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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

MicroRNAs as diagnostic and prognostic biomarkers in colorectal cancer

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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 ^[89] 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?

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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

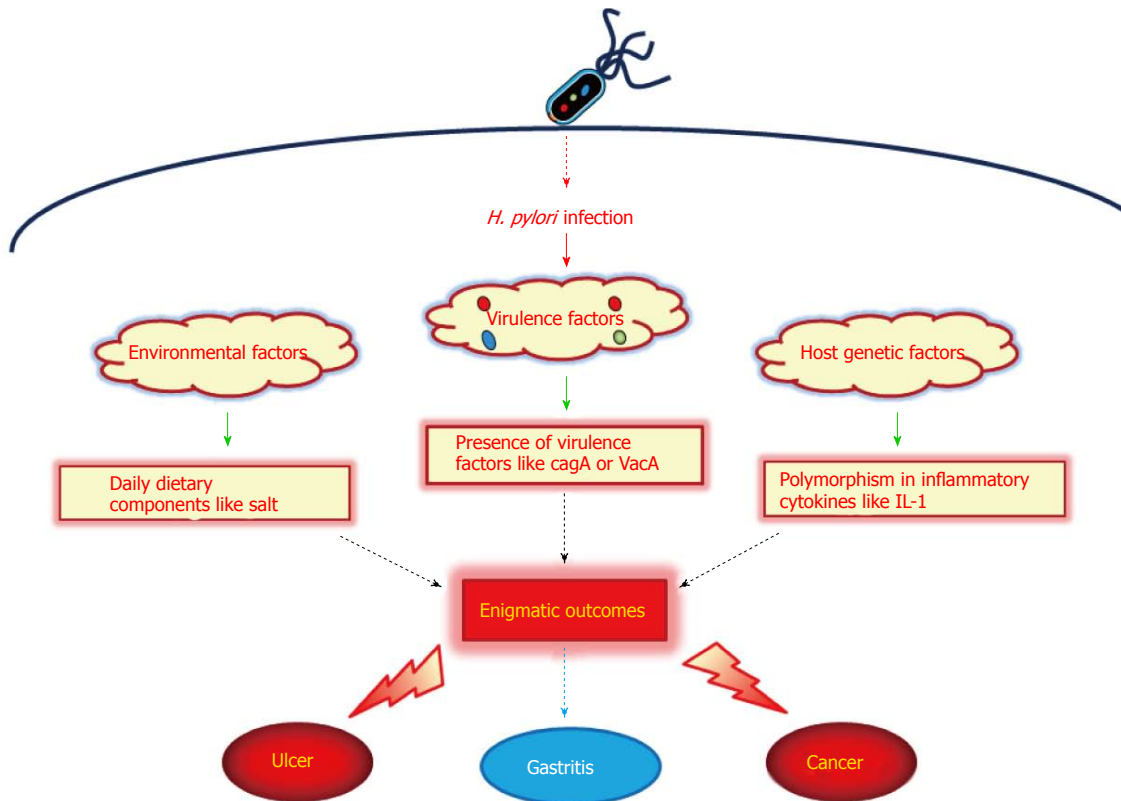


Figure 1 Three main factors behind *Helicobacter pylori* associated enigma namely: (1) Bacterial virulence factors; (2) Host genetic factors; and (3) Environmental factors. *H. pylori*: *Helicobacter pylori*; cagA: Cytotoxin associated gene-A; VacA: Vacuolating cytotoxin A; IL-1: Interleukin-1.

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

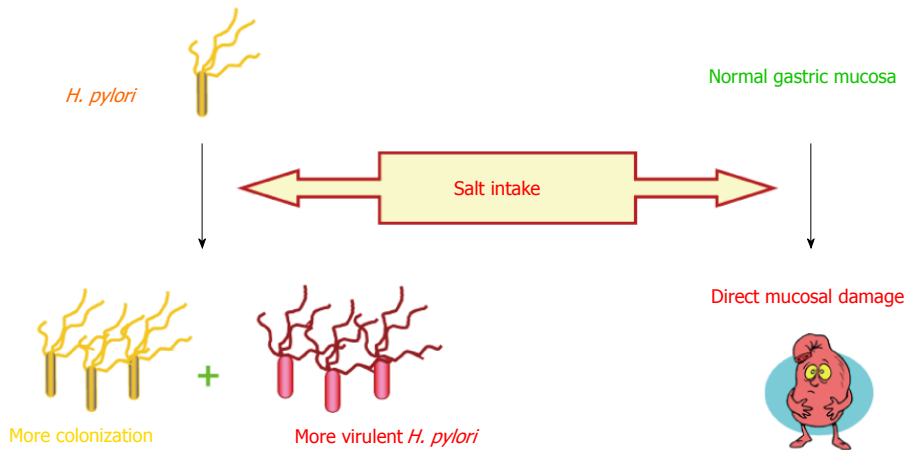


Figure 2 Multiple roles of salt in augmenting *Helicobacter pylori*-associated diseases. Salt can not only cause direct mucosal damage but also increase the colonization as well as virulent *Helicobacter pylori* strains. *H. pylori*: *Helicobacter pylori*.

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

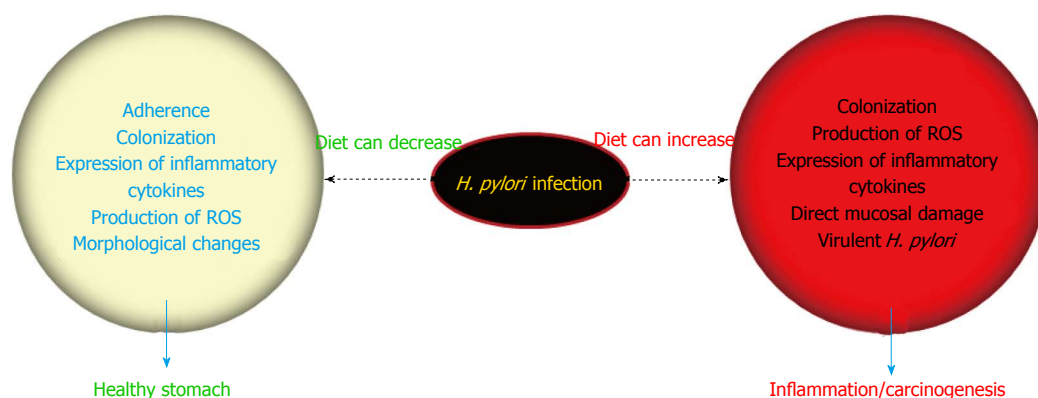


Figure 3 Dual impact of diet in either aggravating or alleviating *Helicobacter pylori* linked diverse disease outcome. Dietary ingredients can equally increase or decrease the inflammatory cascade by modulating the expression of various molecules ultimately modifying the outcome of *H. pylori* infection. *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

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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.

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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

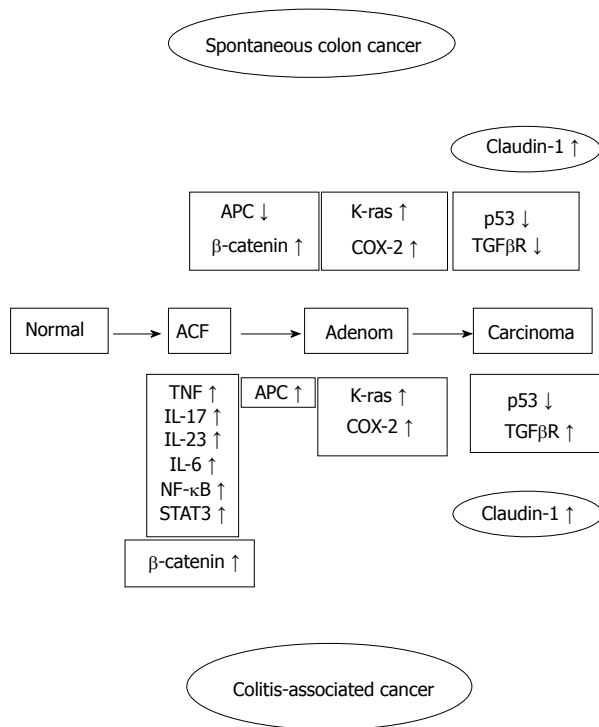


Figure 1 Mechanisms of colorectal cancer and colitis-associated cancer development. APC: Adenomatous polyposis coli; TGFβ: Transforming growth factor beta; NF-κB: Nuclear factor-κB; TNF: Tumor necrosis factor; IL: Interleukin; STAT3: Signal transducer and active of transcription 3.

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

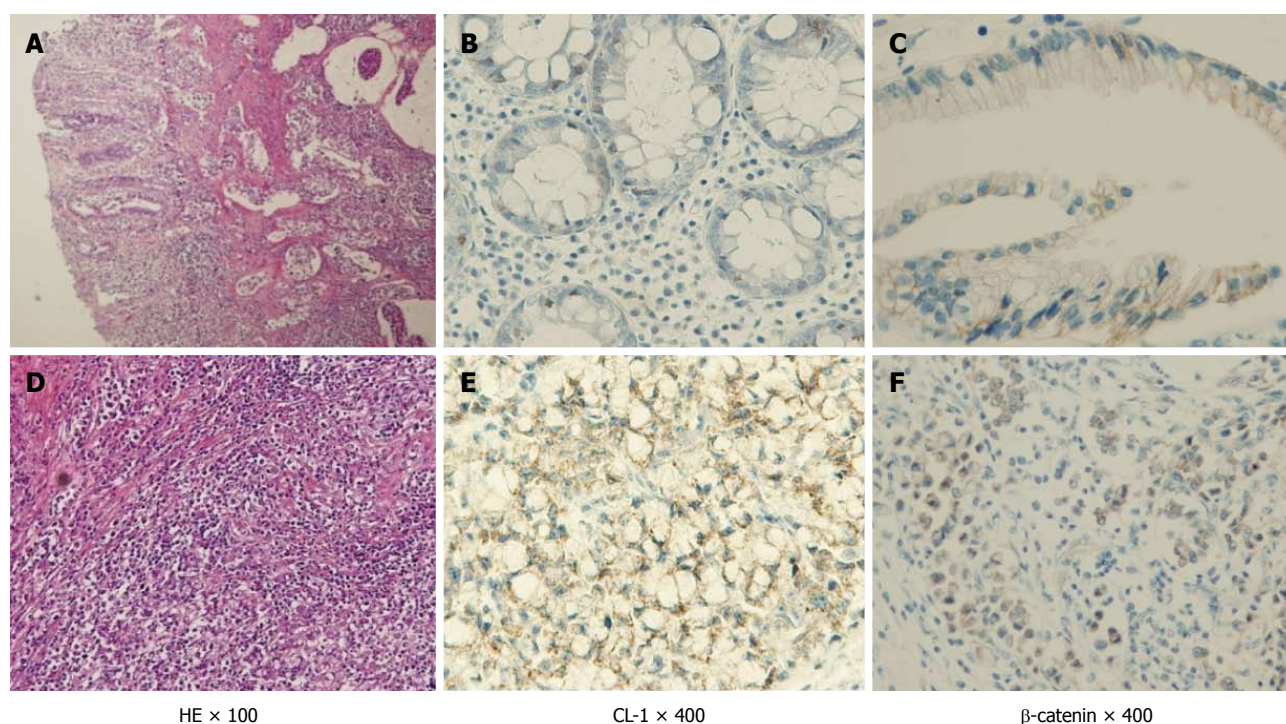


Figure 2 Hematoxylin-eosin (A, D) and immunostaining of claudin-1 (B, E) and β -catenin (C, F) in ulcerative colitis mucosa (A-C) and ulcerative colitis-associated colorectal cancer tissue (D-F) of a patient with ulcerative colitis-associated colorectal cancer.

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

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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

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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 ulocuplumab (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

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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

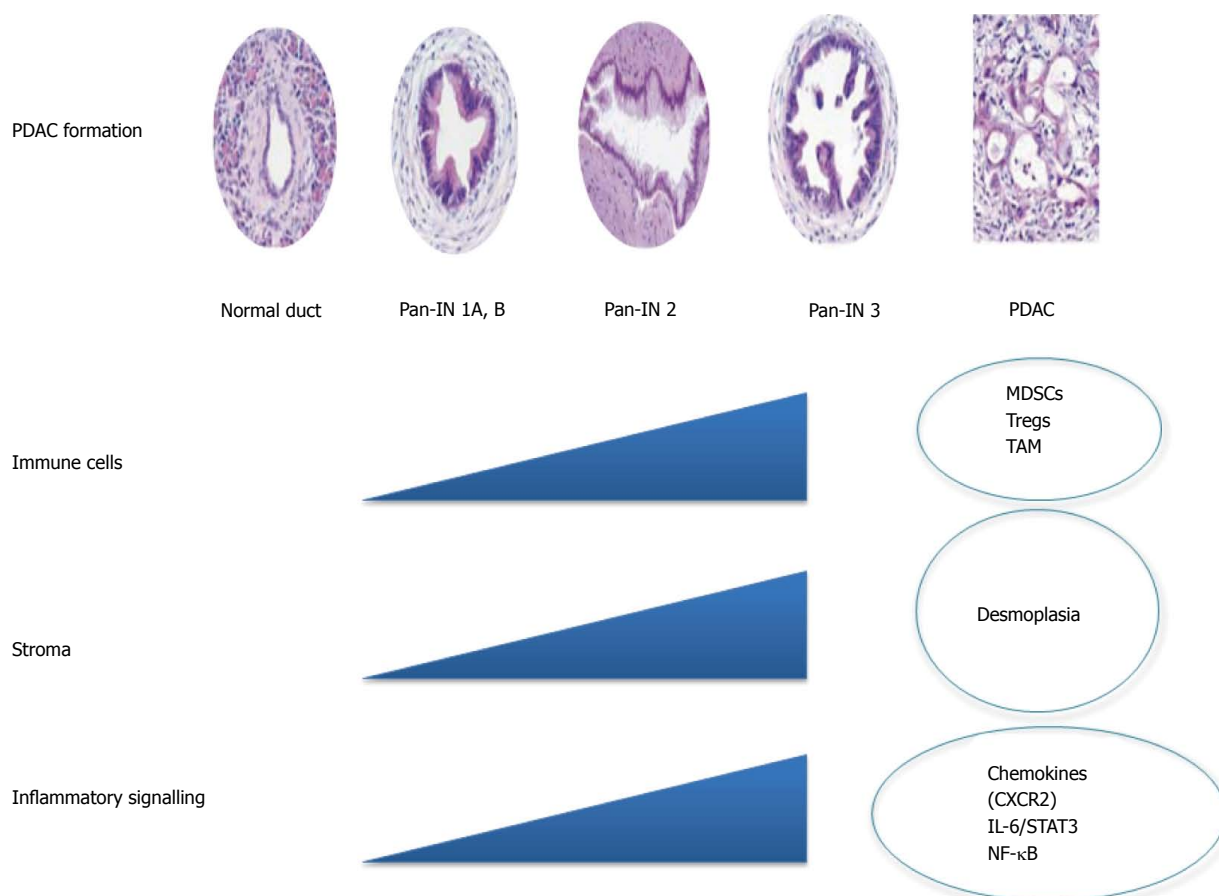


Figure 1 Changes in the pancreatic adenocarcinoma microenvironment during tumor formation. Pancreatic cancer forms from normal tissue via progression through pre-invasive pancreatic intra-epithelial neoplasia (Pan-IN) to invasive PDAC. Changes in immune cell components, stroma, and inflammatory signaling 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

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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].

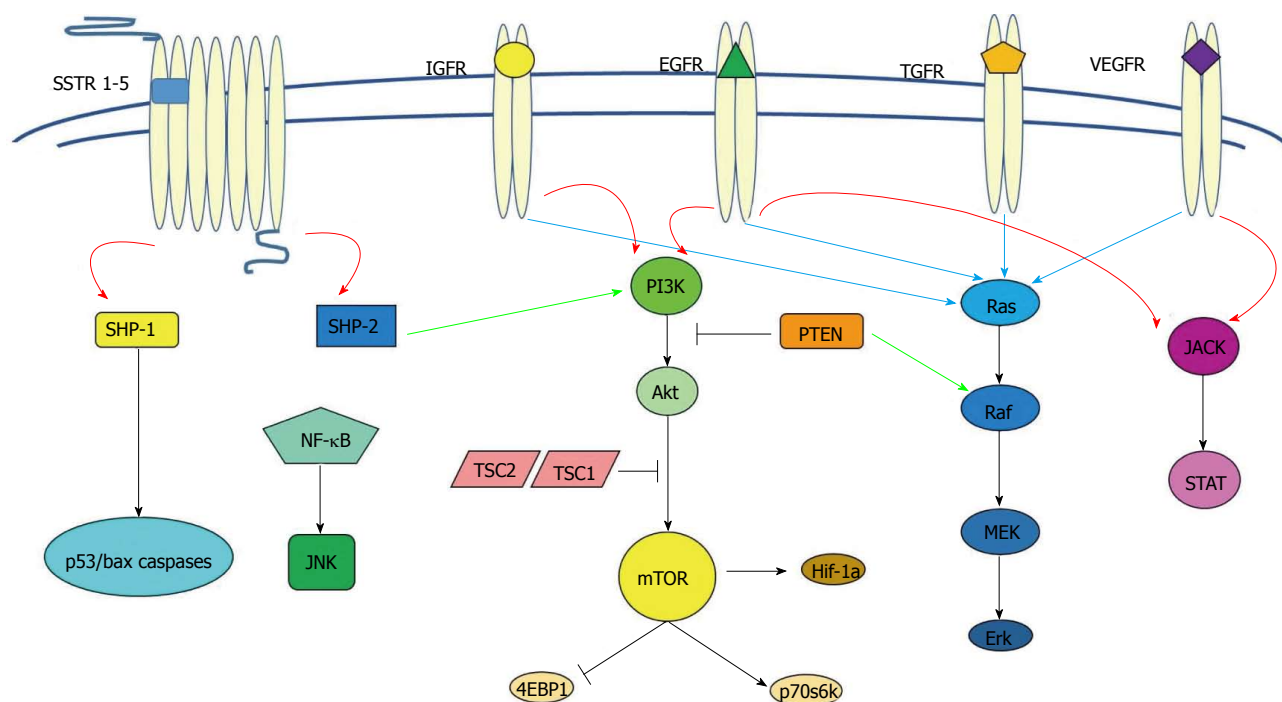


Figure 1 Illustration of principal pathways involved in cellular differentiation, proliferation, survival and apoptosis: Somatostatin receptors, mammalian target of rapamycin protein kinase B, phosphoinositide 3-kinase and phosphatase and tensin homolog, insulin-like growth factor 1 receptor, vascular 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

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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

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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 <i>vs</i> ≥ 65)	1.78	1.24-2.55	0.002
Tumor size (mm) (< 50 <i>vs</i> ≥ 50)	1.51	1.03-2.21	0.036
Serosal invasion (negative <i>vs</i> positive)	2.46	1.45-4.15	0.001
Lymph node metastasis (negative <i>vs</i> positive)	2.48	1.59-3.87	0.000
Curability (curative <i>vs</i> non-curative)	3.46	2.29-5.23	< 0.001

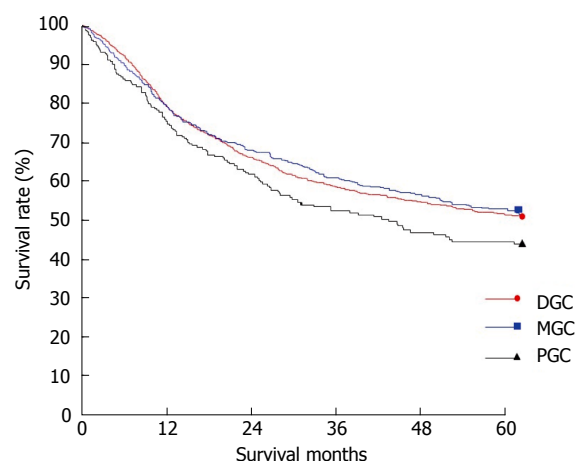


Figure 1 Survival curves for middle third gastric carcinoma, proximal third gastric carcinoma and distal third gastric carcinoma. 5-year survival rate: MGC = 52.8%, PGC = 44.4%, DGC = 51.4%; $P = 0.1138$. MGC: Middle third gastric carcinoma; PGC: Proximal third gastric carcinoma; DGC: Distal third gastric carcinoma.

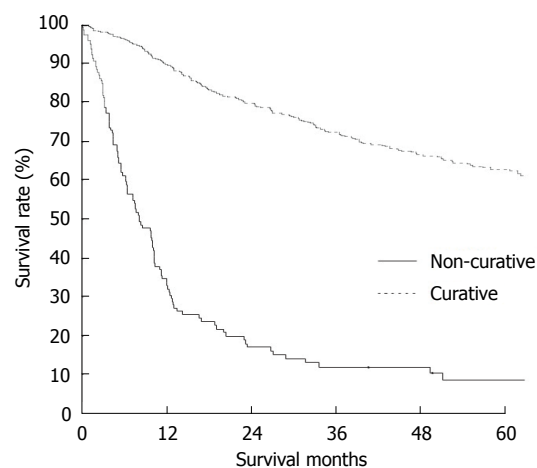


Figure 2 Survival curves for middle third gastric carcinoma according to curability. Five-year survival rate: curative = 62.9%, non-curative = 8.7%; $P < 0.001$.

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,

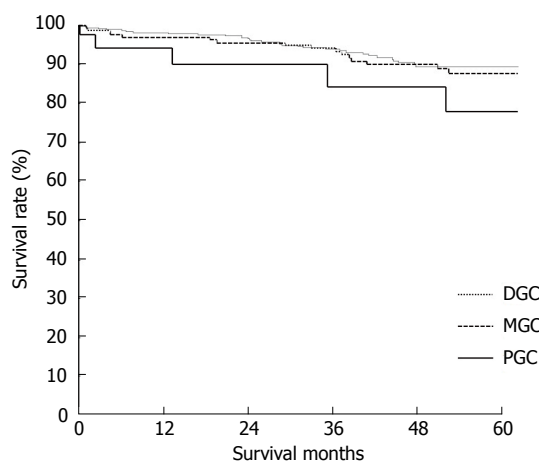


Figure 3 Survival curves of early middle third gastric carcinoma, proximal third gastric carcinoma and distal third gastric carcinoma. MGC = 87.8%, PGC = 77.9%, DGC = 89.5%; $P = 0.0936$. MGC: Middle third gastric carcinoma; PGC: Proximal third gastric carcinoma; DGC: Distal third gastric carcinoma.

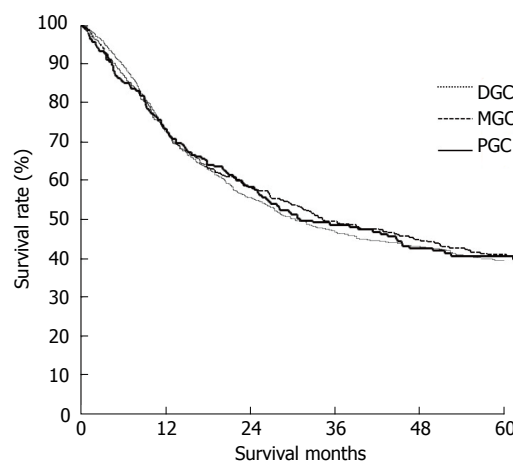


Figure 4 Survival curves of advanced middle third gastric carcinoma, proximal third gastric carcinoma and distal third gastric carcinoma. MGC = 40.8%, PGC = 40.6%, DGC = 39.1%; $P = 0.7586$. MGC: Middle third gastric carcinoma; PGC: Proximal third gastric carcinoma; DGC: Distal third gastric carcinoma.

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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