy before and after surgery have recently been widely investigated^[4,5]. Chemotherapy, usually a standard treatment option for cancer, has not been actively applied as an immune-modulating modality because of concerns about various immunosuppressive effects. However, certain chemotherapeutic agents have recently been shown to improve host immune responses and even break immune tolerance^[6]. Several underlying mechanisms have been clarified, including immunogenic cell death^[7,8], local T-cell infiltration and also the eradication of immune-suppressing regulatory cells such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs), all of which are associated with cells in the tumor microenvironment^[9]. On the other hand, care must be taken that chemotherapy-induced cancer metastasis does occur during treatment through non-immunological pathways^[10].

We review recent advances in chemotherapeutic regimens to improve the tumor microenvironment for pancreatic cancer, and introduce unique features of the tumor microenvironment for pancreatic cancer, interactions between anti-cancer reagents and the constituent cells, and future prospects.

OVERVIEW OF STANDARD CHEMOTHERAPY AND CHEMORADIATION THERAPY FOR PANCREATIC CANCER

Although the only curative option is surgical resection, with the advances in perioperative strategy for pancreatic carcinoma, many cytotoxic agents have proven effective in treating this disease. Chemoradiation therapy had been also adopted aiming at locoregional response and additional effects outside the field of irradiation (abscopal effects)^[11].

Representative cytotoxic agents include historical 5-FU monotherapy, gemcitabine monotherapy, and gemcitabine-based combination therapies^[4]. The following randomized controlled trials are investigations recently undertaken try to improve the chemotherapeutic strategy for pancreatic cancer. Burris *et al.*^[12] had shown for the first time that gemcitabine was superior to 5-FU in terms of overall survival, thus suggesting gemcitabine as a key drug in advanced pancreatic cancer. In 2011, FOLFIRINOX (5-FU, leucovorin, irinotecan and oxaliplatin) had been shown to have survival benefit over gemcitabine alone in patients with metastatic pancreas cancer^[13]. Recently Nab-paclitaxel plus gemcitabine have been reported to have superior efficacy compared with gemcitabine monotherapy in metastatic pancreas cancer (MPACT trial)^[14]. However, MPACT trial, consisting of Gem + Nab-paclitaxel had OS of 8.5 mo compared to 6.7 mo in patients treated with gemcitabine alone) suggesting minimal improvement of survival by current chemotherapy regimens and requiring for further developments.

Taken together, overall survival of patients with metastatic disease extended to nearly 1 year from around 6 mo in the preceding two decades, thanks to recent therapeutic advances. However, although these reagents are promising, median progression-free survival remains limited and the 5-year survival rate of patients is still unsatisfactory, at around 15%-20%, even with these multimodal treatment strategies. This is due in part to dose limiting toxicity of side effects such as neuropathy and bone marrow suppression and due also to chemoresistance, relapse and metastasis even after surgical resection.

TUMOR MICROENVIRONMENT OF PANCREATIC CANCER

Tumor cells alone were initially considered the specific target of chemotherapy, leading to a focus on the cytotoxicity of agents inhibiting DNA repair, tubulin formation and cell proliferation^[15]. However, recent research has identified the tumor microenvironment as comprising tumor cells, host immune cells such as T cells, Tregs and MDSCs, and cancer-associated fibroblasts or stromal cells that support or suppress each

Table 1 Targets of chemotherapy to improve tumor microenvironment

Cellular components	Target molecules	Chemotherapeutic agents	Underlying mechanism	Ref.
TIL	CD4, CD8 positive T lymphocytes RAS/MAPK	GEM, TS-1, MEK inhibitor, PD1/PDL1 immune checkpoint inhibitors	Increase lymphocyte infiltration	[22] [24]
DC		GEM	Proliferation of DC and CTL	[26]
Treg	CD4 and FoxP3 positive T lymphocytes	GEM, cyclophosphamide	Depletion	[21]
MDSC	CCL2, CCR2, GM-CSF	GEM, 5-FU	Increase differentiation	[34,35]
			Depletion	[5]
CAF	Palladin positive fibroblasts	GEM	Depletion	[38]
	mTOR/4E-BP1 pathway	GEM, Pasireotide	Reduce tumor growth and chemoresistance	[39]

TIL: Tumor infiltrating lymphocytes; DC: Dendritic cells; Treg: Regulatory T cells; TAM: Tumor associating macrophages; MDSC: Myeloid derived suppressor cells; CAF: Cancer associated fibroblasts; Gem: Gemcitabine; 5-FU: 5-fluorouracil.

other^[9]. Each of these cellular components contributes to treatment response and patient prognosis, with tumor cells forming a network through direct interactions and cytokines providing important signals to initiate cell invasion into vessels and lymph nodes, leading to distant metastasis. Desmosomes are also one of the specific features of pancreas carcinoma that make drug delivery so difficult and prevent immune cells from infiltrating to tumor nests^[16].

These evidences collectively indicate that tumor cells are thought to grow, interacting with the micro-environment, highlighting the need to clarify the specific mechanisms by which each chemotherapeutic agent improves the tumor microenvironment to contribute to treatment efficacy.

The following sections are arranged to describe recent evidence for the effects of chemotherapeutic agents on the cellular components of the tumor micro-environment (Table 1).

INFILTRATING T LYMPHOCYTES

A number of reports have suggested that the accumulation of CD4 and CD8 lymphocytes in solid tumors offers a good prognostic indicator for patient survival^[17,18]. In terms of pancreatic cancer, Tewari *et al.*^[19] demonstrated a positive correlation between prognosis and the presence of tumor infiltrating T cells. Although the clinical relevance differs among types of cancer, in association with the HLA class I expression level^[20], some agents have been reported to induce T-cell infiltration into pancreas cancers^[21]. Homma *et al.*^[22] showed that CD4⁺ and CD8⁺ cells were significantly increased after neoadjuvant chemotherapy comprising gemcitabine and TS-1 followed by radiotherapy (NACRT), and a high accumulation of CD4⁺ cells offered a good prognostic marker for pancreas carcinoma after NACRT. Teng *et al.*^[23] recently classified the types of tumor microenvironment based on the presence or absence of T-cell infiltration and expression of PD1 along with patient prognosis.

Loi *et al.*^[24] recently suggested that therapeutic cooperation of MEK and PD-1/PD-L1 immune check point inhibitors could increase tumor-infiltrating lympho-

cytes through RAS/MAPK pathways in breast cancer. Great expectations are held for increased control of the tumor microenvironment, especially with tumor-infiltrating lymphocytes enabling further improvements in patient prognosis associated with immune check point inhibitors.

DENDRITIC CELLS

Dendritic cells are the most potent antigen presenting cells and play a crucial part in the initiation, programming, and regulation of antitumor immunity, directing cytotoxic T lymphocytes and natural killer cells to become potent antitumor effectors capable of eradicating malignant cells^[25,26]. Recently it had been reported that dendritic cells are impaired in number and display maturation defects disable to function as antigen presenting cells in pancreatic cancer due to the inflammation of the disease^[27]. Meanwhile, chemotherapy can promote immunogenic cell apoptosis enhancing immunogenicity and mediating efficient phagocytosis by dendritic cell^[7]. Moreover, gemcitabine can enhance the cross presentation of tumor associated antigens by dendritic cells and as well as inducing the proliferation of DC and CTL^[26]. Those strategies utilizing chemotherapeutic agents might be useful to overcome negative microenvironment.

REGULATORY T CELLS

Tregs are defined as T cells expressing both CD4 and forkhead box P3 (FoxP3), and are usually associated with poor prognosis and immunosuppression in various cancers. Transcriptional FoxP3 is a crucial intracellular marker and also a key developmental and functional factor for CD4⁺FoxP3⁺ Tregs^[28]. In terms of pancreatic cancer, multimodal chemotherapy including GEM, cyclophosphamide, and taxane has been demonstrated to decrease Tregs in the tumor microenvironment^[5]. Low Treg percentage in circulation at 1 year after PC resection had been correlated with improved survival^[29]. We have also previously shown that neoadjuvant treatment of pancreatic ductal adenocarcinoma with chemotherapy and chemoradiotherapy can alter the

local Treg balance in favor of antitumor immunity in resected human sections^[21]. Another paper by Keenan *et al*^[30] showed that immunization of mice with *Listeria* Monocytogenes engineered to express k-ras along with depletion of Treg cells reduced progression of early stages PanINs. Also, Shibuya *et al*^[31] recently reported that CD8 effector T cells show marked accumulation in the tumor microenvironment, but are suppressed by Tregs and PD-L1 expressed on T cells. These findings have therefore led to expectations for novel strategies of multimodal chemotherapy in combination with immune checkpoint inhibitors reducing Tregs.

TUMOR-ASSOCIATED MACROPHAGES

Tumor-associated macrophages (TAMs) are derived from CCR2⁺ monocytes in the spleen and peripheral blood, infiltrating into the tumor and developing into macrophages on stimulation by the releasing hormone CCL2 and colony-stimulating factor 1 (CSF-1)^[32,33]. TAMs have recently been reported to limit the effects of chemotherapy and promote tumor chemoresistance^[34]. Michem *et al.* reported that targeting TAMs by inhibiting CSF1R or C-C chemokine receptor 2 (CCR2) could decrease the number of pancreatic tumor-initiating cells and improve chemotherapeutic efficacy *in vivo*. The Denargo group reported that the combination of cytotoxic chemotherapy and blockade of CSF1R, which is prominently expressed by monocytes, Mo-MDSC and macrophages, resulted in improved anti-tumor T-cell responses^[32]. Furthermore, Sanford *et al*^[33] reported that the CCL2/CCR2 chemokine axis plays a crucial role in the recruitment of inflammatory monocytes from bone marrow to peripheral sites of inflammation and an increased ratio of inflammatory monocytes in blood compared to bone marrow offers a novel predictor of decreased patient survival following tumor resection. These lines of evidence clearly show that chemotherapy combined with chemokine blockade might reduce the chemoresistance associated with the exclusion of TAMs.

MYELOID DERIVE SUPPRESSOR CELLS

MDSCs are heterogeneous populations of immune cells derived from progenitor cells in bone marrow. MDSCs with a phenotype of CD33⁺HLA-DR^{low} that are lineage-negative (CD14⁻, CD15⁻) are well described as immunosuppressive in cancer patients contributing to tumor progression by damping T-cell immunity and promoting angiogenesis^[35,36]. Many chemotherapeutic drugs have long been thought to exclude MDSCs from various cancers. Zheng *et al*^[5] showed that GEM and 5-FU have a direct killing effect on MDSCs. In contrast, Takeuchi *et al*^[36] reported that GEM could increase MDSC numbers through increases in GM-CSF levels, converting M2 macrophages into suppressive MDSCs. Therefore MDSC in peripheral blood might be a possible predictive biomarker of chemotherapy failure in PC patients^[37]. Also, GEM and 5-FU have been reported to

activate NLRP3 inflammasomes in MDSCs, leading to interleukin-1 β release, which restrains their antitumor efficacy^[38]. More recently, Hu *et al*^[39] reported TNFB2R as important for its suppressive function. Uniquely, Sanford *et al*^[40] recently reported the clinical utility of zoledronic acid. This agent is usually utilized to improve calcium imbalances in patients osteoporosis, but also prevents tumor-mediated myelopoiesis associated with the generation of MDSC. Further studies are warranted to adjust the balance between direct reduction of MDSCs and indirect promotion of MDSCs by chemotherapy in combination with the multimodal strategies described above.

CANCER-ASSOCIATED FIBROBLASTS

Fibrous stroma associated with cancer in the tumor micro environment has increasingly been recognized as involving cancer-associated fibroblasts (CAFs). These cells are reported to contribute to poorer survival in various tumors, including pancreatic ductal adenocarcinoma, which has been reported to contain large numbers of CAFs^[41]. The characteristically dense desmosome in pancreatic cancer acts as a barrier to drug delivery, thus contributing to chemoresistance^[4]. Among the many markers of CAFs, Sato *et al*^[41] reported that palladin, a CAF marker, could represent an independent marker of poor prognosis and a biomarker to predict the efficiency of chemotherapy or even disease recurrence. Duluc *et al*^[42] recently revealed one of the underlying mechanisms abrogating pancreatic cancer chemoresistance through the mTOR/4E-BP1 pathway, allowing GEM-based chemotherapy combined with sst1 receptor-activating pasireotide to reduce tumor growth and chemoresistance. This kind of anti-stromal targeted therapy could be expected in addition to host immune cell-targeted therapy, as an adjunct to direct killing of cancer cells.

DISCUSSION

With our developing understanding of the cell components in the tumor microenvironment and the effects of chemotherapy in improving this environment, chemotherapeutic agents have gradually been revealed to represent a two-edged sword with effects that both promote and suppress immunity^[5]. Such therapies deplete one factor of immune suppression while at the same time inducing another mechanism to inhibit host immune responses. Some experimental data in this paper show that these problems might be overcome by multimodal combination chemoimmunotherapy in addition to standard chemotherapy, blocking antibodies for cytokine release or utilizing immune checkpoint inhibitors. Beaty *et al*^[43] reported combining chemotherapy with the agonist CD40, as a member of the TNF receptor superfamily, for surgically incurable PDA and observed tumor regression in some patients. Takeuchi *et al*^[36] likewise reported that anti-GM-CSF

antibody blocking could accelerate the formation of immunosuppressive myeloid cells in the tissue microenvironment of human pancreatic cancer.

Chemotherapeutic protocols including timing and dose might also be further explored and modified based on both reductions in tumor size and the induction of anti-tumor-specific immunity. Metronomic chemotherapy or low-dose chemotherapy has been reported to induce anti-tumor T-cell immunity *in vivo*^[44]. One of the underlying mechanisms might be that such low-toxicity doses of cytotoxic agents induce minimal suppression of tumor cells while concomitantly inducing minimal suppression of immune-promoting cells based on altered immune balance.

Lastly, future evidence should be accumulated regarding these balances in the tumor microenvironment during multimodal chemotherapy by measuring biomarkers locally and systematically. Biopsy specimens provide information of infiltrating T lymphocyte levels in the tumor microenvironment, offering possible predictors of beneficial response to chemotherapy in breast and pancreas cancers. SPARC expression levels in the stroma could represent a target for nab-paclitaxel. Although data must continue to be accumulated, miRNA might reflect changes in immune balances and predict the efficacy of chemoimmunotherapy^[45]. Taking all these lines of evidences together in combination with the properties of emerging agents, current problems seem likely to be overcome, at least in part, and the prognosis of pancreas cancer can be expected to continue improving in the coming decades.

CONCLUSION

The chemotherapeutic agents have both immune promoting and suppressing effects in the tumor microenvironment of pancreatic cancer. Based on the better understanding of tumor microenvironment following chemotherapy, the treatment protocol could be modified as personalized medicine and the prognosis of pancreas cancer would be more improved utilizing multimodal chemotherapy.

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Current noninvasive tests for colorectal cancer screening: An overview of colorectal cancer screening tests

Le-Le Song, Yue-Min Li

Le-Le Song, Yue-Min Li, Department of Radiotherapy, the Chinese PLA 309 Hospital, Beijing 100091, China

Le-Le Song, BioChain (Beijing) Science and Technology, Inc., Beijing 100176, China

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Correspondence to: Le-Le Song, MD, PhD, Department of Radiotherapy, the Chinese PLA 309 Hospital, No. 17, Heishanhu Road, Haidian District, Beijing 100091, China. songlele@sina.com
Telephone: +86-10-66775222

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Abstract

Colorectal cancer (CRC) has become the third most common cancer in the world. Screening has been shown to be an effective way to identify early CRC and precancerous lesions, and to reduce its morbidity and mortality. Several types of noninvasive tests have been developed for CRC screening, including the fecal occult blood test (FOBT), the fecal immunochemical test (FIT), the fecal-based DNA test and the blood-based DNA test (the SEPT9 assay). FIT has replaced FOBT and become the major screening test due to high sensitivity, specificity and low costs. The fecal DNA test exhibited higher sensitivity than FIT but its current cost is high for a screening assay. The SEPT9 assay showed good compliance while its performance in screening needs further improvements. These tests exhibited distinct sensitivity and specificity in screening for CRC and adenoma. This article will focus on the performance of the current noninvasive *in vitro* diagnostic tests that have been used for CRC screening. The merits and drawbacks for these screening methods will also be compared regarding the techniques, usage and costs. We hope this review can provide suggestions for both the public and clinicians in choosing the appropriate method for CRC screening.

Key words: Colorectal cancer; Adenoma; Fecal immunochemical test; Fecal DNA; SEPT9; Septin 9

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Core tip: The choice of colorectal cancer (CRC) screening methods is crucial for screening validity and compliance. Currently, the fecal immunochemical test (FIT), fecal DNA and the blood-based SEPT9 assays are the three *in vitro* diagnostic tests for CRC screening. In this article, we reviewed the current application of the three types of assays and compared their performance

in CRC screening. FIT is still the cheapest method with high screening validity, and fecal DNA tests also exhibit high validity but its price is high. In contrast, the SEPT9 assay showed high compliance with acceptable performance. The choice of screening test may depend on the balance of performance, compliance and costs.

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INTRODUCTION

Colorectal cancer (CRC) has become the second and the third leading cause of new cancer cases in Europe and in the United States, respectively^[1]. There were approximately 142820 new cases with 50830 deaths in the United States in 2013, and approximately 447000 new cases of CRC and 215000 deaths in European countries in 2012^[1,2]. The new cases for CRC are approximately 400000 in China in 2012, and it has become the third leading cause of death in the country^[3].

Regular screening can achieve early CRC detection and early treatment. However, 60%-70% of patients are found at middle- or late-stage CRC when they are diagnosed^[4]. Approximately 60% CRC deaths could be avoided and the average 5-year survival rate could be increased from 46% to 73% if healthy people carry out a regular periodic screening each year^[5]. Therefore, an effective early screening method for CRC can reduce CRC morbidity and mortality.

There are four *in vitro* diagnostic (IVD) screening method currently available for CRC screening, including the fecal occult blood test (FOBT), the fecal immunochemical test (FIT), the fecal DNA test and the plasma SEPT9 gene methylation test. This review will provide a detailed analysis on the performance of these tests, and compare their merits and drawbacks in CRC screening. It is our aim for this review that the public and the professionals can choose the appropriate methods for CRC screening.

STOOL-BASED TESTS FOR CRC SCREENING

The FIT test

The guaiac FOBT test (gFOBT) has been used for a long time as a screening test for CRC. It exhibited a sensitivity of 12.9%-79.4% with a specificity of 86.7%-97.7% for CRC screening in many studies^[6-13]. However, its sensitivity and specificity for CRC detection is lower than the more specific FIT (previous called iFOBT) test. This is because the gFOBT relies on peroxidase-like activity

between heme and guaiac, which can be affected by many factors in daily diet without distinguishment between upper and lower gastrointestinal (GI) tract bleeding, while the FIT test targets the hemoglobin in the lower GI tract, as hemoglobin from upper GI tract will be degraded when it arrives at lower GI tract. This characteristic allows FIT test to specifically detect the bleeding from lower GI tract, and therefore detect the diseases with bleeding, such as adenoma, polyps, inflammatory diseases and CRC, etc. As the gFOBT test has many drawbacks in CRC screening, FIT is used more commonly in current CRC screening. We therefore focus on the performance of FIT test in this review.

The performance of FIT test in CRC screening in asymptomatic, average-risk adults has been listed in Table 1. Data from 19 studies showed that the overall sensitivity for CRC was 0.79 (95%CI: 0.69-0.86) and the overall specificity was 0.94 (95%CI: 0.92-0.95)^[12,14-31]. This includes a total of 113360 subjects with 437 CRC cases confirmed by colonoscopy or 2-year follow-up. As the overall sensitivity and specificity are satisfactory for a cancer screening test with low costs, FIT is currently the most commonly-used method for CRC screening. The overall CRC positivity rate of 0.39% (437/113360) appeared to be significantly lower than the other two screening reports with asymptomatic, average-risk adults using fecal DNA (0.65%, 65/9989; $\chi^2 = 15.93$, $P < 0.001$)^[32] and SEPT9 gene methylation assay (0.67%, 53/7941; $\chi^2 = 14.66$, $P < 0.001$)^[33], respectively, indicating that the use of 2-year follow-up as a confirmatory methods may result in underestimation of CRC cases.

The use of qualitative FIT or quantitative FIT has always been an issue in choosing the FIT test for screening. A strip test (colloidal gold immunochromatographic method) is currently the main technique for qualitative FIT. It does not need specific instruments and the interpretation of test results relies on human recognition of test bands, although instruments are available to digitize the chrominance of the bands. In contrast, immunoturbidimetry is the main method for quantitative FIT, and the current devices include automated instrument for samples processing and colorimetry. Therefore, the current qualitative FIT appears to be faster, more convenient, less costly while more subjective than the quantitative FIT.

The performance between the qualitative and quantitative FIT showed significant differences. As shown in Table 1, the overall sensitivity of the qualitative FIT was 0.82^[12,15-21], which was significantly higher than that of the quantitative FIT (0.73) ($\chi^2 = 3.933$, $P = 0.047$)^[22-31], while the qualitative FIT exhibited significantly lower specificity than the quantitative FIT (0.93 vs 0.95) ($\chi^2 = 81.64$, $P < 0.001$), although the difference was small. This comparison needs to be interpreted with caution, as different studies used different cutoff values and resulted in distinct sensitivity and specificity. Ideally, they should be compared under the same cutoff value so that the sensitivity and specificity can be directly

Table 1 The sensitivity and specificity of qualitative and quantitative fecal immunochemical test

Qualitative FIT					Quantitative FIT				
Ref.	Total cases	CRC cases	Sensitivity	Specificity	Ref.	Total cases	CRC cases	Sensitivity	Specificity
Allison <i>et al</i> ^[12] , 1996	7493	35	0.69	0.94	Sohn <i>et al</i> ^[22] , 2005	3794	12	0.25	0.99
Allison <i>et al</i> ^[15] , 2007	5356	14	0.86	0.97	Levi <i>et al</i> ^[23] , 2011	1204	6	1.00	0.88
Cheng <i>et al</i> ^[16] , 2002	7411	16	0.88	0.91	Levi <i>et al</i> ^[24] , 2007	80	3	0.67	0.83
Nakama <i>et al</i> ^[17] , 1999	4611	18	0.56	0.97	Morikawa <i>et al</i> ^[25] , 2005	21805	79	0.66	0.95
Nakama <i>et al</i> ^[18] , 1996	3365	12	0.83	0.96	Launoy <i>et al</i> ^[26] , 2005	7421	28	0.86	0.94
Parra-Blanco <i>et al</i> ^[19] , 2010	1756	14	1.00	0.93	Itoh <i>et al</i> ^[27] , 1996	27860	89	0.87	0.95
Chiu <i>et al</i> ^[20] , 2013	8822	13	0.85	0.92	Nakazato <i>et al</i> ^[28] , 2006	3090	19	0.53	0.87
Chiang <i>et al</i> ^[21] , 2011	2796	28	0.96	0.87	Park <i>et al</i> ^[29] , 2010	770	13	0.77	0.94
					de Wijkerslooth <i>et al</i> ^[30] , 2012	1256	8	0.75	0.95
					Brenner <i>et al</i> ^[31] , 2013	2235	15	0.73	0.96
					Brenner <i>et al</i> ^[31] , 2013	2235	15	0.60	0.95
Overall (pooled data)	41610	150	0.82	0.93		71750	287	0.73	0.95

CRC: Colorectal cancer; FIT: Fecal immunochemical test.

Table 2 Comparison of sensitivity and specificity between Cologuard and fecal immunochemical test

Pathological categories		Cologuard	FIT
Sensitivity ^[32]	CRC	92.3%	73.8%
	Advanced precancerous lesions	42.4%	23.8%
	Polyps with high-grade dysplasia	69.2%	46.2%
	Serrated sessile polyps	42.4%	5.1%
Specificity ^[32]	Nonadvanced or negative findings	86.6%	94.9%
	Negative results on colonoscopy	89.8%	96.4%

CRC: Colorectal cancer; FIT: Fecal immunochemical test.

compared. The pooled data analyzed here provides a reference for comparing the two types of FIT tests. It can be suggested that the quantitative FIT may be a good choice for CRC screening tests that do not need high accuracy or are performed in hospitals where automated instruments are not available.

However, it should be mentioned that the cutoff value for qualitative FIT is preset, while the cutoff value for quantitative FIT can be adjusted to balance the sensitivity with specificity. Therefore, the data format for qualitative FIT is "positive" or "negative" without traceability, while the results from quantitative FIT are digitized with traceability. This is extremely useful when the relationship between the amount of bleeding in a certain disease and the population/personal information (such as diet, age, habit, sex, etc.) is investigated. Future model for predicting CRC incidence might partially relies on the data from quantitative FIT.

The fecal DNA test

The detection of abnormal DNA or epigenetic markers from colorectal lesions is based on natural exfoliation of cancerous or precancerous cells into the colorectal tract. The fecal DNA test aims at detecting the DNA mutations, microsatellite instability, impaired DNA mismatch repair and abnormal methylation. There are many studies focusing on the detection of CRC by fecal DNA markers^[34,35], and the overall sensitivity for CRC

detection by various fecal DNA marker combinations ranged from 53% to 87%, with specificities beyond 76%^[34,35]. Although there are a large number of fecal DNA markers available in these studies, the first commercial fecal DNA test was not available until the approval of Cologuard (Exact Sciences, Madison, WI, United States) by the United States FDA in 2014. Imperiale *et al*^[32] published the leading study on Cologuard in 2014. By randomizing subjects to Cologuard or FIT screening, it showed that the sensitivity of Cologuard was superior to that of FIT in CRC, advanced precancerous lesions, polyps with high-grade dysplasia and serrated sessile polyps, while its specificity appeared to be lower than that of FIT (Table 2).

The Cologuard DNA test includes quantitative molecular assays for KRAS mutations, aberrant NDRG4 and BMP3 methylation, and β -actin, plus a hemoglobin immunoassay. As the hemoglobin immunoassay is essentially a FIT test, Cologuard is actually a combination of gene mutation, methylation and occult blood tests. The multitarget stool DNA test provides a new way that combines various detecting technology to detect CRC and early colorectal lesions with high sensitivity and specificity. The high detection of precancerous lesions, HGD and serrated sessile polyps is extremely useful for a screening test, as these lesions may develop into CRC if they are not resected. The only obstacle for broad application of Cologuard is the cost, as the detection of multitargets increased the cost of the test. Its current expense of \$599 per test is high for a routine screening assay.

BLOOD-BASED TESTS FOR CRC SCREENING

The plasma SEPT9 gene methylation assay

An ideal screening test for cancer could be a simple blood test in the foreseeable future. The plasma SEPT9 gene methylation test Epi proColon (Epigenomics AG, Berlin, Germany) is currently the only commercially

Table 3 The reported positive detection rate for each colorectal cancer stage using 1/3 algorithm

Ref.	Positive detection rate for each colorectal cancer stage			
	I	II	III	IV
deVos <i>et al</i> ^[38] , 2009	52.6% (10/19)	75.0% (30/40)	77.8% (21/27)	100.0% (4/4)
Warren <i>et al</i> ^[40] , 2011	71.4% (5/7)	90.3% (28/31)	100.0% (7/7)	100% (5/5)
Tóth <i>et al</i> ^[41] , 2012	84.0% (21/25)	100.0% (14/14)	100.0% (35/35)	100.0% (18/18)
Lee <i>et al</i> ^[47] , 2013	30.8% (8/26)	36.7% (11/30)	25.0% (7/28)	64.7% (11/17)
Johnson <i>et al</i> ^[44] , 2014	61.5% (16/26)	80.0% (16/20)	65.2% (15/23)	92.3% (12/13)
Pooled positive detection rate	58.3% (60/103)	73.3% (99/135)	70.8% (85/120)	87.7% (50/57)

Table 4 The reported positive detection rate for each colorectal cancer stage using 2/3 algorithm

Ref.	Positive detection rate for each colorectal cancer stage			
	I	II	III	IV
Grützmann <i>et al</i> ^[37] , 2008	50.0% (11/22)	69.4% (25/36)	79% (42/53)	91% (10/11)
deVos <i>et al</i> ^[38] , 2009	26.3% (5/19)	60.0% (24/40)	66.7% (18/27)	75.0% (3/4)
Tóth <i>et al</i> ^[41] , 2012	60.0% (15/25)	92.8% (13/14)	81.6% (31/35)	77.8% (14/18)
Jin <i>et al</i> ^[46] , 2015	66.7% (12/18)	82.6% (19/23)	84.1% (37/44)	100.0% (5/5)
Pooled positive detection rate	51.2% (43/84)	71.7% (81/113)	80.5% (128/159)	84.2% (32/38)

available blood-test for CRC early detection and screening, and was approved recently by the United States FDA as a CRC screening test for average-risk population over 50 years old. Many clinical studies have proved the test to be a method with acceptable sensitivity and specificity for CRC detection^[33,36-49]. The test was firstly developed by Lofton-Day *et al*^[36] in 2008 as a research kit, and was commercialized by Epigenomics AG as its first generation assay Epi proColon 1.0. At the same time, ARUP lab also developed its SEPT9 assay as a lab-developed test^[40]. Abbott developed its real-time mS9 CRC assay, but there was only one report on its performance and the sensitivity of 36.3% was much lower than other SEPT9 tests^[47]. The 2nd generation test (Epi proColon 2.0) was launched in 2011-2012 with better performance. Till today, most reports on the SEPT9 assay appeared to be case-control study or cohort study investigating the test performance in selected population, exhibiting a sensitivity of 36.6%-95.6% with a specificity of 81.5%-99.0% using 1/3, 2/3, 1/2 or 1/1 algorithm^[33,36-49]. In contrast, there is only one study (the PRESEPT trial) investigating the application of the assay in CRC screening in average-risk population, exhibiting a sensitivity of 48.2% and 68.2% with a specificity of 91.5% and 80.0% using 1/2 or 1/3 algorithm, respectively^[33,43].

Detection of early stage CRC is crucial for early intervention and reduction of mortality. The positive detection rate (PDR) of the SEPT9 assay for stage I, II, III and IV was 26.3%-84.0%, 36.7%-100.0%, 25.0%-100.0% and 64.7%-100.0%, respectively, depending on different algorithm, exhibiting a huge variation for each stage. As 1/3 and 2/3 algorithm are the most commonly used methods for result interpretation, we calculated the PDR for each stage using the two algorithms. The pooled PDR for stage I, II, III and IV was 58.3%, 73.3%, 70.8% and 87.7%, respectively, using 1/3 algorithm (Table 3), and was

51.2%, 71.7%, 80.5% and 84.2%, respectively, using 2/3 algorithm (Table 4)^[36-47]. No statistical difference in PDR in any stage between the two algorithms has been found. It can be clearly seen that the PDR for early stage CRC (stage I) was above 50% and fell into 70%-80% for stage II and III, which is acceptable for a blood-based CRC test. However, these PDRs were from case-control or cohort studies, and more studies should be performed at screening settings.

Although the SEPT9 assay was designed for CRC detection, researchers also studied its detection sensitivity for precancerous adenoma. The pooled PDR for non-advanced adenoma and advanced adenoma (AA) was 10.0% and 18.2%, respectively, from six studies, in which the PDR for AA was significantly higher than the PDR of normal control group (11.8%, χ^2 test, $P < 0.001$)^[30,33,37,40,43,46]. However, as PDR of 18.2% was still too low for an effective test, the SEPT9 assay may not be applicable in adenoma detection.

The SEPT9 assay exhibited high compliance in screening. One recent report showed that 63% of subjects in a CRC screening study refused colonoscopy. 97% of subjects who refused colonoscopy accepted a noninvasive screening test, and 83% chose the SEPT9 test and 15% chose FIT test. The majority of patients who refused colonoscopy chose the SEPT9 assay due to its convenience and less time-consuming procedure^[50].

CEA and other serum glycoprotein markers

CEA and carbohydrate antigen 199 (CA199) are the two most common serum-based glycoprotein CRC markers, however, they are not appropriate for CRC screening due to their low sensitivity and the lack of CRC specificity, especially for early-stage CRC^[41,51-53]. For example, CEA test exhibited a sensitivity of 40.9%-51.8% and a specificity of 85.2%-95% for CRC detection in three studies^[41,51,52]. Therefore, it is more appropriate to be used in monitoring the CRC recurrence or response from

Table 5 Sensitivity and specificity of fecal immunochemical test, fecal DNA and SEPT9 tests in colorectal cancer and advanced adenoma screening

	FIT ^[12,15-31]	Fecal DNA ^[32]	SEPT9 ^[43]
Sensitivity (CRC)	79%	92%	68%
Specificity	94%	87%	80%
Sensitivity (AA)	24%	42%	18%

FIT: Fecal immunochemical test; CRC: Colorectal cancer; AA: Advanced adenoma.

patients to surgical or systemic therapy, rather than screening^[53].

The main drawback of serum glycoprotein markers in CRC screening is that the sensitivity and specificity of any single marker is not high enough to make it a reliable indicator. These markers have been found in various cancers other than CRC with low sensitivity for early stage lesions. Combined use of multiple markers may be a way to achieve diagnostic significance in CRC detection. In one report, five glycoprotein markers, including CEA, CA199, CA242, CA72-4, and CA125, are used together as indicators for CRC. It showed that the sensitivity of any single marker was low (18.8%–52.2%) for detecting CRC in stages I and II, while the combination of the five exhibited a sensitivity of 85.3% at the specificity of 95%^[54].

COMPARISON OF NONINVASIVE TESTS FOR CRC SCREENING

The sensitivity for CRC and AA, and the specificity in asymptomatic average-risk population for FIT, fecal DNA and SEPT9 tests are shown in Table 5. It can be seen that the fecal DNA test exhibited the best performance in terms of sensitivity for CRC and AA, while its specificity was slightly lower than that of the FIT. It is noteworthy that the fecal DNA test can detect 42% AA, which may reduce the number of subjects progressing to CRC, *i.e.*, reducing the CRC morbidity. The SEPT9 assay is the only blood-base CRC screening assay currently. Although its screening performance was not satisfactory at the moment, it showed very high compliance^[50]. The blood-based CRC screening assay may be more popular if the sensitivity and specificity in screening setting could be improved to the level of those in case-control studies (ideally sensitivity > 70% and specificity > 90% for CRC screening).

The current costs for FIT, fecal DNA and the SEPT9 test are \$10-50, \$599 and approximately \$170, respectively. As the recommended screening frequency for FIT, fecal DNA and the SEPT9 is once per year, once per three years and once per year, respectively, FIT might be the cheapest test considering the balance between performance and costs. However, the quality adjusted life year of the three tests should be compared under the same setting to evaluate the cost-effectiveness of them, although some studies have been performed for each

Table 6 Positive detection rate of SEPT9, fecal immunochemical test and carcino-embryonic antigen tests and various combined tests

SEPT9 alone	FIT alone	CEA alone	SEPT9 + FIT	SEPT9 + CEA	FIT + CEA	SEPT9 + FIT + CEA
77.00%	74.6%	41.3% ^e	94.4% ^c	86.4% ^c	84.5%	97.2% ^e
(181/235)	(53/71)	(97/235)	(67/71)	(203/235)	(60/71)	(69/71)

^c*P* < 0.01; ^e*P* < 0.001 *vs* SEPT9 alone. FIT: Fecal immunochemical test; CEA: Carcino-embryonic antigen; NS: Not significant.

individual method in different settings, such as different health systems.

CRC SCREENING WITH COMBINED TESTS

The combination of fecal DNA (mutation and methylation) with a hemoglobin immunoassay in Cologuard has provided a good example for CRC screening when multiple markers are analyzed together to enhance the detection sensitivity. There are merits and drawbacks for this strategy. First, combination of multiple markers enhances sensitivity at the price of reducing specificity. The number of false positive cases will increase with the increased number of markers. Therefore, to identify the markers with high sensitivity and specificity and to find the best combination of markers remain a challenge for combined screening test development. Ideally, the number of markers should be kept to minimum, while the sensitivity and specificity can be balanced to provide the best performance. Secondly, the detection of multiple markers with distinct methods increases the technical difficulties in an assay. For example, the detection of mutation in Cologuard may use sequencing or PCR method, while the detection of methylation needs to use the methylation specific PCR method containing bisulfite conversion. In contrast, immunoassay is used in the detection of hemoglobin. Furthermore, the sample preparation procedure may also be different for detecting different abnormalities. Therefore, a good combined test needs not only optimization of each individual test, but also an accurate algorithm to maximize the performance of each test. The optimization and interpretation of the combined test must come from clinical trials with large number of cases. Thirdly, a screening test should be accurate, fast, convenient, simple and cheap. These features allow large-scale screening in a certain period of time, and allow easy test in areas where test instruments are not available. In addition, low costs ensure screening tests for average-risk population, in which the CRC incidence could be lower than 1% in people over 50 years old. All the above considerations need to be addressed in future development of combined screening test.

As FIT, SEPT9 and CEA tests are all CRC detection tests with high specificity, the combination of them may provide higher sensitivity with no significant compromise

in specificity. We recently tested this assumption in an opportunistic screening setting, in which blood and stool samples were collected from outpatients and inpatients coming to the GI departments of three Chinese hospitals^[55]. Table 6 shows the test results from the screening. SEPT9, FIT or CEA alone detected 77.0%, 74.6% and 41.3% of CRC cases, respectively, while the combination of the three increased the sensitivity to 97.2%, and SEPT9 plus FIT exhibited a sensitivity of 94.4%. Since CEA is more sensitive to late-stage CRC than early-stage CRC, and no significant difference was found between SEPT9 + FIT + CEA and SEPT9 + FIT, we recommend SEPT9 + FIT as a routine method for CRC screening.

CONCLUSION

The FIT, fecal DNA and the SEPT9 tests are IVD tests currently used for CRC screening. FIT tests exhibited satisfactory sensitivity and specificity with low costs and therefore become the major screening test for CRC at the moment. The sensitivity of the fecal DNA test appeared to be very high due to combination of multiple methods while its high cost is an obstacle preventing the test from broad use. Both sensitivity and specificity for the SEPT9 test in CRC screening were lower than those of the FIT and fecal DNA test, but it showed high compliance with promising future if its accuracy can be improved. Combined tests with multiple markers should be a future direction in CRC screening, however, some hurdles, such as technical integration, test/interpretation optimization, and high costs, etc, need to be overcome before they can be used in large-scale CRC screening aiming at asymptomatic average-risk population.

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Case of pseudo-Meigs' syndrome caused by gastric cancer-related metastatic ovarian tumor with prolonged survival

Masaru Okamoto, Kazunori Maeda, Atsushi Yanagitani, Kiwamu Tanaka

Masaru Okamoto, Department of General Internal Medicine, Tottori Prefectural Central Hospital, Tottori 680-0901, Japan

Kazunori Maeda, Atsushi Yanagitani, Kiwamu Tanaka, Department of Gastroenterology, Tottori Prefectural Central Hospital, Tottori 680-0901, Japan

Author contributions: Okamoto M, Maeda K, Yanagitani A and Tanaka K equally discussed the clinical manifestations; Maeda K, Yanagitani A and Tanaka K reviewed the manuscript critically; Okamoto M wrote the paper.

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Correspondence to: Masaru Okamoto, MD, Department of General Internal Medicine, Tottori Prefectural Central Hospital, Tottori, Edu, Tottori City, Tottori 680-0901, Japan. okamotoma@pref.tottori.jp
Telephone: +81-857-262271
Fax: +81-857-293227

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Abstract

A 48-year-old woman presented with bilateral enlarged ovaries, ascites, bilateral pleural effusion, and advanced gastric cancer. Pleural fluid cytology did not reveal malignant cells. Oophorectomy, performed as a palliative procedure, was followed by rapid resolution of the pleural effusion and ascites. The patient was diagnosed with pseudo-Meigs' syndrome, and underwent chemotherapy followed by partial gastrectomy. At the last follow-up, 84 mo following oophorectomy, she was alive, and free of disease recurrence, despite not receiving any further treatment. Pseudo-Meigs' syndrome should be considered in patients with bilateral ovarian tumors, ascites and pleural effusion, and treatment such as oophorectomy may result in symptomatic improvement and better prognosis in similar patients.

Key words: Pseudo-Meigs' syndrome; Ovarian tumor; Gastric cancer; Pleural effusion; Ascites

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Core tip: In general, the prognosis of gastric cancer with distant metastases is poor. On the other hand, oophorectomy for gastric cancer-related metastatic ovarian tumors may improve survival, especially in the absence of metastasis to other organs. We here report a long-term survival case of pseudo-Meigs' syndrome caused by gastric cancer following oophorectomy. We conclude that pseudo-Meigs' syndrome should be considered in patients with gastric cancer with enlarged

ovaries, pleural effusion, and ascites.

Okamoto M, Maeda K, Yanagitani A, Tanaka K. Case of pseudo-Meigs' syndrome caused by gastric cancer-related metastatic ovarian tumor with prolonged survival. *World J Gastrointest Oncol* 2016; 8(11): 801-804 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v8/i11/801.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v8.i11.801>

INTRODUCTION

Meigs' syndrome is defined as the presence of pleural effusion and ascites in association with benign ovarian tumors such as fibromas. In these patients, pleural effusion and ascites typically disappear following oophorectomy. Pseudo-Meigs' syndrome is similar to Meigs' syndrome, except that the ovarian tumor may be malignant rather than benign. Pseudo-Meigs' syndrome caused by gastric cancer with metastatic ovarian tumors is very rare. Herein, we report a case of pseudo-Meigs' syndrome caused by gastric cancer with metastatic ovarian tumors, with prolonged survival following oophorectomy.

CASE REPORT

A 48-year-old woman was admitted to our hospital with a 3-mo history of lower abdominal fullness. She had no significant medical history. She smoked half a pack of cigarettes and consumed 1 L of beer a day for 28 years. On physical examination, her height and weight were 161 cm and 49 kg, respectively. Her blood pressure (103/60 mmHg), pulse rate (92 beats/min), body temperature (37.3 °C), and arterial oxygen saturation at room air (96%) were almost normal. The right chest breath sounds were decreased, her abdomen was distended, and a hard fist-sized mass was palpable in her lower abdomen. Her serum levels of carbohydrate antigen 19-9 and carbohydrate antigen 125 (CA 125) were elevated at 170.4 U/mL (normal, < 35 U/mL) and 897 U/mL (normal, < 37 U/mL), respectively. Computed tomography demonstrated bilateral enlarged ovaries, ascites, and bilateral pleural effusion (Figure 1). Upper gastrointestinal endoscopy revealed an ulcerated lesion with raised margins on the greater curvature of the body of the stomach (Figure 2). Biopsy of this lesion confirmed the diagnosis of poorly differentiated adenocarcinoma. Based on the above findings, a diagnosis of gastric cancer with metastatic ovarian tumors, malignant ascites, and pleural effusion, was made, although pleural fluid cytology failed to identify any malignant cells. Consequently, bilateral oophorectomy was performed as a palliative procedure. The resected ovarian tumors measured 15 cm (right) and 8 cm (left) in diameter. The tumors were solid with multiple mucus-containing cysts (Figure 3A and B). There was no evidence of tumor dissemination in

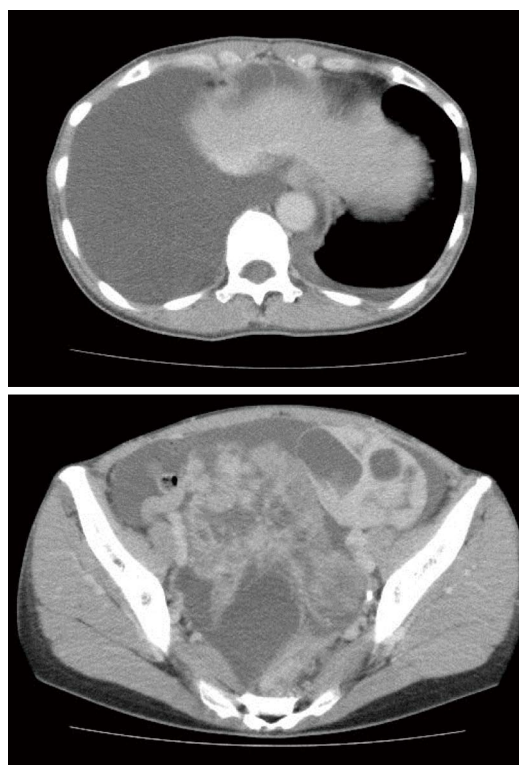


Figure 1 Whole body computed tomography-scan demonstrating bilateral enlarged ovaries, ascites and bilateral pleural effusion.

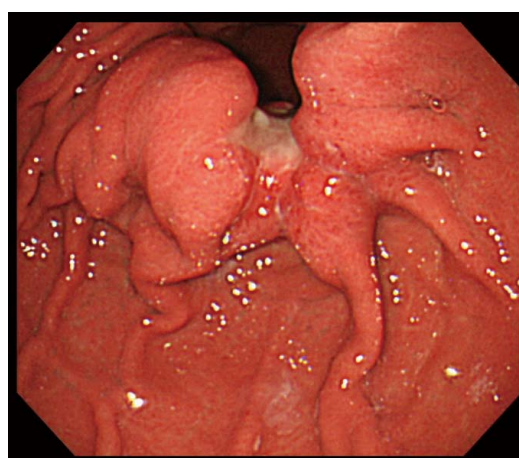


Figure 2 Upper gastrointestinal endoscopy revealing an ulcerated lesion with raised margins on the greater curvature of the body of the stomach.

the abdomen and the ascitic fluid was negative for cancer cells on cytology. Histological examination of the resected ovarian specimens confirmed that the tumors comprised poorly differentiated adenocarcinoma similar to the gastric tumor (Figure 3C), suggesting that the bilateral ovarian tumors were secondary to the gastric cancer (Krukenberg tumors).

The patient's pleural effusion and ascites resolved completely within 2 wk of her surgery. In view of these findings, we considered a diagnosis of pseudo-Meigs' syndrome. Following the oophorectomy, she received chemotherapy with docetaxel and S-1, with one course comprising docetaxel 40 mg/m² as an intravenous

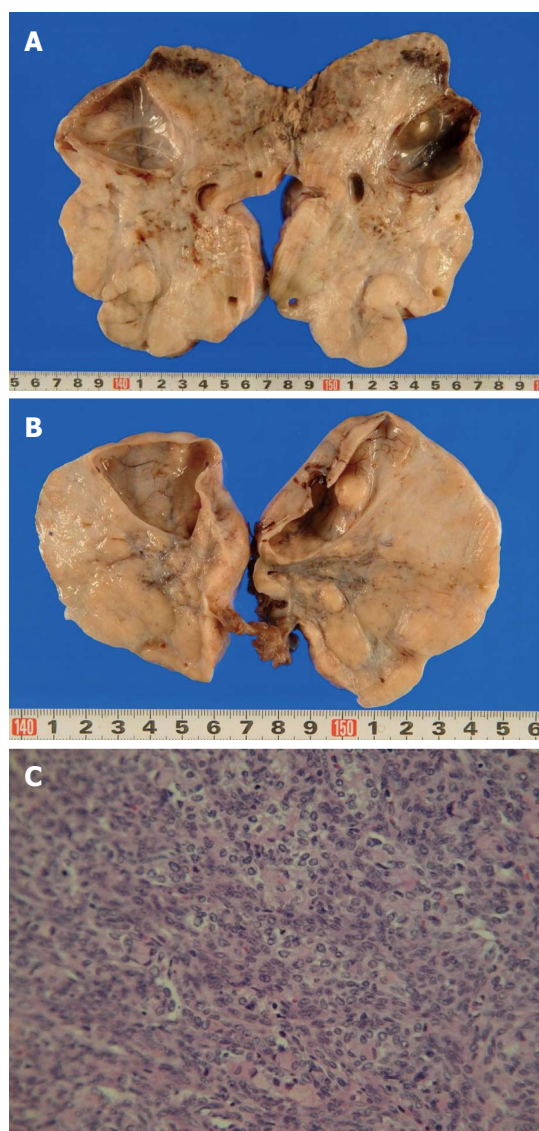


Figure 3 Resected ovarian tumors measured 15 cm (right) (A) and 8 cm (left) (B) in diameter. The tumors were solid with multiple mucus-containing cysts. Histological examination of the resected ovarian specimens confirmed that the tumors were composed of poorly differentiated adenocarcinoma similar to the gastric tumor (C).

infusion on day 1 and oral S-1 80 mg/m² on days 1-14 of a 21-d cycle. After 10 cycles of chemotherapy, approximately 9 mo after her first hospital visit, since no further metastases were detected, she underwent distal gastrectomy. The final pathological diagnosis was Stage IV [pT3, pN1, cM1(Ovary)] according to the TNM classification of gastric carcinoma (UICC fifth edition). At the last follow-up, 84 mo following oophorectomy, she was alive and free of disease recurrence, despite not receiving any further treatment.

DISCUSSION

In 1937, Meigs and Cass^[1] first reported a case series of seven patients with ovarian fibroma with pleural effusion and ascites, in whom the pleural effusion and

ascites disappeared following removal of the ovarian tumors. Subsequently, Rhoads *et al.* reported similar cases and coined the term "Meigs' syndrome"^[1,2]. A similar presentation associated with primary malignant or metastatic ovarian tumors, instead of benign ovarian tumors, is referred to as pseudo-Meigs' syndrome^[3].

Ascites may be secondary to ovarian tumors or fluid secretion from the peritoneum, develop as a result of tumor stimulation or in response to cytokines, or may be secondary to tumor-related lymphatic obstruction^[4]. Pleural effusion is explained as occurring secondary to the movement of ascitic fluid to the pleural cavity *via* transdiaphragmatic lymphatics and diaphragmatic foramen^[5]. It is uncommon for malignant tumor cells to be identified in the pleural or ascitic fluid in patients with pseudo-Meigs' syndrome.

Metastatic ovarian cancer comprises 6%-30% of all ovarian malignancies. The most common primary sites are the gastrointestinal tract, breast, and reproductive organs. In Japan, gastric cancer is the most common primary site because of its relatively high prevalence^[6]. Pseudo-Meigs' syndrome is more frequently caused by primary ovarian malignant tumors than ovarian tumors metastases from gastrointestinal cancer, and gastric cancer as the primary source for pseudo-Meigs' syndrome is particularly rare. In fact, to our knowledge, only 10 cases have been described so far, including five in the literature^[5,7-10] and five in Japanese conference proceedings. The mean age of the patients in the published reports was 51.8 (range, 32-76) years. Two and three cases had unilateral and bilateral ovarian metastases, respectively, and the ovarian tumor size ranged from 10 to 25 cm in diameter. Pleural effusion was bilateral in three cases and unilateral in two. Moreover, the serum CA 125 levels are often elevated in patients with Meigs' syndrome and decrease following oophorectomy, as in the present case. Benjapibal *et al.*^[11] explained that CA 125 may be produced from the peritoneal epithelium by a biomedical factor, secondary to mechanical irritation by a large tumor, or owing to an increase in the intra-peritoneal pressure related to the large volume of ascites.

The prognosis of gastric cancer with distant metastasis is poor, with a median survival time of 8.6-13.8 mo when treated by chemotherapy alone or in combination with molecular targeted therapy^[12]. On the other hand, Lu *et al.*^[13] reported that oophorectomy for gastric cancer-related metastatic ovarian tumors may improve survival, especially in the absence of metastasis to other organs. The overall survival times of patients who did and did not undergo metastasectomy were reported as 14.1 and 8 mo, respectively, in their study. Furthermore, Briau *et al.*^[14] reported that oophorectomy along with current chemotherapy regimens, such as taxane- or platinum-based therapy, improved survival even if the patients had extra-ovarian metastatic sites. In the present case, we selected a docetaxel and S-1 combination regimen with the expectation of efficacy for non-measurable lesions other than in the ovaries^[15,16].

The increased survival of the patient reported herein may be owing to her oophorectomy in conjunction with chemotherapy. Accordingly, this case emphasizes the need to be aware of pseudo-Meigs' syndrome, and supports the recommendation of oophorectomy in cases where metastases are limited to the ovaries.

COMMENTS

Case characteristics

A 48-year-old woman with a 3-mo history of lower abdominal fullness.

Clinical diagnosis

Gastric cancer with metastatic ovarian tumors, malignant ascites, and pleural effusion.

Differential diagnosis

Gastric cancer with primary ovarian cancer, reactive ascites and, pleural effusion.

Laboratory diagnosis

The serum levels of carbohydrate antigen 19-9 and carbohydrate antigen 125 were elevated, at 170.4 U/mL (normal, < 35 U/mL) and 897 U/mL (normal, < 37 U/mL), respectively.

Imaging diagnosis

Computed tomography demonstrated bilateral enlarged ovaries, ascites, and bilateral pleural effusion.

Pathological diagnosis

The resected ovarian specimens comprised poorly differentiated adenocarcinoma, similar to the gastric tumor, suggesting that the bilateral ovarian tumors were secondary to the gastric cancer; there was no evidence of tumor dissemination in the abdomen and the ascitic fluid was negative for cancer cells on cytology.

Treatment

Oophorectomy and gastrectomy in conjunction with chemotherapy.

Related reports

Gastric cancer, as the primary source for pseudo-Meigs' syndrome, is particularly rare. This case is one of only a few reports of pseudo-Meigs' syndrome caused by gastric cancer with prolonged survival.

Term explanation

Meigs' syndrome is defined as the presence of pleural effusion and ascites in association with benign ovarian tumors such as fibromas. In these patients, pleural effusion and ascites typically disappear following oophorectomy. Pseudo-Meigs' syndrome is similar to Meigs' syndrome, except that the ovarian tumor may be malignant rather than benign.

Experiences and lessons

The authors experienced a long-term survival case of pseudo-Meigs' syndrome caused by gastric cancer. If the metastases are limited to the ovaries, it is important not to automatically assume that the tumor is unresectable.

Peer-review

This is an interesting case report in terms of the disappearance of ascites and pleural effusion after oophorectomy, and prolonged survival despite of the extent of the disease at diagnosis.

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- 805** From traditional serrated adenoma to tubulovillous adenoma and beyond

Kalimuthu SN, Chelliah A, Chetty R

- 810** Role of circulating free DNA in colorectal cancer

Matikas A, Voutsina A, Trypaki M, Georgoulas V

ORIGINAL ARTICLE**Retrospective Study**

- 819** Signet ring colorectal carcinoma: Do we need to improve the treatment algorithm?

Tamhankar AS, Ingle P, Engineer R, Bal M, Ostwal V, Saklani A

SYSTEMATIC REVIEWS

- 826** Colon adenoma features and their impact on risk of future advanced adenomas and colorectal cancer

Calderwood AH, Lasser KE, Roy HK

CASE REPORT

- 835** Esophageal liposarcoma: Well-differentiated rhabdomyomatous type

Valiuddin HM, Barbetta A, Mungo B, Montgomery EA, Molena D

Contents

World Journal of Gastrointestinal Oncology
Volume 8 Number 12 December 15, 2016

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From traditional serrated adenoma to tubulovillous adenoma and beyond

Sangeetha N Kalimuthu, Adeline Chelliah, Runjan Chetty

Sangeetha N Kalimuthu, Adeline Chelliah, Runjan Chetty, Department of Pathology, Laboratory Medicine Program, University Health Network and University of Toronto, Toronto M5G 2C4, Canada

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Correspondence to: Runjan Chetty, MD, Professor, Department of Pathology, Laboratory Medicine Program, University Health Network and University of Toronto, 200 Elizabeth Street, 11th Floor, Eaton Wing, Toronto M5G 2C4, Canada. runjan.chetty@gmail.com
Telephone: +1-416-3405319
Fax: +1-416-3405517

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Abstract

It is well established that colorectal cancer develops

from a series of precursor epithelial polyps, including tubular adenomas, villous/tubulovillous adenomas (VA/TVA), sessile serrated adenomas (SSA) and traditional serrated adenomas (TSA). Of these, TSAs are least common and account for only 5% of all serrated polyps. TSAs are characterised by the presence of a "pinecone-like" architecture, granular eosinophilic cytoplasm, luminal serrations, ectopic crypt foci (ECF) and elongated, pencillate nuclei. However, the distinct slit-like luminal serrations, reminiscent of small bowel mucosa, appear to be the most unique and reproducible feature to distinguish TSAs from other polyps. There is a contention that TSAs are not inherently dysplastic and that the majority do not show cytological atypia. Two types of dysplasia are associated with TSA. Serrated dysplasia is less well recognised and less commonly encountered than adenomatous dysplasia. In addition, it is now becoming increasingly evident that TSAs can be admixed with HP, SSA and VA/TVA. At a genetic level, polyps may switch phenotype as they accumulate genetic changes, evolving from a serrated pathway to a more conventional one, which could be the basis for a spectrum theory starting out with a TSA with serration and ECF evolving into a TSA with conventional dysplasia and, eventually, to a well-developed conventional adenoma. Nevertheless, there is an exigency for future studies to provide further illumination and bridge the gaps in our present understanding.

Key words: Serrated polyps; Traditional serrated adenoma; Tubulovillous adenoma; Serrated pathway; Fusion pathways

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Core tip: Traditional serrated adenoma (TSA) is the least common type of the serrated polyps and is characterized by a constellation of distinct cytomorphological features. TSAs are thought to be precursors to the biologically aggressive, *BRAF* mutated, microsatellite stable,

colorectal cancer. It is becoming increasingly evident that TSAs can co-exist with other serrated polyps including hyperplastic polyps and sessile serrated adenomas. In addition, TSAs may also be seen with adenomatous polyps. In this review, we wish to highlight the issues around nomenclature, diagnostic criteria, coexistence with other polyp types, the occurrence of dysplasia and molecular pathways involved in the neoplastic progression of TSAs.

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INTRODUCTION

It is well established that colorectal cancer (CRC) develops from a series of precursor epithelial polyps^[1-5], which include conventional adenomas, incorporating tubular adenomas and villous/tubulovillous adenomas (VA/TVA) and serrated polyps, incorporating hyperplastic polyps (HP), sessile serrated adenomas (SSA) and traditional serrated adenomas (TSA)^[1,2,4,6,7]. CRC is known to develop through three putative molecular pathways with the conventional adenoma-carcinoma sequence (chromosomal instability pathway), the mismatch repair and serrated pathways, accounting for the molecular pathogenesis of most CRCs^[2,4]. VA/TVAs are thought to be the advanced precursors in the "adenoma-carcinoma" pathway^[2]. Conversely, CRCs arising from serrated polyps (which can be visible at the edge or intimately associated with the invasive tumour), are thought to be portentous of the "serrated pathway"^[1,4,8-10]. All three serrated polyps stay true to their epithet by characteristically demonstrating luminal serrations. Of these, TSAs are the least common and account for only 5% of all serrated polyps^[1].

NOMENCLATURE AND CLINICOPATHOLOGICAL CRITERIA

The historical provenance and journey taken to recognise TSAs in their present guise has been an interesting one. TSAs were first recognised by Longacre and Fenoglio-Preiser^[11,12] and were grouped together under the broad term of "serrated adenomas", as these polyps were thought to be conventional adenomas with a serrated luminal profile. Torlakovic *et al.*^[13] further refined this definition by appending the term "traditional" to the appellation, specifically to distinguish TSAs from SSAs and later highlighted several distinct morphological features specific to TSAs^[14].

TSAs are equally distributed between the genders and usually present in the sixth to seventh decade of

life^[11]. They range from 9-14 mm in maximum dimension and endoscopically have a pinecone-like appearance or may exhibit a fernlike/stellate pit pattern on chromoendoscopy^[11].

TSAs can be both sessile and pedunculated, the former being more common in the more proximal lesions^[11]. Histologically, TSAs are exemplified by a constellation of characteristic cytomorphological features, which include a tubulovillous, "pinecone-like" architecture, striking granular eosinophilic cytoplasm, presence of ectopic crypt foci (ECF), distinct luminal serrations, elongated, pencillate nuclei with evenly dispersed chromatin and small inconspicuous nucleoli and haphazardly distributed goblet cells (Figure 1)^[2,4,6,9,10,14]. Of these lineaments, the distinct slit-like luminal serrations with mushroom/jigsaw puzzle-like broad luminal fronds, reminiscent of small bowel mucosa, appear to be the most unique and reproducible feature to distinguish TSAs from other polyps^[6,8,11]. Interestingly, Bettington *et al.*^[5] have recently described a "serrated" TVA, which occurs more frequently in a proximal location and in essence, morphologically resembles a conventional TVA but at least > 50% of the polyp displays prominent serrations. The authors argue that this represents a distinct entity, with more frequent *KRAS* mutations and CpG island methylation; however, we surmise that the "undulating" or "maze-like" serrations described may merely represent a morphological spectrum seen within TVAs and could possibly be secondary to mechanical compression due to luminal spatial constriction. This latter conjecture could possibly elucidate why this particular type of serration may, at least focally, be observed in larger polyps harbouring a villous configuration, albeit almost never seen in TSAs. Nevertheless, it is important to distinguish serrated TVA from TSA, particularly in the scenario when TSAs co-exist with conventional TVAs^[6].

ECF have been defined as abnormal development of crypts, secondary to inactivating mutations in the bone morphogenetic protein 4 signalling pathway, causing loss of orientation towards the muscularis mucosae resulting in these short disorientated abortive crypts that fail to reach the muscularis mucosae (Figure 1)^[2,6,14]. ECF were previously touted to be a prerequisite for the diagnosis of TSA^[14]. However, these "maelstrom-like" or whorled clusters may not always be seen in TSAs^[2,6,8,15], particularly in smaller lesions (< 10 mm) and have also been documented in VA/TVAs (34%)^[2], albeit to a lesser frequency than observed in TSAs.

TSA WITH OTHER COEXISTENT POLYPS

When most of the aforementioned hallmark features are represented, a diagnosis TSA can be made with minimal difficulty. However, it is becoming increasingly evident that TSAs can be admixed with HP, SSA and VA/TVA^[1,2,6,16,17]. The documented rate of co-existent HP/SSA and VA/TVA with TSA range from 31%-52%,

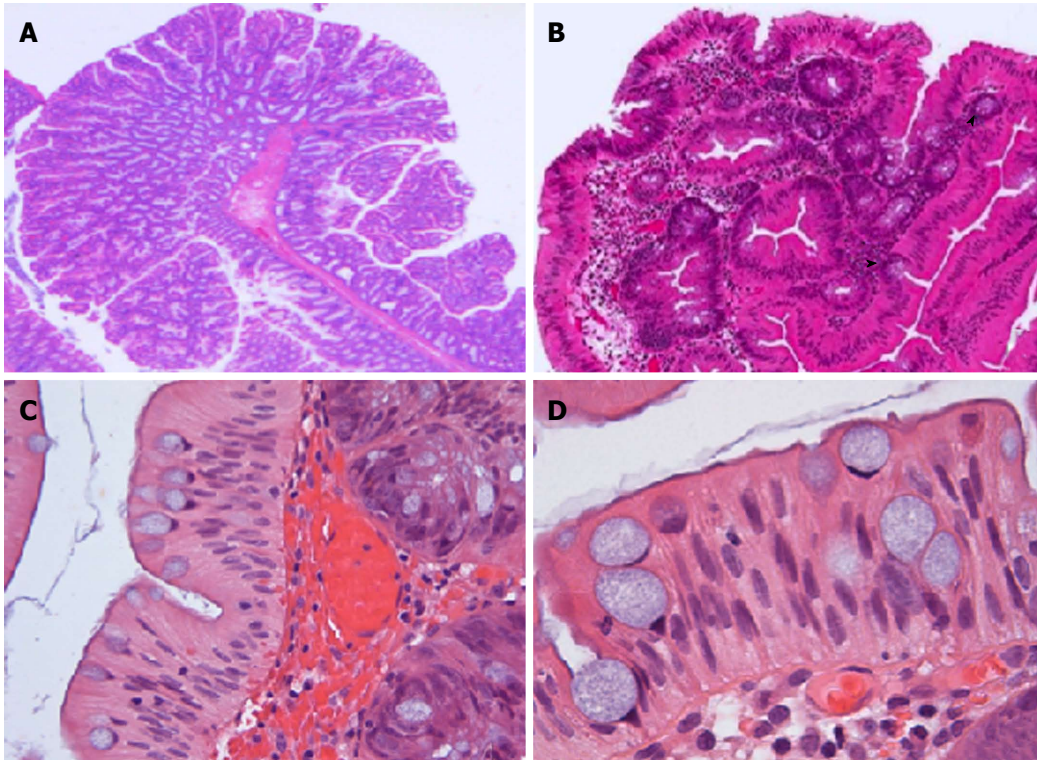


Figure 1 Traditional serrated adenoma. A: Traditional serrated adenoma (TSA) demonstrating an arborizing, "pinecone-like" pattern; $\times 12.5$; B: TSA replete with abortive crypts or ectopic crypt foci (ECF) (arrowhead); $\times 100$; C: Characteristic slit-like luminal serrations with deep clefts and indentations, resulting in mushroom-like or jigsaw puzzle-like appearance, which resemble the apical brush border of small bowel; $\times 200$; D: The epithelial cells have intensely eosinophilic cytoplasm with centrally located palisaded, regular, pencil nuclei and nuclear grooves. In addition, there are haphazardly distributed goblet cells with apical mucin and basally located nuclei; $\times 400$ (all H and E).

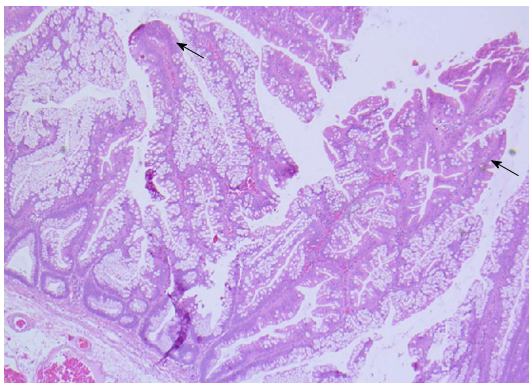


Figure 2 Sessile serrated adenoma with the characteristic dilated crypts. It is a horizontal growth along the muscularis mucosa and deep serration, seamlessly merging with foci of traditional serrated adenomas like areas (arrow); $\times 100$, H and E.

14%-17% and 17%-43%^[1,2,15,16], respectively. There is presently sufficient evidence to suggest that a significant proportion of TSAs contain precursor lesions in the form SSAs (Figure 2)^[3,4,17], the former being more common of the two. These lesions are often seen intimately associated with the TSA. In contrast, the adenomatous polyps are sharply delineated from the TSAs and appear separate and morphologically distinct (Figure 3A). It is important not to over-document a co-existent adenomatous component when adenomatous

dysplastic glands occur within a TSA (Figure 3). It is helpful to recognise that dysplastic glands in TSAs more or less retain the characteristic serrations and will be more replete with ECFs than expected in VA/TVAs.

TSA AND DYSPLASIA

The subject of dysplasia in TSA has long been a contentious one. Historically, TSAs were considered to be inherently dysplastic, owing to the close cytological resemblance to tubular adenomas or TVA^[12]. However, this axiom has since been challenged and there is an alternate view proposed^[8,10,11,15]. TSAs are unquestionably neoplastic; however, the absence of overt cytological atypia, infrequent or absent mitoses, low Ki-67 proliferation index, consistent B-catenin and p53 negativity, and retention of p16 staining, suggest TSAs are not intrinsically dysplastic^[6,10,11,15]. Instead, the cells of TSAs may represent metaplastic or senescent cells^[10,15]. It is noteworthy that there are two forms of dysplasia that can occur in TSAs and indeed in the other two serrated polyps^[4,9,10,17]. The first is the well-accepted conventional adenomatous dysplasia, which can be readily recognised with minimal difficulty. The second, less well recognised and controversial, is serrated dysplasia. Serrated dysplasia manifests secondary to activation of the serrated pathway, which is initiated by *BRAF* mutations^[4,7,10,11,18]. Similar to

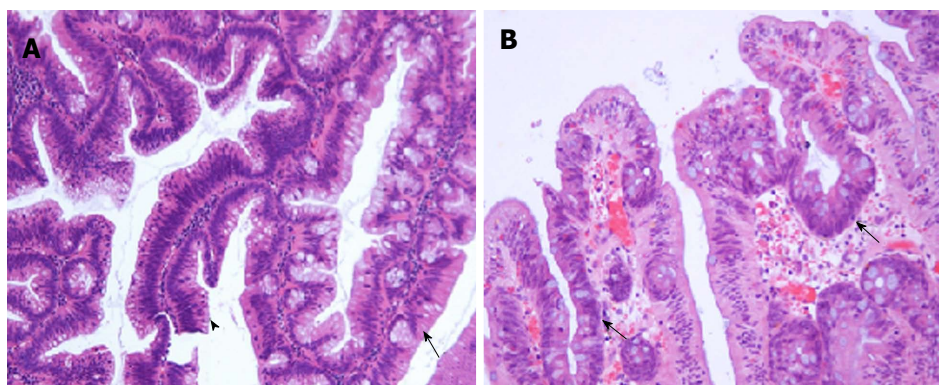


Figure 3 Traditional serrated adenoma and dysplasia. A: Traditional serrated adenoma (TSA) (arrow), sharply demarcated from more a more conventional tubulovillous adenoma (TVA) (arrowhead); $\times 100$; B: TSA with conventional dysplasia with some preservation of the characteristic architecture; $\times 200$ (both H and E).

adenomatous dysplasia, this form of can be graded as low grade and high grade based on both cytological and architectural features. Low grade serrated dysplasia can be subtle and is characterised by ovoid enlarged nuclei, vesicular dispersed chromatin with low mitotic activity. In addition, scattered dystrophic goblet cells may also be observed^[6,1,18]. In contrast, high grade dysplasia is readily recognisable as a result of severe cytological and architectural atypia. Currently, the biological significance of low grade serrated dysplasia is poorly understood. As such, in practice, it is recommended that only high grade serrated dysplasia be reported.

While the majority of the adenomatous polyps encountered may only display a single phenotype, in our experience, closer scrutiny often reveals isolated TSA-like glands or SSA/HP like areas. However, from a practical point of view, the sensible approach would be to name the polyp after the most dominant histological type, followed by any secondary or tertiary component identified with an accompanying comment as to the presence or absence of dysplasia.

MOLECULAR PATHWAYS

At a molecular level, the morphological alteration in phenotype may possibly be secondary to the transition from the serrated pathway to a more conventional one. For example, when conventional dysplasia ensues in a TSA, it is possible that the Wnt signalling pathway typically associated with chromosomal instability alters its morphology^[4]. Hence, the classic TSA with serrations and ECF, evolves into a TSA with conventional dysplasia and eventually, focally or entirely, resembling a conventional well-differentiated adenoma. This presupposes that molecular aberrations result in consistent morphological appearances. Jass *et al*^[3] was the first to propose the theory where separate to the adenoma-carcinoma and serrated pathway, there may be “fusion” pathways that combine mechanisms associated with both adenomatous and serrated polyps^[3,4]. However, further studies are required to consolidate this assertion.

CONCLUSION

Overall, it stands to reason that there is a considerable morphological overlap between different polyp types, albeit the molecular basis to which remains to be elucidated. Although conjectural, recent evidence raises the important question as to whether these polyps truly represent separate entities or are they merely manifestations at different stages of a morphological and/or molecular continuum. Nevertheless, there is an exigency for future studies to provide further illumination and bridge the gaps in our present understanding.

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Role of circulating free DNA in colorectal cancer

Alexios Matikas, Alexandra Voutsina, Maria Trypaki, Vassilis Georgoulas

Alexios Matikas, Alexandra Voutsina, Maria Trypaki, Vassilis Georgoulas, Laboratory of Tumor Cell Biology, School of Medicine, University of Crete, Voutes, Heraklion, 71110 Crete, Greece

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Correspondence to: Alexios Matikas, MD, Msc, Laboratory of Tumor Cell Biology, School of Medicine, University of Crete, Voutes, PO Box 1352, Heraklion, 71110 Crete, Greece. georgsec@med.uoc.gr
Telephone: +30-2810-392783
Fax: +30-2810-543601

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Abstract

The gradual elucidation of the underlying biology of colorectal cancer has provided new insights and therapeutic options for patients with metastatic

disease which are selected according to predictive biomarkers. This precision medicine paradigm, however, is incomplete since not all eligible patients respond to these agents and prognostic stratification is largely based on clinicopathologic variants. Importantly, no robust data exist to help properly select patients with localized disease at high risk for recurrence and most likely to benefit from adjuvant chemotherapy. There is a rapidly expanding body of literature regarding the role of the qualitative and quantitative analysis of circulating free DNA in various neoplasms, which consistently outperforms traditional tumor markers both as a predictive and as a prognostic marker. Several lines of evidence suggest that circulating free DNA may exhibit a complementary role to existing modalities for the early diagnosis of colorectal cancer, the selection of patients for adjuvant chemotherapy, for the follow-up of treated patients, for the selection of treatment for advanced disease and the assessment of response and for determining the prognosis of patients. These data, which are reviewed here, illustrate the important role that circulating biomarkers may soon have at the daily clinical practice.

Key words: Cell-free DNA; Circulating tumor DNA; Colorectal cancer; Biomarker; KRAS

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Core tip: Published studies clearly indicate that cell-free DNA levels and the detection of specific molecular events in the plasma of colorectal cancer patients is a relevant prognostic and predictive biomarker, with clinically meaningful value at various disease settings such as asymptomatic screening, follow-up after curative surgery and metastatic disease. Further randomized studies are needed before these techniques are implemented at the daily practice.

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INTRODUCTION

Globally, colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females; there is a significant regional variation in incidence and mortality rates in Western countries, especially the United States, where both are decreasing as a result of the widespread adoption of effective screening policies and of the evolution of treatment strategies at the adjuvant setting. Approximately 8% of cancer deaths are caused by CRC^[1,2]. Twenty percent of newly diagnosed patients have *de novo* clinically overt metastases; moreover, 10% of patients diagnosed with local and 30% with regional disease will eventually relapse, most commonly with disseminated disease^[3]. These patients presumably already harbor occult micrometastases, thus identifying them and administering systemic treatment following local excision may improve their chance for cure. Moreover, despite significant advances in the understanding of underlying molecular mechanisms and in the development and regulatory approval of several active agents during the past 15 years, 5-year survival rates of patients with metastatic CRC (mCRC) remain poor at 13%^[3], with the majority of these patients receiving palliative systemic treatment without a curative intent. Thus, it is clear that earlier diagnosis when interventions may be curable and also better predictive and prognostic biomarkers both for localized and advanced disease are highly needed.

Liquid biopsy is a minimally invasive process based on a simple venipuncture that potentially addresses several issues, since it can be safely implemented on a wide scale basis and can be repeated with minimal risks for the patient. Moreover, liquid biopsy may illustrate the molecular diversity of the underlying disease process and serial testing facilitates the monitoring of its spatial and temporal genomic evolution and at the same time it circumvents the need for re-biopsy, which is invasive, cumbersome and not always feasible^[4]. Moreover, re-biopsy is subject to sampling bias and it may not be representative of the intratumoral heterogeneity. These biomarkers may be protein-based, such as cancer antigens [carcinoembryonic antigen (CEA)], cell-based, such as circulating tumor cells (CTC) and disseminated tumor cells and nucleic acid-based, such as circulating cell-free DNA (cfDNA) and micro RNAs. CEA has been the only circulating biomarker in clinical use for decades, but its usefulness is limited by suboptimal sensitivity and specificity^[5].

CIRCULATING cfDNA

cfDNA may originate from normal or from tumor

cells and it can be detected in healthy subjects, with increased levels noted in benign conditions such as inflammatory processes and infections^[6]. Necrotic and apoptotic cells may release DNA fragments passively, depending on the tumor burden, its growth kinetics and the effects of antineoplastic treatment, but it is also believed that cfDNA may be actively shed by tumor cells with the goal to transform cells in distant sites^[7]. Finally, CTCs and micrometastases may also be the source of cfDNA, along with the primary tumor.

Several technical challenges hamper the ability to standardize the identification and measurement of cfDNA in oncology patients. First of all, the low concentration of highly fragmented DNA molecules renders their identification, amplification and quantification rather challenging^[8]. Several DNA extraction methodologies have been developed with no one appearing to clearly improve yields and pre-analytical sample processing and storage could potentially influence results. Moreover, the abundance of available methodologies such as digital polymerase chain reaction (PCR), real-time quantitative PCR (qPCR) and emerging next-generation sequencing technologies result in a lack of comparability between results reported in various translational and clinical studies^[9,10]. Despite these challenges, an exponentially increasing body of literature has clearly demonstrated the promise that the measurement of cfDNA holds in various clinical settings and in multiple different neoplasms and advances in technology aim to tackle both the problems of detecting diluted tumor DNA and determining its origin based on the presence of known aberrations found on the primary tumor.

EARLY DIAGNOSIS OF COLORECTAL CANCER

Screening for CRC has been consistently shown to reduce disease specific mortality^[11]. An abundance of screening modalities is available and recommended by clinical practice guidelines, which implies that no one is clearly superior to the others since no randomized comparisons have been performed^[11,12]. Colonoscopy is regarded as the preferred technique^[13]. However, it is costly and invasive. Thus, enriching the population that undergoes colonoscopy with subjects at the highest risk for the development of CRC with a minimally invasive selection procedure is a matter of active research.

A large number of studies, reviewed elsewhere^[14], have evaluated the efficacy as screening tools of the detection of several molecular events, such as kirsten rat sarcoma viral oncogene homolog (KRAS), adenomatosis polyposis coli, TP53 and mismatch repair genes (*MMR*) mutations, DNA methylation and miRNA signatures in body fluids, mainly stool but also plasma and serum. In summary, the reported sensitivity and specificity rates vary widely, owing to the small quantity of hyperfragmented DNA, often of low quality, retrieved from body fluids (especially stool) and the different

techniques and biomarkers tested. Regarding plasma cfDNA, preliminary results are promising. In a high risk population with positive fecal occult blood test that subsequently underwent colonoscopy, Perrone *et al.*^[15] demonstrated that the quantification of cfDNA by qPCR was predictive for CRC but not premalignant lesions (area under curve, 0.709; 95%CI: 0.508-0.909). This important finding clearly illustrates that cfDNA can have a complementary role to traditional screening modalities for CRC. In the same study, the detection rate of KRAS mutations in the plasma by mutant-enriched PCR was low at 3%, compared to tissue-based analysis (45%). However, KRAS mutations can also be detected in patients with inflammatory bowel disease, complicating the interpretation of its significance^[16]. Significant barriers to the implementation of cfDNA-based strategies include the relatively low sensitivity especially for premalignant lesions and the probability of over-diagnosis.

MONITORING MINIMAL RESIDUAL DISEASE

Following curative surgery for localized CRC, approximately 50% of stage III patients according to the American Joint Committee on Cancer (node-positive disease) and 20% of stage II patients (T3N0 and T4N0) are expected to experience disease relapse without adjuvant chemotherapy, possibly due to the presence of occult micrometastases. Therefore, identifying these high risk patients could optimize adjuvant treatment strategies. The benefit derived from chemotherapy is well established for stage III patients, with the results of large, well-conducted randomized trials demonstrating a benefit for overall survival (OS) for patients treated with the combination of a fluoropyrimidine (5-fluorouracil or capecitabine) and oxaliplatin (FOLFOX and XELOX, respectively)^[17,18]. The management of stage II patients is much more controversial, as clinical trials and meta-analyses indicate that the absolute risk reduction is marginal and sometimes non-significant^[19-21]. Clinical practice guidelines recommend the use of clinic-pathological risk factors for the selection of eligible patients for adjuvant chemotherapy, such as T4 stage, perforation, obstruction, number of lymph nodes resected, presence of lymphovascular or perineural invasion, poor grade, preoperative CEA levels and positive or indeterminate margins^[22-24]. Additionally, molecular markers such as the presence of microsatellite instability^[25] and gene signatures^[26] have also been shown to have prognostic and/or predictive value. These data clearly underscore that robust decision making tools are needed for the selection of patients that will enjoy improved outcomes from additional chemotherapy, thus sparing from its toxic effects those not likely to benefit. Consequently, cfDNA has been evaluated in this setting. Reinert *et al.*^[27] recently showed that using droplet digital PCR-based (ddPCR)

personalized assays, the quantification of plasma cfDNA had almost 100% sensitivity and specificity for the prediction of relapse after surgery, with a mean lead time of 10 mo. Furthermore, the value of cfDNA measurement specifically in stage II CRC patients is being prospectively evaluated, with preliminary results showing that 7.7% of patients who had detectable cfDNA after curative surgery had higher relapse rates, 5/6 patients with detectable and 5/72 of those with undetectable cfDNA^[28]. The selection of the proper mutation markers to be monitored in cfDNA is as yet unresolved. Sato *et al.*^[29] monitored preoperative and postoperative cfDNA levels based on a panel of 50 genes, using ddPCR; only markers with an allele frequency above 0.1% in plasma DNA correlated with the clinical course. Interestingly, cfDNA has also been shown to be useful in the early detection of relapse after metastasectomy of liver metastases, significantly outperforming both CEA and imaging in one study^[30].

PREDICTION OF RESPONSE TO TREATMENT

The demonstration of the efficacy of monoclonal antibodies (moAbs) targeting the epidermal growth factor receptor (EGFR), such as cetuximab and panitumumab, in specific molecular subtypes of mCRC, marked a significant breakthrough towards the delivery of precision medicine. V-Ki-ras2 KRAS has a critical role in EGFR signaling transduction. Activating KRAS mutations at exon 2 are detected in approximately 40%-45% of patients with CRC and have been shown to confer resistance to treatment with cetuximab^[31,32] and panitumumab^[33]. Moreover, less common KRAS mutations at exons 3 and 4 and NRAS mutations at exons 2, 3, 4 identified at a further 17% of patients have been shown to also correlate with resistance to anti-EGFR moAbs^[34]. On the other hand, mutations at the B-Raf proto-oncogene, serine/threonine kinase (BRAF), which is further downstream at the mitogen-activated protein kinase (MAPK) pathway have been shown to have significant prognostic, but not predictive value as a marker of poor response to anti-EGFR antibodies when combined with chemotherapy, although evidence for the latter is not compelling due to the small number of patients and low statistical power of these analyses^[35,36]. To further complicate matters regarding the role of BRAF mutations, several lines of evidence suggest that their presence is a marker of resistance to monotherapy with anti-EGFR moAbs^[37-39].

Availability of adequate tissue is rarely a problem in CRC patients. Additionally, there is a high rate of concordance between the primary tumor and metastases regarding the mutational status of mCRC but, importantly, approximately 10% of patients with KRAS wild type (WT) primary tumors have KRAS mutated metastases and *vice versa*^[40]. Qualitative analysis of cfDNA for the presence of activating mutations is an

emerging method which has been consistently shown to closely correlate with the primary tumor status. For example, Morgan *et al*^[41] utilized a commercial PCR kit and demonstrated that the detection of KRAS mutations in cfDNA is highly concordant with their presence at the primary tumor and they could also be detected in the plasma of a patient with KRAS WT primary. Accordingly, Thierry *et al*^[42] showed in a prospectively evaluated patient cohort that qPCR-based detection of KRAS and BRAF mutations in mCRC patients is exquisitely sensitive and specific when using the primary tumor status as a reference standard, results that have been confirmed by others^[30]. Taken together, these data clearly imply that the detection of predictive molecular events in mCRC patients is an excellent surrogate for their presence at the primary tumor and could be used in cases where archival tumor is not available or when the acquisition of fresh tissue is not feasible.

MONITORING RESPONSE TO THERAPY

The quantitative measurement of a known aberration in the plasma may be used for disease monitoring of mCRC patients while under treatment and data show that cfDNA levels robustly correlate with tumor burden and response to treatment, raising the possibility of its use at the near future as an adjunct to anatomical imaging. Several investigators have established the correlation between tumor burden, cfDNA and levels of specific mutations such as KRAS in circulation^[42-44]. Moreover, high levels of cfDNA were shown to be predictive for diminished disease control rates when mCRC patients were treated with irinotecan and cetuximab in a study by Spindler *et al*^[43]. In a prospective trial of mCRC patients undergoing first-line chemotherapy, a 10-fold or higher reduction in circulating tumor DNA (ctDNA) before cycle 2 correlated well with CT imaging at 8-10 wk while lesser degrees of reduction were associated with a trend for improved progression free survival (PFS)^[45]. Although the quantification of cfDNA levels and their temporal changes seem appealing for clinical use, two issues need to be considered: First of all, benign conditions such as infections may also lead to raised cfDNA levels. Also, the genomic evolution of the tumor could potentially lead to alterations in cfDNA levels regardless of tumor burden. Until more specific and sensitive methods that measure ctDNA are available, it is prudent that cfDNA levels are used in combination with imaging scans for response assessment.

DETECTION OF RESISTANCE

Despite appropriate patient selection according to molecular testing, approximately 10% of treatment naïve patients experience disease progression as best response when receiving first line treatment with a chemotherapy doublet and an anti-EGFR antibody^[46];

importantly, virtually all patients will eventually develop disease progression while receiving such treatment. The resistance mechanisms of mCRC to anti-EGFR moAbs can be broadly categorized in three categories and it should be mentioned that there is significant overlapping between primary and secondary resistance, with a few notable exceptions: (1) events that disrupt the binding of cetuximab or panitumumab at the EGFR. These events may be point mutations, with the best characterized being the S492R mutation which interferes with the binding of cetuximab but not panitumumab^[47], a decrease of EGFR copy number or differential expression of the EGFR ligands epiregulin and amphiregulin^[48]; (2) activation of downstream kinases, effectively bypassing the inhibition of EGFR. The best described events include the KRAS, NRAS and BRAF mutations whose role at the primary resistance was previously described. Although less common, KRAS gene amplification has also been implicated in the development of resistance to cetuximab and panitumumab^[49]; and (3) activation of parallel, bypassing pathways. The most commonly described mechanisms are phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha exon 20 mutations in approximately 10%-15% of patients and phosphatase and tensin homolog (PTEN) mutations in 18%, both leading to activation of the PI3K/AKT/mTOR pathway^[39,50]. However, since these mutations frequently coexist with other resistance mechanisms such as KRAS mutations, it is difficult to establish a potentially direct causative role. Other genomic events that have been implicated in the development of secondary resistance are HER2 amplification^[51], IGF-1R activation^[52] and MET amplification^[53].

It is clear that CRC exhibits significant spatial and temporal heterogeneity while under the selective pressure of treatment, which is further complicated by the extensive crosstalk, shared downstream pathways and bypass signaling that is activated during treatment with anti-EGFR moAbs which allows pre-existing resistant clones to emerge. The early recognition of these molecular mechanisms, before disease progression is clinically or radiologically apparent, may offer a better chance at intercepting them, thus improving patient outcomes.

The best studied in cfDNA mechanism of acquired resistance is the emergence of KRAS mutations, which has been shown to occur while under treatment both with cetuximab^[54] and panitumumab^[55]. Notably, in a series of CRC patients receiving treatment it was shown that KRAS mutations developed in 38% of patients previously responding to anti-EGFR moAbs, 96% had newly acquired activation of the MAPK pathway and 70 new somatic mutations were described in total^[56]. Interestingly, in a series of 108 mCRC patients pretreated with a fluoropyrimidine, irinotecan and oxaliplatin, who received irinotecan and cetuximab, Spindler *et al*^[57] demonstrated that the emergence of detectable KRAS mutations in the plasma may precede

radiological progression and that patients with a KRAS mutant primary but no detectable mutations in the plasma could benefit from treatment despite previous exposure to and progression after irinotecan.

Apart from KRAS mutations, other molecular events that emerge under the selective pressure of anti-EGFR treatment and that drive resistance to these agents have been detected in cfDNA, such as EGFR mutations^[58] and MET amplification^[53]. The relative importance of each specific aberration, in light of the frequent co-existence of several mechanisms in a single patient^[59] needs to be elucidated in further studies.

CELL-FREE DNA AS A PROGNOSTIC MARKER

The prediction of survival in mCRC patients relies heavily on clinic-pathologic characteristics, such as hepatic tumor burden, node positive primary, CEA levels, microsatellite stability status, BRAF mutation status, resectability of metastatic disease and tumor grade^[60]. Objective and reproducible biomarkers are needed in order to optimize risk stratification and guide treatment decisions. The prognostic capacity of quantitative and qualitative cfDNA characteristics have been investigated in several studies. cfDNA levels were indeed shown to correlate with survival in mCRC^[56], including at the second line setting where increased cfDNA levels predicted shorter PFS and OS compared to lower levels, with a hazard ratio (HR) of 1.4 (95%CI: 1.1-1.7, $P = 0.03$) for PFS and 1.6 (95%CI: 1.3-2.0, $P < 0.0001$) for OS for each quartile of increase^[61]. Qualitative characteristics, such as methylated cfDNA levels have also been shown to independently predict OS in mCRC^[62]. In the largest published prospective mCRC patient cohort ($n = 97$), El Messaoudi *et al.*^[63] used qPCR and showed that cfDNA levels, higher specific mutation loads and the level of cfDNA fragmentation are strong prognostic factors; cfDNA levels were independent prognostic factors for the entire patient cohort and the level of fragmentation only for the KRAS/BRAF mutated subset. Specifically, a difference in OS of 10 mo was reported between the groups of high vs low cfDNA levels (18.07 mo vs 28.5 mo respectively, $P = 0.0087$)^[63]. Finally, Spindler *et al.*^[64] compared the prognostic value of the detection of KRAS mutations in the plasma compared to the primary tumor with the use of qPCR, with the former being an independent predictor for OS (HR = 2.98, 95%CI: 1.53-5.80, $P = 0.001$) and PFS (HR = 2.84, 95%CI: 1.46-5.53, $P = 0.002$), whereas the latter had no correlation with outcomes, which underscores the value of cfDNA qualitative testing.

DISCUSSION

There is enormous interest for the discovery, development, clinical evaluation and standardization of

circulating biomarkers in oncology, since liquid biopsy offers the possibility of real-time monitoring of the disease trajectory. Due to the absence of large scale comparisons of the relative efficacy of cfDNA and CTCs and the lack of standardization of clinical assays, no clear recommendations can be made on which is the superior biomarker and published literature suggests that they may be complementary^[65,66]. For example, CTCs have been shown to be useful in determining the level of heterogeneity of the disease, with significant differences compared to the primary tumor demonstrated in single cell whole genome analysis. Moreover, the phenotypic and genotypic characterization of CTCs could be a powerful tool aiding treatment planning and determining the likelihood of drug resistance^[67]. However, the large number of CTC capture and enrichment techniques, which are based on antibody selection or on the physical properties of CTCs and the often times low quantity and quality of DNA extracted from these rare cells represent significant drawbacks^[68].

Contrary, cfDNA exhibits several important advantages: Its extraction is less cumbersome compared to CTCs, but the preanalytical process is equally non-standardized. Also, multiple studies across various neoplasms have shown that its qualitative and quantitative measurement is representative of the overall tumor burden and of the mutational load of the disease and its heterogeneity. Therefore, its future potential role as a complement in asymptomatic screening and disease staging, as a tool for disease monitoring even without the use of anatomic imaging, as a prognostic marker and for the long term follow-up of disease free patients with CRC is exciting^[69]. Nevertheless, the aforementioned lack of standardization is a significant obstacle for the commercialization and widespread adoption of cfDNA in oncology. Moreover, cfDNA, in contrast with CTCs, does not provide information regarding RNA and protein profiling of the tumor. However, sensitivity may be improved since cfDNA has been detected both in patients with and without detectable CTCs, but CTCs were not detected in the absence of cfDNA^[56]. These observations clearly hint towards a combinatory approach to circulating biomarkers.

It is conceivable that in the near future serial cfDNA testing will become a component of routine clinical practice regarding the management of CRC. Its already established prognostic power may be integrated in novel staging schemes and its predictive capacity may influence treatment decisions. Importantly, its use may spare patients from unnecessary toxicity caused by ineffective treatments. It is imperative, however, that cost-effectiveness analyses be conducted since these costly techniques require specific equipment and are often labor-intensive.

CONCLUSION

cfDNA has already shown promise in CRC in multiple

clinical settings. Its prospective evaluation in randomized trials is of paramount importance before it can be considered as standard practice and cost-effectiveness analyses are also needed. Until then, translational studies continue to underscore its clinical utility and offer insights on the continuously evolving understanding of the disease complexity.

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Retrospective Study

Signet ring colorectal carcinoma: Do we need to improve the treatment algorithm?

Anup Sunil Tamhankar, Parag Ingle, Reena Engineer, Munita Bal, Vikas Ostwal, Avanish Saklani

Anup Sunil Tamhankar, Parag Ingle, Department of Surgical Oncology, Tata Memorial Centre, Mumbai, Maharashtra 400012, India

Reena Engineer, Department of Radiotherapy, Tata Memorial Centre, Mumbai, Maharashtra 400012, India

Munita Bal, Department of Surgical Pathology, Tata Memorial Centre, Mumbai, Maharashtra 400012, India

Vikas Ostwal, Department of Medical Oncology, Tata Memorial Centre, Mumbai, Maharashtra 400012, India

Avanish Saklani, Department of Gastro-Intestinal Surgery, Tata Memorial Centre, Mumbai, Maharashtra 400012, India

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Correspondence to: Dr. Avanish Saklani, MS, FRCS,

Associate Professor, Department of Gastro-Intestinal Surgery, Tata Memorial Centre, Mumbai, Maharashtra 400012, India. asaklani@hotmail.com
Telephone: +91-996-9506719
Fax: +91-222-4177000

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Abstract

AIM

To elaborate about this peculiar variant from a tertiary cancer center from India.

METHODS

It's a retrospective study (2011-2014) of all patients diagnosed with signet ring colo-rectal cancer (SRCC). Various clinico-pathological variables were studied.

RESULTS

One hundred and seventy consecutive patients with SRCC were diagnosed (11.4% of all colorectal cancers). Median Age of the cohort was 41 years. Most common location was recto-sigmoid area (54.7%). Majority patients presented in stage III and IV (91.2%). Most of the stage IV patients had isolated peritoneal metastases (86.5%). Colonic tumors had higher incidence of peritoneal metastases (91.8% vs 83.3%) as well as isolated peritoneal recurrences (37.5% vs 16.7%) than rectal primaries. Thirty-seven point five percent of patients recurred after curative surgery. Amongst them 63.63% patients had isolated peritoneal recurrences. Circumferential resection margin (CRM) was involved in 17.9% patients. Median relapse free survival (RFS) and overall

survival (OS) of the cohort were 14.9 and 18.13 mo respectively. CRM involvement, colonic primary were associated with poorer RFS and OS.

CONCLUSION

SRCC has predilection for peritoneal dissemination. More aggressive and/or extended chemotherapy schedules as well as prophylactic hyperthermic intra-peritoneal chemotherapy at the time of primary surgery may be attempted in these patients.

Key words: Colorectal cancer; Signet ring cell carcinoma; Peritoneal metastases; Hyperthermic intra-peritoneal chemotherapy

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Core tip: The incidence of Signet Ring Colo-Rectal Cancer appears to be higher in Indian subcontinent than the world literature. It has predilection for peritoneal lining. It affects younger age group. Majority cases present in stage III and IV. Recto-sigmoid region is affected commonly. The most common metastatic site and site of recurrence is peritoneal cavity. Probably it should be treated with a different protocol than the conventional adenocarcinoma with focus on aggressive peritoneal cytoreductions and hyperthermic intra-operative intraperitoneal chemotherapy (HIPEC). Further research is needed to evaluate molecular biology of this variant and utility of prophylactic HIPEC during curative surgery.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers worldwide^[1]. There are three subtypes described in the literature based on the amount and location of mucin in the tumor. These are conventional adenocarcinoma (AC), mucinous carcinoma (MC) and signet ring cell carcinoma (SRCC)^[2,3]. SRCC constitutes 1% of all colorectal carcinomas^[4-9]. It is an aggressive variant which affects younger population and has poorer prognosis^[5]. The literature explaining the biology as well as the optimum treatment algorithm of this particular variant is scarce due to its low incidence. So we look into the incidence, demographics, clinico-radiological presentation and outcome of treatment of this peculiar variant from a tertiary cancer centre from India (Tata Memorial Centre, Mumbai).

MATERIALS AND METHODS

All patients diagnosed with colorectal carcinoma from 1st January 2011 to 31st December 2013, registered under the Department of Gastro-intestinal Oncology services, Tata Memorial Centre, were included. The data was collected retrospectively from Electronic database as well as case files from Department of Surgical Oncology. The histopathology specimens of all these patients were reviewed at Department of Surgical pathology, Tata Memorial Centre. Signet ring cell colorectal cancers were defined as per WHO criteria (AC with more than 50% of signet-ring cells). Patients were staged as per AJCC classification (7th edition). Response to neoadjuvant chemoradiotherapy (NACTRT) was assessed as per RECIST criteria. The decision about the same was taken in the multidisciplinary meeting held for every patient. Pathological complete response was defined as absence of viable tumor cells in the primary, the lymph nodes and peri-rectal soft tissue. Circumferential resection margin (CRM) positivity was defined as presence of viable tumor cells at or within 1 mm of it. Follow up data was obtained from electronic medical records and/or telephonic questionnaire. Recurrences were based on biopsy or strong clinico-radiological evidence. Peritoneal metastases or recurrences constituted peritoneal deposits, malignant ascites, omental deposits and ovarian deposits. Relapse free survival (RFS) was assessed from the date of cancer directed surgery to date of recurrence. Overall survival (OS) was measured from the date of diagnosis of malignancy to date of death. SPSS-21 (IBM corporation) was used for the statistical analysis. Categorical variables were compared with χ^2 test. Survival functions were analyzed with Kaplan Meir curves and compared with log rank test.

RESULTS

From 1st January 2011-31st December 2013, 1487 patients with colorectal cancer got registered under the department of Gastrointestinal Services Tata Memorial Centre. Amongst them, signet ring cell carcinoma was diagnosed in 170 consecutive patients (11.4%). Follow up of 18 of 170 patients (10.58%) was inadequate (< 1 mo) (Table 1). Median Age of the cohort was 41 years. Males were affected nearly twice more than females (M: F = 1.8:1). Most tumors were located in the rectum and sigmoid colon (Rectum: 41.2% and Sigmoid: 13.5%). Majority patients presented in stage III (51.8%) and stage IV (39.4%). Most of the stage IV patients had isolated peritoneal metastases (58/67, 86.5%) (Table 2). Curative surgery was feasible only in 51.76% (88/170) patients. Thirty-seven point five percent (33/88) patients recurred after curative surgery. Twenty-one thirty-thirds (63.63%) patients had isolated peritoneal recurrences (Table 3). Most patients had high nodal burden, pN1 being 23.2% (22/95), pN2 being 57.9% (55/95). Amongst node positive patients, 66.3% (53/77)

Table 1 Demographic parameters

Parameter	Statistics
Total No.	170
Sex ratio	
Male	110
Female	60
Age (median), yr	41
Stage, <i>n</i> (%)	
II	6 (3.5)
III	88 (51.8)
IV	67 (39.4)
Not available	9 (5.3)
Location, <i>n</i> (%)	
Right colon	49 (28.8)
Transverse colon	13 (7.6)
Descending colon	11 (6.5)
Sigmoid colon	23 (13.5)
Rectum	70 (41.2)
Appendix	1 (0.6)
Not available	3 (1.8)

Table 2 Pattern of metastases in stage IV patients

Site of metastases	<i>n</i> (%)
Liver	1 (1.5)
Lung	1 (1.5)
Isolated peritoneal	58 (86.5)
Retropitoneal lymphnodes	2 (3.1)
Others	5 (7.4)

had perinodal extension. The rate of lymph node metastases and lympho-vascular invasion increased progressively with increasing pathological T stage.

Median RFS and OS of the cohort were 14.9 mo and 18.13 mo respectively. OS of peritoneal and non-peritoneal metastases were equivalent (16 mo vs 13 mo, $P = 0.729$) (Table 4).

Forty-eight rectal cancers were operated. Data for patients undergoing NACTRT was available for 37 cases only. Pathological complete response was seen in 21.6% (8/37) patients. CRM was involved in 17.9% (7/39) patients (data on CRM was not available for 9 cases). CRM involvement was associated with poorer RFS (15 mo vs 37.2 mo, $P = 0.060$) and OS (19.9 mo vs 41.5 mo, $P = 0.018$) as compared to patients with uninvolved CRM (Tables 4 and 5).

The location of primary had a significant impact on the clinico-pathological outcome of the patient. As compared to rectal primaries, colonic tumors had higher incidence of peritoneal metastases (83.3% vs 91.8%, $P = 0.074$) as well as isolated peritoneal recurrences (16.7% vs 37.5%, $P = 0.062$). Colonic primaries were associated with poorer OS than rectal tumors after curative resection (32.298 mo vs 40.089 mo, $P = 0.058$) and RFS (24.74 mo vs 34.02 mo, $P = 0.048$) (Table 6).

DISCUSSION

CRC is one of the most common cancers worldwide. Worldwide, it leads to 10% and 9.2% of cancers in

Table 3 Pattern of recurrence after curative surgery

Pattern of recurrence	<i>n</i> (%)
Locoregional	4 (12.12)
Distant	4 (12.12)
Isolated peritoneal	21 (63.63)
Peritoneal + second primary	2 (6.06)
Local + peritoneal	2 (3.4)

Regional recurrences: Regional lymph node recurrences; Distant recurrences: Non-regional lymph nodal and visceral recurrences.

Table 4 Factors affecting overall survival

Parameter	OS (mo)	Significance
Location (After curative surgery)		
Colon	32.3	0.058
Rectum	40.1	
CRM		
Positive	19.9	0.018
Negative	41.5	
Metastases		
Peritoneal	14.85	0.729
Non-peritoneal	11.14	

CRM: Circumferential resection margin; OS: Overall survival.

males and females respectively. It is a cause of 8% and 9% of cancer related deaths in males and females respectively^[1]. Several histological subtypes have been reported^[2,3]. It has two different subgroups apart from classical AC. They are classified based on varying amounts of signet-ring cell and/or mucinous component. Signet-ring cell carcinoma (SRCC) is characterized by intra-cytoplasmic mucin which displaces the nucleus aside. MC is characterized by extracellular mucin pools. SRCC or MC (defined as carcinoma with more than 50% of signet-ring cells or mucinous component, respectively as per WHO classification) constitutes approximately 1% or 5%-15% of CRC cases, respectively in the world literature^[4-9]. As compared to the world literature, the incidence of SRCC is much higher in our study (11.4% vs 1%). The median age of the cohort in our study was also lower than world literature (41 years vs 50-55 years)^[5,6,10,11]. This could represent either a referral bias being a tertiary cancer centre in India or definite distinct disease biology in the Indian population. Further studies regarding the demographic profile of this particular variant in Indian population are under consideration currently.

The literature is divided about the most common site of colorectal cancer in young population with some indicating proximal colon^[12] and others suggesting it to be recto-sigmoid region^[13,14]. In our study, rectum and sigmoid colon region was most commonly affected. This may be related to preferential referral of locally advanced rectal cases to our institute. One of the studies has shown that colorectal cancers affecting younger age group (< 40 years) have significantly higher incidence of signet ring cell cancer. Such tumors also affect rectosigmoid area more commonly than rest of the colon in

Table 5 Factors affecting relapse free survival in operated patients

Parameter	RFS (mo)	Significance
CRM		
Positive	15.003	0.060
Negative	37.202	
Location		
Colon	24.74	0.048
Rectum	34.02	

RFS: Relapse free survival; CRM: Circumferential resection margin.

young patients^[15].

SRCC has been associated with peculiar genomic changes such as high-degree microsatellite instability (MSI-high) (up to 40%), high-frequency of CpG island methylator phenotype, higher methylation level of long interspersed nucleotide element-1 and frequent BRAF mutation and low COX-2 expression^[8,16-20]. Due to high frequency of MSI-H mutations^[21] and associated poor prognosis, tumors with signet ring histo-morphology are recommended to be screened for MSI-H mutations as per revised Bethesda guidelines^[22]. The serrated adenoma-carcinoma pathway has been proposed for development of these tumors. Terada *et al*^[23] found that epithelial membrane antigen was downregulated in colorectal SRCC. Kim *et al*^[24] showed that focal loss of epithelial cell adhesion molecule was associated with development of SRCC in colonocytes. These molecular changes may be related to preferential peritoneal spread of this subtype. Currently the studies are under consideration at our institute to assess genomic changes related to this specific phenotype which may be the cause of higher incidence of signet ring colorectal cancer in Indian population than the world literature.

Our study revealed that, though SRCC has an aggressive biology in general, it seems to respond well to NACTRT with pathological complete response rate of 21%. Literature assessing response of SRCC to NACTRT is scarce due to low incidence worldwide. Jayanand *et al*^[25] showed that these tumors respond well to RT with high pathological complete response rates. It may be related to their aggressive nature and higher mitotic index. So potentially NACTRT should be included in the treatment protocol of rectal SRCC for improved outcomes.

Patients with SRCC are more likely to present in advanced stages (Stage III/IV) than AC. SRCC patients more often present with metastatic disease and are more likely to develop peritoneal metastases. This may be related to their peculiar molecular origin which is yet to be proven. It is also shown that SRCC metastasizes to the lymph nodes, whereas AC metastasizes primarily to the liver^[6,9,11]. Our study also showed similar findings.

SRCC has been associated with a poor prognosis compared with AC^[5,6,10,11]. Studies have shown that peritoneal metastases of SRCC are associated with a poorer prognosis, and survival is even worse if other

Table 6 Impact of location on outcome

Parameter	Colon	Rectum	Significance
Recurrence after curative resection			
Peritoneal, <i>n</i> (%)	15/40 (37.5)	8/48 (16.7)	0.062
Non-peritoneal, <i>n</i> (%)	3/40 (7.5)	7/48 (14.6)	
Pattern of Metastases at presentation			
Peritoneal, <i>n</i> (%)	45/49 (91.8)	15/18 (83.3)	0.074
Non-peritoneal, <i>n</i> (%)	4/49 (9.2)	3/18 (16.7)	
RFS (mo)	24.74	34.02	0.048
Overall Survival (mo)	26.011	30.32	0.062
OS after curative surgery (mo)	32.298	40.089	0.058

RFS: Relapse free survival; OS: Overall survival.

organs are also affected^[26]. But in our study, patients with peritoneal metastases had similar OS as compared to those with non-peritoneal metastases. This may be due to small sample size of the study. Often, these metastases cannot be treated with curative intent. As of now, curative surgery is an option mainly limited to liver and lung metastases, which are the most common metastatic sites in AC patients. Systemic chemotherapy for peritoneal metastases may not yield the same results compared with hematogenous metastases due to blood-peritoneal barrier. As a result, outcome is poor in advanced SRCC cases^[27].

The incidence of synchronous and metachronous peritoneal metastases in colorectal carcinoma (AC) seems to be in the range of 4%-5% (much lower than with SRCC)^[26,28]. Studies have revealed that peritoneal carcinomatosis among patients with metastatic colorectal cancer is associated with a 30% reduction in overall survival (10.7 mo vs 17.6 mo)^[29]. The overall survival of these patients is found to be less than 6 mo despite the use of 5FU and leucovorin based chemotherapy^[30,31]. But palliative surgery and systemic chemotherapy, together have been shown to improve survival upto 12 mo in patients with isolated peritoneal metastases^[29,32].

Hyperthermic intra-operative intra-peritoneal chemotherapy (HIPEC) has shown promising results for peritoneal metastases of colorectal origin^[29]. Verwaal *et al*^[29] reported outcome of 1427 patients with peritoneal metastases of colorectal origin treated with cytoreductive surgery (CRS) and HIPEC. Peri-operative morbidity and mortality were 34% and 3% respectively. Median hospital stay was 16 d. Median PFS was 15 mo and OS was 33 mo. Three- and five-year survival rates were 46% and 31% respectively. So authors concluded that CRS and HIPEC seems to be safe and beneficial in peritoneal metastases of colorectal origin^[33]. But literature assessing benefit of HIPEC for SRCC is scarce and controversial with studies denying^[34,35] and implying^[36] benefit of HIPEC in this subgroup. But these reports are retrospective and are fraught with small sample sizes.

Recently, Hao *et al*^[37] have proposed a study assessing the benefit of monoclonal antibody blocking EpCAM in CRC. This may be relevant in the further management

of SRCC as EpCAM also has altered expression in this subtype.

Klaver *et al.*^[38] have proposed a randomized controlled trial (COLOPEC) for assessing benefit of prophylactic HIPEC in patients at high risk of peritoneal carcinomatosis. They have included patients (non-metastatic) with T4 disease or on table tumor site perforation for prophylactic HIPEC followed by routine adjuvant chemotherapy. It has been postulated in assumption that very few patients with peritoneal carcinomatosis become eligible for CRS and HIPEC; as a result they have poor prognosis. So if a prophylactic HIPEC reduces the occurrence of peritoneal metastases in future, it may result in benefit in OS. The investigators have not considered signet ring cell pathology as inclusion criteria for the study; probably because of low incidence (1%-2%) of it in the western literature. A similar study may be considered in Indian patients with signet ring cell carcinoma to assess benefit of prophylactic HIPEC at the time of primary surgery as it has a peculiar tendency for isolated peritoneal recurrences and the incidence of this particular histopathological subtype seems to be higher in them (11.4%) as suggested by present study.

It is unclear whether different histological subtypes should influence treatment decisions, since it is often not addressed in clinical trials. In the literature, studies concerning outcome after adjuvant or palliative chemotherapy for SRCC are rare. However, due to the aggressive behavior and high incidence of SRCC in young patients, it is imperative to develop understanding of potential adjuvant treatment options as it is likely to alter quality of life and have significant socio-economic impact. Colonic SRCC are more likely to have peritoneal dissemination and poorer survival than rectal SRCC. So more aggressive treatment options, like HIPEC may be useful in these patients at the time of primary surgery or after peritoneal limited recurrence in order to improve survival and quality of life. This can only be addressed in a randomized control trial setting. Due to high nodal disease burden and high incidence of failure after curative surgery (up to 40%), more extended and/or aggressive adjuvant chemotherapy options should also be explored in this subset of population which is younger and is likely to tolerate the aggressive treatment better.

Signet ring colorectal cancer has poor prognosis. It has a higher incidence in Indian subcontinent. It affects young patients and has predilection for peritoneal dissemination.

Isolated peritoneal metastases as well as isolated peritoneal recurrences are very frequent in these patients. SRCC responds well to radiation. So whenever indicated, neoadjuvant radiation should be included in the treatment protocol for rectal SRCC.

More aggressive and/or extended chemotherapy schedules as well as prophylactic HIPEC at the time of primary surgery, especially for colonic tumors, should be explored in a trial setting in order to improve dismal

survival in these patients.

COMMENTS

Background

Signet ring colorectal cancer (SRCC) is a subtype of colorectal adenocarcinoma. It tends to affect younger age group. Most of the patients present in stage III or IV. The most common site affected is rectosigmoid region. It has a peculiar affection for peritoneal lining. Most of the metastases and recurrences happen exclusively in the peritoneal cavity. Visceral metastases are rare. Average prognosis of these patients is poor. There is no effective adjuvant or palliative treatment for this entity. Early studies in the field of cytoreduction and hyperthermic intra-operative intraperitoneal chemotherapy (HIPEC) have shown promising results and prolongation of survival in peritoneal carcinomatosis of colorectal cancer. The trials are underway to test the impact of prophylactic HIPEC during primary surgery for cT4N1/2 diseases. Since SRCC has a different natural course than the conventional adenocarcinoma of colon, it may be worthwhile to evaluate the possible role of extended chemotherapy or prophylactic HIPEC at the time of curative surgery for SRCC.

Research frontiers

Currently trials are underway (COLOPEC and Prophylchip) to assess efficacy of prophylactic HIPEC in high risk colorectal cancers to prevent occurrence of peritoneal metastases and prolongation of survival. Though aggressive, SRCC has shown its peculiar nature to remain confined to peritoneal cavity in majority patients. This makes it a potential target for peritoneum directed therapies (Cytoreduction and HIPEC). Also monoclonal antibodies blocking EpCAM are being evaluated in CRC. This may be relevant in the further management of SRCC as EpCAM also has altered expression in this subtype.

Innovations and breakthroughs

Cytoreduction and HIPEC has shown survival benefit in peritoneal carcinomatosis of colorectal origin in a large randomized trial by Verwaal *et al* SRCC has not been evaluated widely in the western literature, probably due to lower incidence. But in Indian subcontinent, the incidence of this disease entity appears to be higher than rest of the world. It also affects younger population; as a result has significant bearing on the socioeconomic outcome of entire family. There is a strong need to develop a modified treatment protocol for this disease than conventional adenocarcinoma as the disease biology appears to be different and standard chemotherapy doesn't act well on the peritoneal disease. Certain molecular abnormalities are also noted in SRCC such as high microsatellite instability, EpCAM mutations, high-frequency of CpG island methylator phenotype, higher methylation level of long interspersed nucleotide element-1 and frequent *BRAF* mutation and low COX-2 expression. Further research needs to be carried out to understand the biology of this disease entity well which might give us an insight into potential treatment options for the same.

Applications

To summarize, SRCC seems to be a suitable target for peritoneum directed therapies which include aggressive cytoreduction and HIPEC. Extended/modified chemotherapy protocols may improve survival. Further understanding of molecular biology of this disease may open new methods for its treatment.

Peer-review

It is a retrospective study of an uncommon subtype of colorectal carcinoma. The author statized some information of this cancer including the age, location, stages, metastasis, recurrence and survival.

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Colon adenoma features and their impact on risk of future advanced adenomas and colorectal cancer

Audrey H Calderwood, Karen E Lasser, Hemant K Roy

Audrey H Calderwood, Section of Gastroenterology, Department of Medicine, Dartmouth-Hitchcock Medical Center, Lebanon, NH 03756, United States

Karen E Lasser, Section of General Internal Medicine, Department of Medicine, Boston University School of Medicine, Boston, MA 02118, United States

Hemant K Roy, Section of Gastroenterology, Department of Medicine, Boston University School of Medicine, Boston, MA 02118, United States

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Correspondence to: Audrey H Calderwood, MD, MS, Section of Gastroenterology, Department of Medicine, Dartmouth-Hitchcock Medical Center, One Medical Center Drive, Lebanon, NH 03756, United States. audrey.h.calderwood@hitchcock.org
Telephone: +1-603-6505261
Fax: +1-603-6505225

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Abstract

AIM

To review the evidence on the association between specific colon adenoma features and the risk of future colonic neoplasia [adenomas and colorectal cancer (CRC)].

METHODS

We performed a literature search using the National Library of Medicine through PubMed from 1/1/2003 to 5/30/2015. Specific Medical Subject Headings terms (colon, colon polyps, adenomatous polyps, epidemiology, natural history, growth, cancer screening, colonoscopy, CRC) were used in conjunction with subject headings/key words (surveillance, adenoma surveillance, polypectomy surveillance, and serrated adenoma). We defined non-advanced adenomas as 1-2 adenomas each < 10 mm in size and advanced adenomas as any adenoma ≥ 10 mm size or with > 25% villous histology or high-grade dysplasia. A combined endpoint of advanced neoplasia included advanced adenomas and invasive CRC.

RESULTS

Our search strategy identified 592 candidate articles

of which 8 met inclusion criteria and were relevant for assessment of histology (low grade *vs* high grade dysplasia, villous features) and adenoma size. Six of these studies met the accepted quality indicator threshold for overall adenoma detection rate > 25% among study patients. We found 254 articles of which 7 met inclusion criteria for the evaluation of multiple adenomas. Lastly, our search revealed 222 candidate articles of which 6 met inclusion criteria for evaluation of serrated polyps. Our review found that villous features, high grade dysplasia, larger adenoma size, and having ≥ 3 adenomas at baseline are associated with an increased risk of future colonic neoplasia in some but not all studies. Serrated polyps in the proximal colon are associated with an increased risk of future colonic neoplasia, comparable to having a baseline advanced adenoma.

CONCLUSION

Data on adenoma features and risk of future adenomas and CRC are compelling yet modest in absolute effect size. Future research should refine this risk stratification.

Key words: Colon adenoma; Colorectal cancer screening; Surveillance; Colonoscopy

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Core tip: The data on adenoma size, adenoma multiplicity and serrated polyps in terms of risk for future adenomas and colorectal cancer are compelling, however, the absolute effect size is relatively modest. Current guideline recommendations to perform colonoscopy surveillance at 3-5 years after baseline adenomas and serrated polyps appear appropriately tailored to the risk of future neoplasia.

Calderwood AH, Lasser KE, Roy HK. Colon adenoma features and their impact on risk of future advanced adenomas and colorectal cancer. *World J Gastrointest Oncol* 2016; 8(12): 826-834 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v8/i12/826.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v8.i12.826>

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer death among men and women in the United States^[1]. The lifetime probability of developing CRC is approximately 5%, with 90 percent of cases occurring after age 50. In 2016, an estimated 134500 people will be diagnosed with CRC and 49200 will die of the disease^[1].

The vast majority of CRCs arise from a histologically-specific type of colon polyp, the adenoma, which forms as a result of sporadic mutation in the adenomatous polyposis coli pathway or DNA mismatch repair and by definition contains low-grade dysplasia. Over many

years, a minority of adenomas may grow in size and progress from low-grade dysplasia to high-grade dysplasia, to carcinoma-*in-situ* to invasive carcinoma. More recently, serrated adenomas (named for the "sawtooth" pattern in the crypts) have been identified as accounting for approximately 20%-30% of CRCs. In this review, we will use the term "adenoma" to describe adenomatous and serrated colon polyps and "serrated polyps" to specify polyps with serrated histology.

Colonoscopy is the most widely used modality for CRC screening^[2,3]. Advantages of colonoscopy include the ability of endoscopists both to identify and remove adenomas, which decreases the risk of subsequent CRC^[4]. By definition, "screening colonoscopy" occurs in patients without a history of adenomas and "surveillance colonoscopy" occurs at set intervals (usually 3-5 years) in patients with a history of adenomas to survey for new adenomas^[5]. It is important to understand the existing evidence upon which surveillance colonoscopy recommendations are made to help inform shared decision making with patients who have co-morbid conditions or limited life expectancy^[6]. This review will focus on the association between specific adenoma features and future colonic neoplasia.

MATERIALS AND METHODS

We searched the National Library of Medicine through PubMed for articles from 1/1/2003 to 5/30/2015. We did not search prior to 2003 because of technological advances in colonoscopy optics in 2002, which dramatically improved the diagnostic accuracy of colonoscopy; data prior to 2003 were not considered relevant to the current risk estimates of CRC after colonoscopy. We used the following filters: English language, human, age > 18, clinical trial, multicenter, prospective observational, meta-analysis. Specific MESH terms were used: Colon, colon polyps, adenomatous polyps, epidemiology, natural history, growth, cancer screening, colonoscopy, colorectal cancer. The MESH terms were used in conjunction with subject headings/key words: Surveillance, adenoma surveillance, polypectomy surveillance, and serrated adenoma. We excluded reviews, guidelines, editorials, case-control, cross-sectional and case series or reports.

We excluded studies of patients with inflammatory bowel disease, personal history of CRC, or family history of genetic CRC syndromes. We reviewed all abstracts for relevance. Full articles of the relevant abstracts were then reviewed with the quality of evidence graded by all three authors using the American Heart Association Evidence-Based Scoring System for Level of Evidence as follows: A: Data derived from multiple randomized clinical trials (RCTs); B: Data derived from a single randomized trial or nonrandomized studies; C: Consensus opinion of experts. The bibliographies of all included articles were also evaluated for additional articles by a single author [histology and size (AHC), multiple adenomas and serrated polyps (HKR)] then

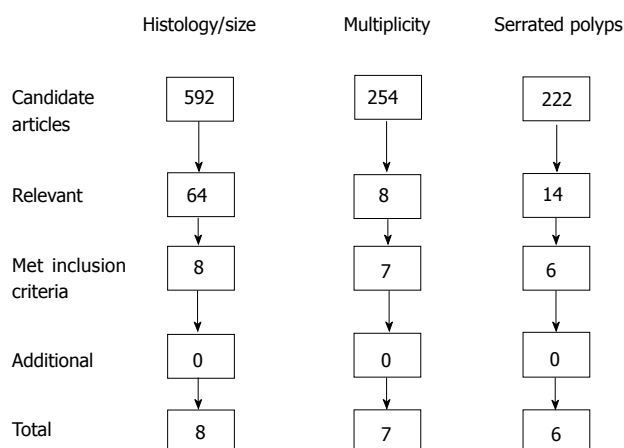


Figure 1 Results of the literature search. Literature search results evaluating the impact of histology and size of adenomas, number of adenomas, and serrated polyps on the risk of future advanced adenomas and colorectal cancer.

reviewed by all three authors for consensus.

We defined non-advanced adenomas as 1-2 adenomas each less than 10 mm in size^[5]. We defined advanced adenomas as any adenoma ≥ 10 mm size or with $> 25\%$ villous histology or high-grade dysplasia^[5]. A combined endpoint of advanced neoplasia included advanced adenomas and invasive CRC.

RESULTS

Histology (low grade vs high grade dysplasia, villous features)

Our search strategy identified 592 candidate articles (Figure 1), of which 64 were relevant. We excluded 56 based on study design or absence of relevant primary outcome or predictors, leaving 8 studies (Table 1). Six of these studies met the accepted quality indicator threshold for overall adenoma detection rate (ADR) $> 25\%$ among study patients^[7], including one study that explicitly described that ADR was $> 25\%$ for each individual endoscopist in study^[8]. ADR was only 22% in the study by Bonithon-Kopp *et al.*^[9] and the meta-analysis by Saini *et al.*^[10] did not present information on ADR.

A small to moderate association between adenoma histology and risk of future advanced adenomas and CRC with variable significance was found among the 8 studies. Four studies^[11-14], including a pooling project of 8 prospective RCTs (evidence level A), found that villous histology was a significant risk factor for future advanced neoplasia (adjusted OR = 1.3; 95%CI: 1.1-1.5)^[14]. Of note, the relative risk (RR) in Lieberman's study (6.1; 95%CI: 2.5-14.7) is higher compared to the other studies because the comparator was subjects without any neoplasia, in contrast to subjects with adenomas without villous histology used in the other studies. In addition, Lieberman studied a Veteran's Affairs (VA) population who are known to have higher rate of baseline adenomas compared to non-VA patients^[12]. A

prospective cohort study of 1086 patients with a median of 10.5 years of follow-up found that villous histology within an adenoma increased the relative risk of any future adenoma (1.8; 95%CI: 1.2-2.6) (evidence level B)^[15]. A primary RCT^[9], a meta-analysis of 5 studies^[10], and a prospective cohort study^[8] found no association of villous histology with future neoplasia (evidence level B).

Similarly, histological findings of high-grade dysplasia had a small and variable association with risk of advanced neoplasia. The meta-analysis by Saini *et al.*^[10] found an increased RR of 1.8 (95%CI: 1.1-3.2)^[10], whereas the primary RCT^[9], pooling project^[14], and prospective registry study^[13] found no association (evidence level A). A prospective cohort study found that compared to an external control population, patients with high-grade dysplasia at baseline had an elevated SIR for CRC of 2.8 (95%CI: 0.3-10.2) compared to the reference group without high grade dysplasia (SIR 0.52; 95%CI: 0.3-0.95)^[15]. In Lieberman's prospective study of 1193 VA patients, he found a RR of advanced neoplasia of 6.8 (95%CI: 2.6-18.1) compared to those with no neoplasia at baseline^[12].

In summary, villous histology within an adenoma may have a small association with future advanced neoplasia, however this was not seen uniformly across all studies. Compared to having no adenomas at baseline, adenomas with high-grade dysplasia are associated with an increased risk of future advanced neoplasia; however, compared to having adenomas that do not contain high-grade dysplasia, the association with future advanced neoplasia is small and variable depending on the study.

Size

We used the same search strategy for histology to evaluate the impact of adenoma size on risk of future colonic neoplasia, finding the same 8 studies (Table 2)^[8-15]. Larger adenoma size at baseline increased the risk of future advanced neoplasia. In Martinez's pooling project of 8 prospective RCTs, the risk of advanced neoplasia increased for each increase in size category (evidence level A). When adenomas < 5 mm were considered the reference group, those with adenomas 10-19 mm and adenomas ≥ 20 mm had a RR of 2.3 (95%CI: 1.8-2.8) and 3.0 (95%CI: 2.2-4.0), respectively^[14]. Similarly, four other prospective studies found that adenomas ≥ 10 mm imparted an increased RR of future advanced neoplasia ranging from 1.7 (95%CI: 1.2-2.3) to 3.0 (95%CI: 1.8-5.1) and 6.4 (95%CI: 2.7-14.9) (level of evidence B)^[8,12,13]. On the other hand, Saini's meta-analysis of 5 studies and a primary RCT, the European Fiber-Calcium Intervention trial (in which 552 patients with resected adenomas randomized to calcium and soluble fiber underwent surveillance colonoscopy at 3 years) failed to show any association between adenoma size and future advanced neoplasia (evidence level B)^[9,10]. A prospective study by Bertario of 1086 patients did not show an association between polyp size ≥ 10 mm and SIR of advanced

Table 1 Articles summarizing the risk of neoplasia based on the histology of polyps seen at baseline colonoscopy

Ref.	Sample size	Median follow-up, yr	Predictor	Primary outcome	Absolute risk of outcome (%)	RR ¹ [95%CI]
RCT						
Laiyemo <i>et al</i> ^[11]	1905	4	Villous	ACN	9 (7-11) vs 5 (4-6)	2.3 [1.5-3.4]
Bonithon-Kopp <i>et al</i> ^[9]	552	3	HGD	ACN	9.8 vs 5.5	1.9 [1.0-3.6]
			Villous		10.3 vs. 6.8	1.7 [0.8-3.7]
Pooled analysis						
Martínez <i>et al</i> ^[14]	8 studies 9167	3.9	HGD	ACN	16.0 (13.2-18.7) vs 10.6 (9.8-11.3)	1.1 [0.8-1.4]
			Villous		16.8 (15.1-18.5) vs 9.7 (9.0-10.4)	1.3 [1.1-1.5]
Meta-analysis						
Saini <i>et al</i> ^[10]	5 studies	3	HGD	ACN	4% risk difference (0-8)	1.8 [1.1-3.2]
			Villous		2% risk difference (-1 to 4)	1.3 [1.0-1.7]
Prospective						
Bertario <i>et al</i> ^[15]	1086	10.5	HGD	CRC	2.8 SIR (0.3-10.2) vs 0.52	Not available
			Tubulovillous	Any adenoma	Not available	1.3 [1.0-1.6]
			Villous			1.8 [1.2-2.6]
² Lieberman <i>et al</i> ^[12]	1193	5.5	No adenomas	ACN	2.4	Ref
			HGD		17	6.8 [2.6-18.1]
			Villous		16	6.1 [2.5-14.7]
Chung <i>et al</i> ^[8]	3808	4.5	Villous	ACN	Not available	1.5 [0.7- 3.0]
Registry						
Van Heijningen <i>et al</i> ^[13]	2990	2	HGD	AA	13	1.2 [0.8-1.8]
			Villous		8	2.0 [1.2-3.2]
			HGD	ACN	11	Not available
			Villous		17	

¹Relative risk compared to patients adenomas without the predictor characteristics. Adenomas with villous compared to those without adenomas with villous features (as opposed to those without any adenomas). ²Relative risk compared to those with no neoplasia. ACN: Advanced colonic neoplasia (includes advanced adenomas and colorectal cancer); AA: Advanced adenomas; CRC: Colorectal cancer; HGD: High grade dysplasia; RCT: Randomized control trial.

Table 2 Articles summarizing the risk of future colonic neoplasia based on the size of polyps seen at baseline colonoscopy

Ref.	Sample size	Median follow-up, yr	Predictor	Primary outcome	Absolute risk of outcome (%)	RR [95%CI]
RCT						
Laiyemo <i>et al</i> ^[11]	1905	4	≥ 10 mm	ACN	9 (7-11) vs 5 (4-6)	0.9 [0.6-1.4]
Bonithon-Kopp <i>et al</i> ^[9]	552	3	≥ 10 mm	ACN	7.1 vs 7.8	1.1 [0.5-2.1]
Pooled analysis						
Martínez <i>et al</i> ^[14]	8 studies 9167	3.9	< 5 mm	ACN	8.7 (7.7-9.7)	Ref
			10-19 mm		15.9 (14.5-17.4)	2.3 [1.8-2.8]
			≥ 20 mm		19.3 (16.4-22.3)	3.0 [2.2-4.0]
Meta-analysis						
Saini I <i>et al</i> ^[10]	5 studies	3	≥ 10 mm	ACN	2% risk difference (-2 to 6)	1.4 [0.9-2.3]
Prospective						
Bertario <i>et al</i> ^[15]	1086	10.5	≥ 20 mm	Any adenoma CRC	Not available SIR	1.5 [1.1-2.1] Not available
			Baseline < 10 mm		0.52 [0.3-0.9] 0.33 [0.1-0.9]	
			≥ 10 mm		0.82 [0.3-1.8]	
Lieberman <i>et al</i> ^[12]	1193	5.5	≥ 10 mm	ACN	15.5 vs 2.4	6.4 [2.7-14.9]
Chung <i>et al</i> ^[8]	3808	4.5	≥ 10 mm	ACN	Not available	3.0 [1.8-5.1]
Registry						
Van Heijningen <i>et al</i> ^[13]	2990	2	≥ 10 mm	AA	8 vs 4	1.7 [1.2-2.3]

ACN: Advanced colonic neoplasia (includes advanced adenomas and colorectal cancer); AA: Advanced adenomas; CRC: Colorectal cancer; RCT: Randomized control trial; SIR: Standard incidence ratio.

neoplasia (evidence level B)^[15].

In summary, adenoma size ≥ 10 mm appears to be associated with future advanced neoplasia and the magnitude of risk increases for larger adenomas ≥ 20 mm in size.

Multiple adenomas

Our search strategy revealed 254 articles of which 7 met inclusion criteria (Figure 1). Van Heijningen *et al*^[13] noted that among 2990 consecutive colonoscopies in the Netherlands, there was an increased risk of

Table 3 Articles summarizing the risk of colonic neoplasia based on the number of polyps seen at baseline colonoscopy

Ref.	Sample size	Median follow-up, yr	Predictor	Primary outcome	Absolute risk of outcome (%)	RR [95%CI]
RCT						
Laiyemo <i>et al</i> ^[11]	1905	4	≥ 3 adenomas	ACN	10 (7-14) vs 6 (5-7)	1.5 [1.0-2.2]
Bonithon-Kopp <i>et al</i> ^[9]	552	3	≥ 3 adenomas	ACN	18.1 vs 5.0	2.7 [1.2-6.4]
Meta-analysis						
Saini I <i>et al</i> ^[10]	5 studies	3	≥ 3 adenomas	ACN	5% risk difference (1-10)	2.5 [1.1-6.0]
Prospective						
¹ Lieberman <i>et al</i> ^[12]	1193	5.5	1-2 ≥ 3	ACN	4.6 11.9	1.9 [0.8-4.4] 5.0 [2.1-12.0]
Chung <i>et al</i> ^[8]	3808	4.5	≥ 3 adenomas	ACN	Not available	3.1 [1.5-6.6]
Registry						
Van Heijningen <i>et al</i> ^[13]	2990	2	1 2 3 4 ≥ 5	AA	4 7 8 12 18	Ref 1.6 [1.1-2.4] 2.1 [1.3-3.4] 2.0 [0.9-4.6] 3.3 [1.7-6.6]
Ng <i>et al</i> ^[18]	4989	2	1 2 3	AA	Not available	Adjusted OR 3.6 [2.6-5.0] 7.1 [4.9-10.4] 13.7 [0.9-4]

¹Relative risk compared to those with no neoplasia. ACN: Advanced colonic neoplasia (includes advanced adenomas and colorectal cancer); AA: Advanced adenomas; OR: Odds ratio; RCT: Randomized control trial.

advanced adenomas on surveillance exams depending on number of adenomas at initial screening colonoscopy (Table 3)^[13]. Using participants with one adenoma as the reference group, those with 2, 3, 4 and ≥ 5 adenomas at baseline colonoscopy had 1.6 (95%CI: 1.1-2.4), 2.1 (95%CI: 1.3-3.4), 2.0 (95%CI: 0.9-4.6) and 3.3 (95%CI: 1.7-6.6) times the relative risk of future advanced adenomas, respectively (evidence level B)^[13]. Lieberman *et al*^[12] evaluated 1193 Veterans undergoing surveillance colonoscopy 5 years after baseline colonoscopy. Compared to those who were neoplasia-free at baseline, patients with 1-2 small adenomas and ≥ 3 adenomas had a RR of advanced adenoma at follow-up of 1.9 (95%CI: 0.83-4.4) and 5.0 (95%CI: 2.1-12.0), respectively, the latter of which was comparable to the risk of having a single advanced adenoma at baseline (evidence level A).

Bonithon-Kopp *et al*^[9] found that in the European Fiber-Calcium Intervention trial, patients with ≥ 3 adenomas had a HR of 5.5 (95%CI: 2.4-12.6) of developing advanced adenomas at three year colonoscopy but only if one of the adenomas was proximal - if all the adenomas were distal, there was no increase in risk of advanced adenomas (0.83; 95%CI: 0.18-3.9) (evidence level A)^[9]. Analysis of 4 year surveillance colonoscopy data from the Polyp Prevention Trial (*n* = 1905) found that compared to having one non-advanced adenoma, individuals with 2 or ≥ 3 adenomas had a RR for advanced neoplasia of 1.38 (95%CI: 0.92-2.1) and 1.84 (95%CI: 1.2-2.8), respectively^[11]. Finally, a meta-analysis of older literature found that those with ≥ 3 adenomas at index colonoscopy were more likely to have recurrent advanced adenomas than were patients with 1 to 2 adenomas (RR = 2.5; 95%CI: 1.1-6.0) (evidence

level B)^[10]. In summary, these data suggest that adenoma number may confer a risk of future neoplasia comparable to adenoma size and as discussed below may be further influenced by the quality of the performance of colonoscopy.

Serrated polyps

Our search revealed 222 candidate articles of which 6 met inclusion criteria (Figure 1). Four were mainly cross-sectional studies, which evaluated the presence of concurrent adenomas and two evaluated the correlation with future neoplasia (Table 4). In a study of 10199 subjects, having a large serrated polyp ≥ 1 cm (LSP) was associated with an increased odds of concurrent advanced neoplasia (adjusted OR = 4.0; 95%CI: 2.8-5.7) and CRC (adjusted OR = 3.3; 95%CI: 2.2-5.0) compared to those who were neoplasia-free (evidence level B)^[16]. Álvarez *et al*^[17] reported that in 5059 patients randomized to undergo screening colonoscopy vs stool test, LSPs were associated with concurrent proximal (OR = 4.2; 95%CI: 1.7-10.2) and distal (OR = 2.6; 95%CI: 1.5-4.6) advanced neoplasia. Several other studies corroborate the relationship between proximal LSPs and concurrent advanced adenomas^[18,19].

With regard to future lesions, a secondary analysis of a large randomized flexible sigmoidoscopy study from Norway that included a median follow-up of 10.9 years provides some insights (evidence level A)^[20]. Having a LSP was associated with an increased risk of future CRC (adjusted OR = 3.3; 95%CI: 1.3-8.6), comparable to having a baseline advanced adenoma. Interestingly, none of the other serrated polyps left *in situ* developed CRC in that tumor, suggesting the serrated polyps might be a marker of field carcinogenesis rather than a precursor lesion^[20]. Schreiner *et al*^[21] found that

Table 4 Risk of concurrent and future advanced adenomas and colon cancer based on serrated polyps

Ref.	Sample size	Median follow-up, yr	Predictor	Primary outcome	Absolute risk	Risk [95%CI]
RCT						
Holme <i>et al</i> ^[20]	100210	10.9	≥ 10 mm serrated polyp	Future CRC	3.4 vs 1.4 cases/1000 patient years	HR 3.3 [1.3-8.6]
Registry						
Álvarez <i>et al</i> ^[17]	5059	None	Proximal l ≥ 10 mm	ACN	Not available	4.2 [1.7-10.2]
			Distal l ≥ 10 mm			2.6 [1.5-4.6]
			Proximal HP			1.6 [1.3-2.3]
Hiraoka <i>et al</i> ^[16]	10199	None	≥ 10 mm serrated polyps	ACN	Not available	4.0 [2.8-5.7]
				CRC		3.3 [2.2-5.0]
				Proximal CRC		4.8 [2.5-8.4]
Hazewinkel <i>et al</i> ^[19]	1426	None	Proximal SP	ACN	Not available	2.4 [1.6-3.8]
			Proximal HP			2.0 [1.1-3.4]
			Prox SSA/P			3.0 [1.5-6.2]
			≥ 10 SP			4.0 [1.9-8.6]
			≥ 10 mm HP			3.2 [1.1-9.1]
			≥ 10 mm SSA/P			5.0 [1.7-14.9]
Ng <i>et al</i> ^[18]	4989	None	SSA	ACN	Not available	4.5 [2.4-8.5]
			Proximal SP			2.2 [1.4-3.6]
			≥ 10 mmSP			59.3 [18.9-186.2]
			≥ 3 SP			4.9 [1.2-19.2]
			≥ 3 non-advanced adenomas			3.6 [2.6-5.0]
Schreiner <i>et al</i> ^[21]	3121	None	Proximal SP	AA	17.3 vs 10.0	1.9 [1.3-2.7]
			≥ 1 cm SP		27.3 vs 10.3	3.4 [1.7-6.7]
	1371	5.5	Proximal SP	Future		
			without adenomas	AA	5.1 vs 2.7	3.1 [1.6-6.2]
			Proximal SP		7.9 vs 6.3	1.2 [0.5-3.8]
			with nonadvanced adenoma		28.9 vs 14.7	2.3 [1.0-5.0]
			Proximal SP			
			with advanced adenoma			

AA: Advanced adenomas; ACN: Advanced colonic neoplasia (includes advanced adenomas and cancer); HP: Hyperplastic polyp; HR: Hazard ratio; RCT: Randomized control trial; SP: Serrated polyp; SSA: Sessile serrated adenoma.

patients with proximal non-dysplastic serrated polyps followed for median of 5.5 years had an increased odds for future adenomas of 3.1 (95%CI: 1.6-6.2) compared to those who were polyp free at baseline (evidence level B). Thus, while it is clear that certain serrated polyps can progress to CRC, those that are right sided and/or ≥ 1 cm are associated with future neoplasia and are a marker for concurrent adenomas and need to be considered as equivalent to an adenoma from a surveillance perspective.

DISCUSSION

Our review found that specific histologic features of adenomas (*i.e.*, high grade dysplasia and villous features) are associated with a small risk of future advanced adenomas though data was inconsistent across studies (level B evidence). In particular, villous features did not confer a consistent or significant association, suggesting it may not be an important risk factor for future advanced adenomas. Data was even more inconsistent for adenoma size, although the linear association between size and risk is compelling (level B evidence). Size itself is challenging to determine reliably because of the lack of a standardized method for estimating adenoma size and the inter-observer variability among size estimation endoscopically as well

as differences in estimations between endoscopic and pathology measurements^[22,23]. Use of an open biopsy forceps as a reference standard for measurement during colonoscopy was accurate to the millimeter only 37% of the time^[24]. The variability in estimating size of adenomas is concerning given that a 1 mm difference in size can change surveillance by 2 years. In a prospective study using size on pathology as gold standard, endoscopists mis-sized polyps 63% of the time, leading to inappropriate surveillance intervals 35% of the time^[25]. Relying on pathology reports for size estimates is challenging, given that polyps are often removed piecemeal and can be fragmented during retrieval. Thus, the accuracy of size estimates for determining surveillance intervals should be viewed cautiously.

Having ≥ 3 adenomas at baseline is associated with an increased risk of future colonic neoplasia (level B evidence), particularly if at least one adenoma is located in the proximal colon, although more supporting data is needed. The findings of our study echo those of the seminal prospective randomized National Polyp Study^[26], in which multiple adenomas (≥ 3; OR = 6.9; 95%CI: 2.6-18.3) and large adenomas (OR = 2.2; 95%CI: 0.6-7.8) were associated with future advanced adenomas at surveillance. In that study, however only multiplicity was a significant risk factor ($P < 0.001$). The risk conferred by villous features or high grade dysplasia

Table 5 Current guideline recommendations for surveillance based on from United States Multi-society Task Force on colorectal cancer, British Society of Gastroenterology, and European Society of Gastrointestinal Endoscopy^[5,27,28]

Organization and year of guidelines	Recommendations for surveillance of adenomas	
	Baseline finding	Timing of next exam, yr
USMSTF on CRC ^[5] , 2012	1-2 small adenomas	5-10
	Adenoma with villous histology	3
	Adenoma with high grade dysplasia	3
	Adenoma ≥ 10 mm	3
	3-10 adenomas	3
	Serrated polyps:	
	< 10 mm no dysplasia	5
	≥ 10 mm	3
	Dysplasia	3
	Traditional serrated adenoma	3
British Society of Gastroenterology ^[28] , 2010	1-2 small adenomas	5-10
	3-4 small adenomas	3
	Adenoma ≥ 10 mm	3
	≥ 5 small adenomas	1
European Society of Gastrointestinal Endoscopy ^[27] , 2010	≥ 3 at least one ≥ 10 mm	1
	High risk adenomas:	3
	Adenoma ≥ 10 mm	
	Adenomas with high grade dysplasia	
	Villous component ≥ 3 adenomas	
	Serrated polyp ≥ 10 mm	
	Serrated polyps with dysplasia	
	Not high risk adenomas	10

CRC: Colorectal cancer; USMSTF: United States Multi-society Task Force.

at baseline was not included.

Current United States and European guidelines recommend repeat colonoscopy in 3 years for patients with ≥ 3 adenomas or any adenoma ≥ 10 mm size or with high grade dysplasia or villous features compared to 5-10 years for those with 1-2 small adenomas (Table 5)^[5,27]. The British guidelines do not take into account advanced histology and recommend earlier follow-up at 1 year for those with at least 5 small adenomas or 3 adenomas if one is ≥ 10 mm in size^[28]. Current level B evidence demonstrates a higher risk of future colonic neoplasia based on having a large serrated polyp (OR ranging from 3.3-4.2) and supports earlier surveillance at 3 years as recommended by guidelines in this group^[5,28]. The recommendations for surveillance of serrated polyps are identical to adenomas^[5]. While surveillance guidelines may be based primarily on adenoma features and risk of future neoplasia, they may also be influenced by national economics and local culture around population-based screening and surveillance, which can vary by country and continent.

The way in which very small differences in adenoma size and number (e.g., 2 vs 3 adenomas) can affect timing of recommended surveillance (from 3 to 5 years) emphasizes the importance of the quality of the colonoscopy performed. Adenoma detection rate (ADR) is considered the most important quality metric

in the performance of colonoscopy because it is a close surrogate measure for interval CRC rates and can be measured feasibly^[7,29]. Other important quality metrics include cecal intubation rates and bowel preparation quality, both of which impact ADR^[7]. A recent simulation study demonstrated that ADR correlates with a lower lifetime risk of CRC without an increase in cost, thus further underscoring the importance of colonoscopy quality^[30]. As ADR improves overall whether from improved endoscope optics or adjunctive techniques (e.g., narrow band imaging, caps, rings)^[31], the association between baseline colonic neoplasia findings and risk of future neoplasia may need to be reassessed.

Our review has certain limitations. We do not address the impact of other factors besides adenoma features on risk for CRC, which are beyond the scope of this article. However, since CRC involves the interactions of genes and the environment, other factors such as family history, age, smoking, diabetes, and obesity have the potential to impact the risk of recurrent neoplasia. Indeed, the NIH risk score looks at a variety of these factors, although its predictive ability has been modest^[32]. We also did not consider the location (proximal vs distal) of adenomas in this review. Location may impart a differential neoplastic risk, with proximal lesions portending a higher risk for recurrence, and merits further clarification in terms of biological underpinnings and clinical strategies. The duration of follow-up for most of the studies ranged from 2 to 5.5 years, which does not allow for the assessment of long-term outcomes. However, this time frame is in line with current surveillance guideline recommendations and provides an adequate follow-up period for the evaluation of the risk of recurrent neoplasia. Lastly, the existing data do not explicitly compare the risk of future advanced adenomas at surveillance based on having multiple different risk factors simultaneously, likely due to limitations of sample size and loss of power with subgroup comparisons. However, if multiple independent risk factors were identified (e.g., multiplicity and size), then having those simultaneously would increase the individual's overall risk of future advanced adenomas.

Future research should continue to evaluate the risk of CRC based on multiple factors incorporating serial colonoscopy information. A few studies have attempted to predict the risk of future neoplasia based on 2 or more examinations^[11,33]. In addition, other biomarkers of the risk of CRC are needed. Since colorectal carcinogenesis involves both genetic and exogenous risk factors of which approximately half are modifiable (i.e., obesity and smoking)^[34], assessment of risk at the level of the colonic mucosa where the interaction between genetics and environment plays out locally may provide a novel approach. While there are a plethora of candidate biomarkers of field carcinogenesis (e.g., molecular alterations such as methylation, gene expression, microRNA in the normal rectal epithelium), the adenoma is currently the only predictor of risk that is robust enough and practical for use in clinical

practice. Future research should also explore the impact of life expectancy on surveillance colonoscopy to guide clinicians who must weigh the risks and benefits for individual patients.

In conclusion, current United States Multi-Society Task Force on CRC recommendations to perform colonoscopy surveillance at 3-5 years after baseline adenomas and serrated polyps appear appropriately tailored to the risk of future neoplasia. The data on adenoma size, adenoma multiplicity and serrated polyps in terms of risk for future adenomas and CRC are compelling, however, the absolute effect size is relatively modest. Future research should identify methods of stratifying a patient's risk for CRC based on serial colonoscopy exams and could include composite risk scores and biomarkers.

COMMENTS

Background

Patients with adenomas of the colon undergo routine surveillance colonoscopy to survey for new adenomas. It is important to understand the existing evidence upon which surveillance colonoscopy recommendations are made.

Research frontiers

This review focuses on the association between specific adenoma features and the risk of future colonic neoplasia.

Innovations and breakthrough

This comprehensive review of the literature shows that adenoma size, adenoma multiplicity and serrated polyps increase the risk for future adenomas and colorectal cancer (CRC), however, the absolute effect size is relatively modest.

Applications

Current United States Multi-Society Task Force on CRC recommendations to perform colonoscopy surveillance at 3-5 years after baseline adenomas and serrated polyps appear appropriately tailored to the risk of future neoplasia.

Terminology

"Surveillance colonoscopy" refers to colonoscopy performed at set intervals (usually 3-5 years) in patients with a history of adenomas to survey for new adenomas.

Peer-review

This is a well-written comprehensive review of current literature on colon adenoma features and CRC risk. To add value to the manuscript, summarising guidelines round the world to provide the readers a more comprehensive review of suggested evidence and protocols would be proposed.

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Esophageal liposarcoma: Well-differentiated rhabdomyomatous type

Hisham M Valiuddin, Arianna Barbetta, Benedetto Mungo, Elizabeth A Montgomery, Daniela Molena

Hisham M Valiuddin, Lake Erie College of Osteopathic Medicine Bradenton, Bradenton, FL 34211, United States

Arianna Barbetta, Daniela Molena, Thoracic Surgery Service, Department of Surgery, Memorial Sloan Kettering Cancer Center, New York, NY 10065, United States

Benedetto Mungo, Division of Thoracic Surgery, Department of Surgery, Johns Hopkins Hospital, Baltimore, MD 21218, United States

Elizabeth A Montgomery, Division of Gastrointestinal and Liver Pathology, Department of Pathology, Johns Hopkins Hospital, Baltimore, MD 21218, United States

Author contributions: Molena D designed the study, surgically retrieved tumor, and followed up findings; Montgomery EA analyzed specimen, detailed pathology, documented findings and reviewed figures; Barbetta A and Mungo B researched literature and reviewed manuscript; Valiuddin HM conducted literature review, collected data and drafted manuscript.

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Correspondence to: Daniela Molena, MD, Director of Esophageal Surgery, Thoracic Surgery Service, Department of

Surgery, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, NY 10065, United States. molenad@mskcc.org
Telephone: +1-212-6393870
Fax: +1-212-6462277106

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Abstract

Rhabdomyomatous well-differentiated esophageal liposarcomas are extremely rare. As of August 2016, only one other such case has been reported in the English-language medical literature. Liposarcomas in general are one of the most common soft tissue neoplasms in adults, but the incidence of primary esophageal liposarcomas is exceptionally low. There have been only 42 reported cases of primary liposarcoma of the esophagus worldwide thus far. These malignancies are harbored within giant fibrovascular polyps, which slowly grow within the esophageal lumen causing obstructing symptoms. We hereby present the case of a 68-year-old male patient who came in with a 2-mo history of worsening intermittent dysphagia, persistent cough, and postprandial retrosternal pain. After an esophagogastroduodenoscopy, a computed tomographic scan, and a diagnostic endoscopy, complete endoscopic resection was performed of the 13 cm × 6 cm × 2.6 cm fibrovascular polyp. A literature review was done and results are presented herein.

Key words: Esophageal cancer; Esophageal surgery; Endoscopy/endoscopic procedures; Pathology esophagus; Liposarcoma; Mesenchymal tumor

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Core tip: This is only the second case of a rhabdomyomatous well-differentiated esophageal liposarcoma to be reported in the literature. Both cases clinically presented as standard esophageal liposarcomas housed in a giant fibrovascular polyp until histological examination by pathology. Given the rarity of the disease, there are only a few studies outlining its optimal management, nevertheless, diagnosis and treatment of this pathology can be approached by customary means, bearing extremely favorable prognosis.

Valiuddin HM, Barbetta A, Mungo B, Montgomery EA, Molena D. Esophageal liposarcoma: Well-differentiated rhabdomyomatous type. *World J Gastrointest Oncol* 2016; 8(12): 835-839 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v8/i12/835.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v8.i12.835>

INTRODUCTION

Primary esophageal liposarcomas are rare, with only 42 cases being reported in English-language literature as of August 2016^[1]. Histologically, liposarcomas can be further classified into 4 subtypes based on their degree of differentiation: Dedifferentiated, well-differentiated, myxoid, or pleomorphic^[2]. We present the case of a patient who had an esophageal well-differentiated liposarcoma containing rhabdomyomatous cells, of which, only one other case - to the best of our knowledge - has ever been reported^[3].

CASE REPORT

A 68-year-old Caucasian male presented with a 2-mo history of worsening intermittent dysphagia, persistent cough, and postprandial retrosternal pain. He also complained of persistent dull pain on the left side of his neck, radiating to his left ear, which was not related to meals.

Suspecting gastroesophageal reflux disease, the patient was started on proton pump inhibitor pharmacotherapy. Upon no relief of symptoms, the patient was referred to an otolaryngologist to evaluate the pharynx with a laryngoscopy; no abnormalities were seen, and therefore the patient was referred to a gastroenterologist. An esophagram was first performed, which showed a voluminous intraluminal lesion within the thoracic esophagus, possibly being a neoplastic process such as a leiomyoma (Figure 1). An esophagogastroduodenoscopy was then performed, identifying a polypoid mass, which started at the level of the upper esophageal sphincter with a single stalk and extended all the way down to the esophagogastric junction. The polyp occupied about a third of the esophageal lumen, was heterogeneous in surface appearance, and consistent with a giant fibrovascular polyp; concurrently a small hiatal hernia was also seen. Biopsies from the head of the polyp

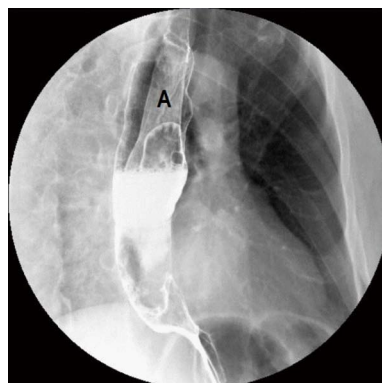


Figure 1 Barium esophagram showing an (A) obstructing intraluminal mass in the thoracic esophagus.

exhibited benign squamous mucosa with mild acute and chronic inflammation. A computed tomographic (CT) scan of the chest showed a large mass along the entire course of the esophagus (Figure 2). After an endoscopic ultrasound excluded the presence of major vessels within the main stalk, endoscopic resection was pursued. While using a flexible esophagoscope to visualize the mass, a snare was passed around the distal end of the polyp on retroflexion and then pulled up around the stalk, which was located on the left side of the esophagus just at the level of the upper esophageal sphincter. The proximal stalk was cauterized and divided with the snare, causing the polyp to drop into the distal esophagus. The polyp was then retrieved transorally using the endoscope and the snare to bring the mass to the level of the upper esophageal sphincter, followed by a laryngoscope and a clamp to extract it from the hypopharynx.

Pathology identified the 13.0 cm × 6.0 cm × 2.6 cm specimen as a well-differentiated liposarcoma arising in a giant fibrovascular polyp. Grossly the polyp had tan uniform surface without stigma of hemorrhage or necrosis (Figure 3). Histologically the polyp showed a central core of adipose and fibrovascular tissue surrounded by overlying squamous mucosa (Figure 4). An immunohistochemical stain for MDM-2 supported the diagnosis of liposarcoma (Figure 5). Focal areas of ossification were noted. In addition, there were scattered atypical cells with abundant eosinophilic cytoplasm, positive for desmin and also focally myogenin positive (Figure 6). The rhabdomyomatous differentiation is considered a low grade lesion (Figure 7). The final resection margin was uninvolved by the tumor. Patient recovered uneventfully and was discharged from the hospital on postoperative day 1. On follow up visit at 4 years, patient still has complete resolution of dysphagia, cough, neck and chest pain. He has been eating well and gained 15 pounds to date. Annual endoscopies and CT scans confirm no recurrence thus far.

DISCUSSION

Esophageal liposarcomas reside in giant fibrovascular

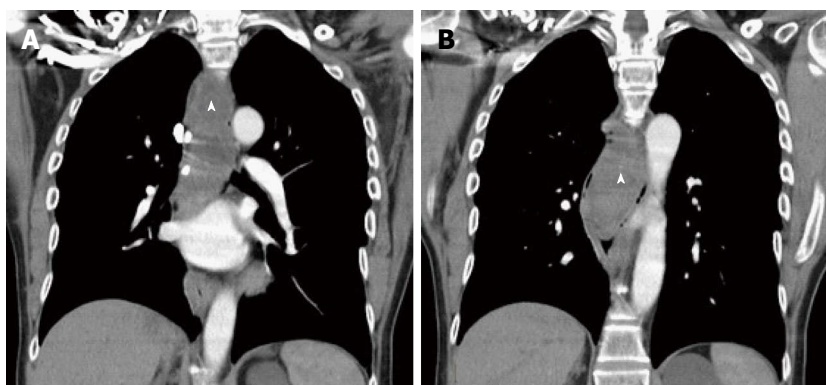


Figure 2 Computed tomographic scan of the chest (A) (B) showing a (arrowhead) large mass traversing the length of the esophagus.

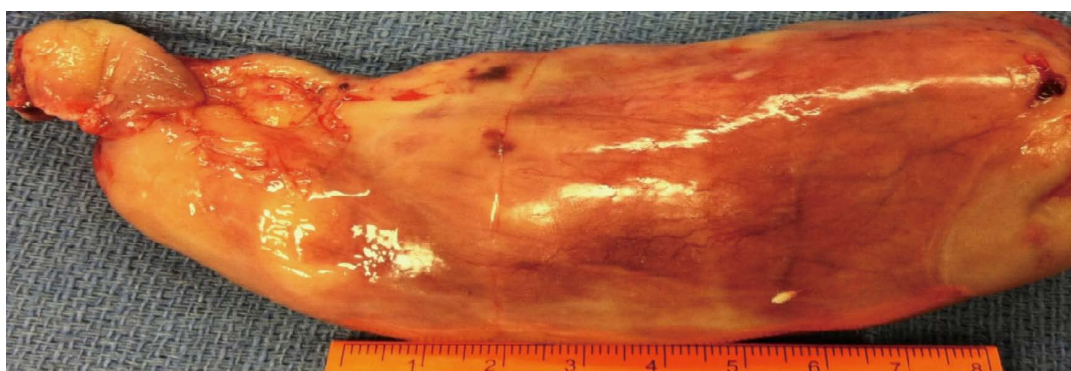


Figure 3 A macroscopic view of the resected giant fibrovascular polyp (13 cm × 6 cm × 2.6 cm) with uniform surface and a large single stalk.

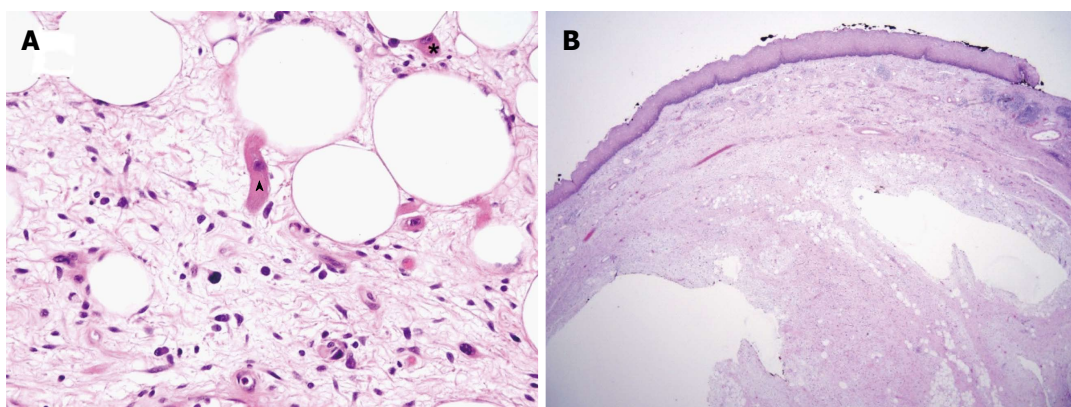


Figure 4 Histologically the polyp showed a central core of adipose and fibrovascular tissue surrounded by overlying squamous mucosa. A: Hematoxylin and eosin stain × 40 identifying (arrowhead) striated muscle cells and adipose tissue within the core of the esophageal liposarcoma; B: The giant polyp is characterized by a central core of adipose and fibrovascular tissue surrounded by overlying squamous mucosa.

polyps and are rare, consisting of 0.5% of all esophageal neoplasms^[4]. Of the histologic sub classifications, well-differentiated type are the most common, with a prevalence of approximately 68%; myxoid being 20%; dedifferentiated and pleomorphic at 6% each^[5]. The pathophysiology of esophageal giant fibrovascular polyps is unknown. The only theory with consensus is that an erroneous out-pouching of loose submucosal tissue undergoes traction and peristaltic forces causing it to insidiously grow and elongate into the lumen^[3].

The average size of a giant fibrovascular polyp is approximately 13 cm in length, and 3.5 cm in width^[3,5]. Liposarcomas are believed to originate from primitive mesenchymal cells rather than mature adipocytes^[2]. The average age of onset of symptoms is 58.4 years, ranged from 38 to 73 years^[4,5]. There has been a 72% male predominance of reported cases^[5]. Almost all lesions were polypoid, except for a couple that were transmural^[5,6]. Eighty percent of the liposarcomas described have been from the cervical portion of the

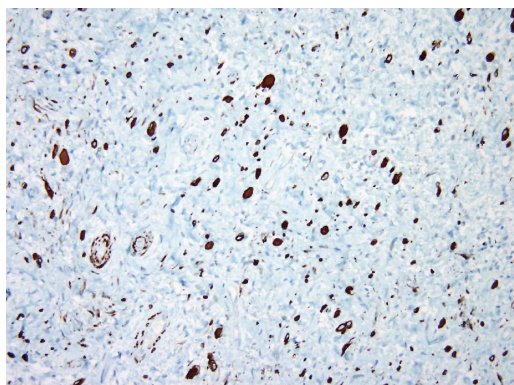


Figure 5 Immunohistology showed positive nuclear staining of lipoblasts with MDM2 confirming the diagnosis of liposarcoma.

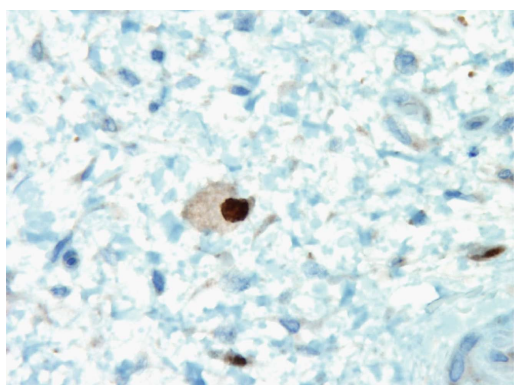


Figure 6 Cell with rhabdomyomatous differentiation were focally positive for myogenin.

esophagus, with the rest originating more distally^[5].

Clinically, patients can present with dysphagia for solids and/or liquids, weight loss, intermittent odynophagia, nausea, globus sensation, cough, emesis and retrosternal pain^[2]. If proper diagnosis and treatment is not administered, there can be drastic complications such as anemia, vomiting of tumor fragments, oral regurgitation of polyp upon emesis, respiratory compromise and fatal asphyxiation^[2,3]. Objective diagnosis can be conducted with barium swallow, esophagogastroduodenoscopy, CT scans, and magnetic resonance imaging (MRI). However, making the diagnosis can require some scrutiny. More specifically, Jakowski and Wakely^[3] described a particular case in which the imaging investigations lead to a differential of achalasia initially, later corrected to a giant pedunculated mass. At best, an accurate esophagram can only identify the presence of a mass; often, other examinations must be used in combination to differentiate, evaluate, and grade the tumor. MRI and CT scans are of great help, not only in recognizing the tumor, but also by calculating the fat component of the tumor, hence providing better characterization of the mass: A 100% fat content is in fact consistent with a lipoma, whereas < 75% signifies atypical lipomas or low grade sarcoma^[2,7].

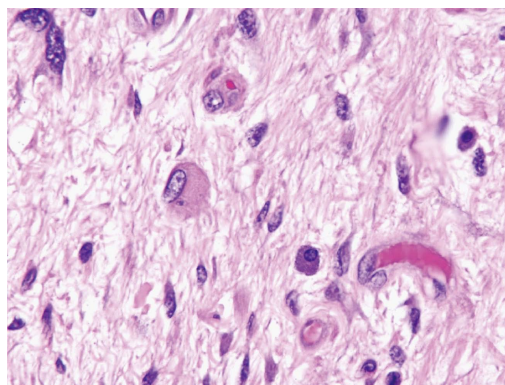


Figure 7 Rhabdomyomatous differentiation is characterized by single and loose aggregates of large round cells with abundant eosinophilic cytoplasm.

The standard of care for giant esophageal polyps is surgical resection, which can be directed by different techniques; including an aggressive open cervical approach, radical three-hole esophagectomy, or local endoscopic resection^[8,9]. Since in our case the endoscopic ultrasound identified a single proximal stalk without a significant feeding vessel, trans oral endoscopic resection was pursued; making sure completeness was achieved by examining margins of specimen to be uninvolved, as cases of reoccurrence after inadequate resection have been reported^[2,5]. Alternatively, resection can also be done through a cervical incision with excision of the polyp from the esophageal lumen, which is in fact the traditional approach, and would have been pursued in presence of a large feeding vessel. Lastly, if the polyps were to have had multiple stalks throughout the entire esophagus, making complete removal through cervical approach unfeasible, an esophagectomy would then be indicated.

In conclusion, given the rarity of the disease, there are only a few studies outlining its optimal management, nevertheless, diagnosis and treatment of this pathology can be approached by customary means, bearing extremely favorable prognosis.

COMMENTS

Case characteristics

A 68-year-old Caucasian male presented with a 2-mo history of worsening intermittent dysphagia, persistent cough, post-prandial retrosternal pain and dull pain on the left side of his neck radiating to his left hear.

Clinical diagnosis

Polypoid mass starting at the level of the upper esophageal sphincter and extending down to the esophagogastric junction, occupying a third of the esophageal lumen.

Differential diagnosis

Neoplastic lesion such as a leiomyoma.

Imaging diagnosis

Esophagogram and computed tomography scan showed a large mass along the

entire course of the thoracic esophagus and an esophagogastroduodenoscopy identified a polypoid mass.

Pathological diagnosis

A 13.0 cm × 6.0 cm × 2.6 cm specimen is identified as a well-differentiated liposarcoma with rhabdomyomatous differentiation arising in a giant fibrovascular polyp and an immunohistochemical stain for MDM-2 supported the diagnosis of liposarcoma.

Treatment

Complete endoscopic resection of the polyp.

Related reports

Esophageal liposarcomas reside in giant fibrovascular polyps and are rare consisting of 0.5% of all esophageal neoplasms. The pathophysiology of esophageal giant fibrovascular polyps is unknown. The only theory with consensus is that an erroneous out-pouching of loose submucosal tissue undergoes traction and peristaltic forces causing it to insidiously grow and elongate into the lumen.

Term explanation

Liposarcoma is a soft tissue neoplasm and is believed to originate from primitive mesenchymal cells rather than mature adipocytes.

Experience and lessons

The standard of care for giant esophageal polyps is surgical resection, which can be directed by different techniques, including a transoral endoscopic resection. A complete resection should be achieved to avoid reoccurrence.

Peer-review

This is only the second case of a rhabdomyomatous well-differentiated esophageal liposarcoma to be reported in literature.

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