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REVIEW

Pancreatic cancer: Are "liquid biopsies" ready for prime-time?

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Abstract

Pancreatic cancer is a disease that carries a poor prognosis. Accurate tissue diagnosis is required. Tumours contain a high content of stromal tissue and therefore biopsies may be inconclusive. Circulating tumour cells (CTCs) have been investigated as a potential "liquid biopsy" in several malignancies and have proven to be of prognostic value in breast, prostate and colorectal cancers. They have been detected in patients with localised and metastatic pancreatic cancer with sensitivities ranging from 38%-100% using a variety of platforms. Circulating tumour DNA (ctDNA) has also been detected in pancreas cancer with a sensitivity ranging from 26%-100% in studies across different platforms and using different genetic markers. However, there is no clear consensus on which platform is the most effective for detection, nor which genetic markers are the most useful to use. Potential roles of liquid biopsies include diagnosis, screening, guiding therapies and prognosis. The presence of CTCs or ctDNA has been shown to be of prognostic value both at diagnosis and after treatment in patients with pancreatic cancer. However, more prospective studies are required before this promising technology is ready for adoption into routine clinical practice.

Key words: Pancreatic; Cancer; Liquid biopsy; Circulating; Tumour; Cells; Circulating tumour DNA

Core tip: Pancreatic cancer is a difficult disease to diagnose and treat. Persistently poor outcomes mean that new biomarkers of disease and treatments are required. Circulating tumour cells and circulating tumour DNA have been investigated as liquid biopsies in pancreatic cancer. Sensitivity is variable but specificity promising. The most effective platform and most informative biomarkers are yet to be identified. There are many potential roles for this technology in the management of patients with pancreatic cancer, including screening, diagnosis, prognosis and monitoring of treatment efficacy; however based on current

available evidence they are not yet ready for routine clinical practice.

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INTRODUCTION

Pancreatic cancer possesses one of the worst prognoses of all malignancies. Overall 5 year survival rates are approximately 5% and mortality rates continue to rise in both sexes[1], in contrast to the survival trends seen in most other malignancies.

The only potentially curative treatment is surgery; however only 15%-20% of patients have resectable disease at presentation and even in those that undergo surgery and adjuvant chemotherapy, the 5-year survival is just 16.3%-28.9%[2]. In the majority of patients the disease presents as locally advanced or metastatic.

The median survival for patients with metastatic disease treated with FOLFIRINOX chemotherapy is 11.1 mo[3] and 5.6 mo in those treated with single-agent gemcitabine[4].

Known risk factors for developing pancreatic cancer include tobacco use, obesity, new-onset diabetes, chronic pancreatitis, hepatitis B and *Helicobacter pylori* (*H. pylori*) infection[5].

Other high-risk groups include: patients with $\geqslant$ 2 first-degree relatives with a history of pancreatic cancer; those with a known mutation of the BRCA2 gene or with other familial syndromes known to be associated with pancreatic cancer, *e.g.*, hereditary pancreatitis, hereditary non-polyposis colorectal cancer, Li-Fraumeni, Peutz-Jeghers, familial melanoma; or those with a recognised pre-malignant lesion, *e.g.*, Intraductal Papillary Mucinous Neoplasms (IPMN)[6,7].

Approximately 95% of pancreatic cancers are ductal adenocarcinomas[8] and tumours contain high percentages of stromal tissue[9]. Over 90% are Kirsten rat sarcoma viral oncogene homolog (*KRAS*)-mutated[10]. Other commonly mutated oncogenes include p16, TP53 and SMAD4[10] and high levels of epidermal growth factor receptor (*EGFR*) mutations have also been noted[11]. The serum biomarker CA19-9 is used in the monitoring of treatment response of pancreatic cancer but it has low specificity and is not recommended for use in primary diagnosis[8]. It is subject to false negatives; where the patient does not produce the Lewis enzyme[12] and false positives; with any cause of a raised bilirubin[8] or other malignancies.

Diagnosis of pancreatic cancer can prove challenging. Histological diagnosis often requires invasive tests because of the anatomical position of the pancreas. Due to the high content of stromal cells within the tumour tissue, biopsies do not always provide sufficient material to confirm a diagnosis.

Endoscopic ultrasound with fine-needle aspiration (EUS-FNA) is recommended in the work-up of patients with pancreatic cancer and is the only recommended method of obtaining a biopsy in patients with potentially resectable disease; although there are concerns about tumour seeding along the biopsy tract[8], the traversed duodenum is resected at the time of surgery, abrogating this risk. It has been shown that EUS has better sensitivity than computed tomography (CT) for detection of pancreatic masses; the sensitivity of EUS-FNA is approximately 85%[2]. However, EUS-FNA is an invasive test that requires the use of sedation and can be difficult to tolerate in a patient group who often present with a poor performance status due to the aggressive nature of the disease.

CIRCULATING TUMOUR CELLS

The use of circulating tumour cells (CTCs) isolated in the peripheral blood of patients with cancer as a potential "liquid biopsy" has been under investigation for some time. Their utility was first demonstrated in breast, prostate and more recently lung cancer where they have been shown to be a prognostic marker both in limited stage and metastatic disease[13].

Given the challenges in the investigation and treatment of pancreatic cancer and the need for better biomarkers, many articles have looked at the potential role of CTCs in the disease management pathway (Figure 1).

Circulating tumour cells are present at a rate of approximately 1 CTC per billion blood cells[14] in patients with a malignancy, therefore enumeration requires a process of enrichment prior to detection. There are several ways of detecting CTCs in peripheral blood.

CellSearch

CellSearch is the only US Food and Drug Administration (FDA) approved method for CTC analysis and therefore the most widely studied. It is dependent on the expression of epithelial markers by the CTC, specifically the Epithelial Cell Adhesion Molecule (EpCAM)[12].

It uses 7.5 mL of peripheral blood drawn into an ethylene-diamine-tetra-acetic acid (EDTA) blood sampling tube. A cellular preservative is immediately added. Specific EpCAM, CD45, and cytokeratin fluorescent antibody labels are applied. Samples are then analysed to give a count per 7.5 mL blood[15]. High levels of CTC heterogeneity with variable expression or down-regulation of EpCAM is a technical challenge within the process [14].

Isolation by size of epithelial tumour cells

An alternative method is isolation by size of epithelial

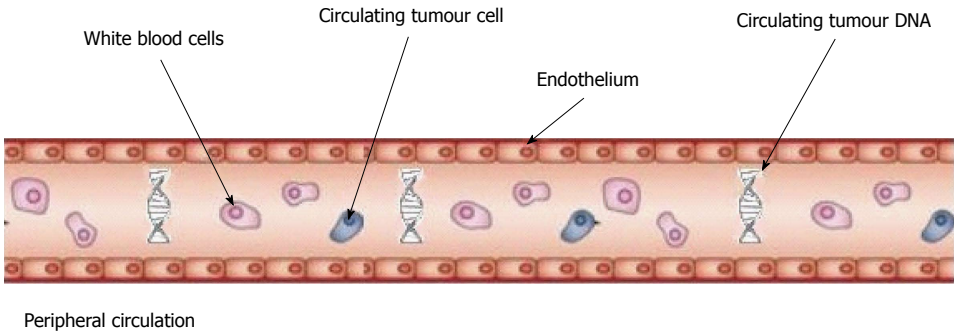

Figure 1 Circulating tumour cells. Tumour cells and DNA fragments circulating in the peripheral bloodstream.

Table 1 Studies reporting detection of circulating tumour cells in patients with pancreatic cancer

Study	CTC platform	Markers	Stage of disease	Time of sampling	Number of patients, *n*	Cut-off for positivity per 7. 5 mL blood	CTCs detected, *n*
Zhang *et al*[16] 2014	Immunocy-togenetics	CK, CD45, DAPI, CEP8	All stages	Pre-operative	22	4	16/22 (72.7%)
Rhim *et al*[17] 2014	Micro-fluidic "GEM" Chip	DAPI, CD45, CK, PDX-1	All stages	Diagnosis	11	⩾ 1	8/11 (73%)
Khoja *et al*[12] 2011	CellSearch and ISET	EpCAM, CK, vimentin, E-cadherin	All stages	Diagnosis or at time of diagnosis of progressive disease > 6 wk from therapy	53	⩾ 1	21/53 (40%) using CellSearch 24/27 (93%) using ISET
Nagrath *et al*[18] 2013	CTC-Chip	Cks	Metastatic	Not stated	15	37.5 (5/mL)	15/15 (100%)
Allard *et al*[15] 2004	CellSearch	CK8, CK18, CK19	Metastatic	Not stated	16	⩾ 1	6/16 ((38%)
Gall *et al*[19] 2013	CellSearch	EpCam, EGFR	Locally advanced	Pre-treatment	75	⩾ 1	4/75 (5%)
Ren *et al*[20] 2011	Immuno-cyto-chemistry	CA 19-9, CK8, CK18	Locally advanced and metastatic	Pre- and post-treatment	41	⩾ 1	33/41 (80.5%) pre-treatment and 12/41 (29.3%) post-treatment

CTC: Circulating tumour cell.

tumour cells (ISET) where blood collected in an EDTA tube is divided into aliquots and diluted with a red cell lysis buffer and then passed through a filtration module. Following washing and staining, CD45-negative cells with features of high nuclear-to-cytoplasmic ratio and hyperchromatic nuclei are designated as CTCs and enumerated[12].

Sensitivity of CTC detection

There have been a number of studies describing CTC detection in patients with pancreatic cancer. Zhang *et al*[16] reported CTCs in 16/22 patients diagnosed with pancreatic cancer at all disease stages, using a combined immunostaining/fluorescence *in situ* hybridisation (FISH) method. They also included 9 patients with pre-malignant or benign lesions and 30 healthy controls. Overall, they reported a sensitivity of 68.18% and specificity of 94.87% using a cut-off of 4 CTCs/7.5 mL of blood.

Rhim *et al*[17] detected CTCs in 73% of patients with pancreatic cancer at all stages at the time of diagnosis and 33% of patients with pre-malignant lesions, using a cut-off of 3 cells/7.5 mL of blood. No CTCs were detected in controls.

Nagrath *et al*[18] were able to detect CTCs in 15/15 patients with a diagnosis of pancreatic cancer across all stages of disease using microchip technology, whilst Khoja *et al*[12] reported detection of CTCs in 21/53 patients with all stages of pancreatic cancer using CellSearch and 24/27 different patients with pancreatic cancer using ISET. Patients were tested at the time of diagnosis or at the time of disease progression following previous treatment. The mean number of cells detected using ISET was 26 compared to a mean of 6 using CellSearch[12].

Allard *et al*[15] found CTCs in 6 of 16 patients with metastatic pancreatic cancer using the CellSearch method. Gall *et al*[19] detected CTCs in 4/75 (5%) patients with locally advanced pancreatic cancer before starting chemotherapy and 5/59 (9%) from the original 75 patients two months after commencing treatment. Ren *et al*[20] detected CTCs in 80.5% of 41 patients with locally advanced or metastatic pancreatic cancer before commencing 5-fluorouracil chemotherapy and in 29.3% after 1 cycle of chemotherapy. These combined results are summarised in Table 1.

Although the sensitivity of CTCs in the trials discussed is variable, the specificity seems to be significantly better. Trials that have compared samples from patients with pancreatic cancer to healthy

controls have shown that healthy controls do not have detectable CTCs[15,18,21,22].

Patients with pancreatic cancer seem to have relatively low numbers of CTCs compared to patients with other tumours including breast, colorectal and prostate cancer[11,14]. This is thought to be due to lower numbers of cells, rather than reduced detection, and it has been hypothesised that this may be related to CTC sequestration as cells pass through the portal circulation in patients with pancreatic cancer[15]. There seems to be no clear consensus on the optimal cut-off for number of CTCs detected in the peripheral circulation of patients with cancer to count as "positive" and similarly there are no significant differences in rate of detection between patients with localised and metastatic disease[2,14,23-25].

Clear data on the absolute sensitivity and specificity of utilising CTCs as a liquid biopsy in patients with pancreatic cancer is still lacking. Studies looking at CTCs in a range of malignancies including breast, colorectal, prostate, and hepatocellular carcinoma have reported very high specificity of around 99%[14,17,22]. In addition, few of the studies including patients with pancreatic cancer have analysed samples from healthy controls, and where they have done, these have always proved negative[14,17,19,21].

CIRCULATING TUMOUR DNA

An alternative method for obtaining a liquid biopsy is to isolate circulating tumour DNA (ctDNA) detected by polymerase chain reaction (PCR) or next generation sequencing (NGS) as a proxy measure.

A multi-disease study (including 155 patients with pancreatic cancer) using PCR assays to search for ctDNA reported that ctDNA was often present where CTCs were not. The study reported detection of ctDNA in > 80% of patients with advanced pancreatic cancer and 48% of patients with localised pancreatic cancer. In a further sub-analysis of the study population, KRAS mutations were detected, using this method, with a sensitivity of 87% and specificity of 99.2% in patients with colorectal cancer[26]. In a pilot study of patients with pancreatic cancer, Earl *et al*[27] were only able to detect KRAS mutations in ctDNA in 26% of patients but they did find a strong correlation between the presence of a KRAS mutation and worse overall survival. Sausen *et al*[28] used digital PCR and detected KRAS mutations in 43% of 51 patients at time of diagnosis with a specificity of > 99.9%. The presence of ctDNA was also analysed following resection and it was reported that disease relapse was detectable at 3.1 mo using ctDNA compared to 9.6 mo using CT. Kinugasa *et al*[29] detected KRAS mutations in ctDNA of 47/75 patients (62%) and reported a concordance in KRAS mutation detections with tissue biopsy of 77.3%. Presence of a KRAS mutation in ctDNA in this study was associated with poorer prognosis.

Zill *et al*[30] compared ctDNA from peripheral blood samples with tumour biopsy samples in 17 patients with both localised and advanced pancreatic cancer and reported a concordance of 90% in genetic mutations; they also demonstrated a correlation between CA19-9 levels and ctDNA percentage across time in a group of 8 patients. Sergeant *et al*[31] used RT-PCR to isolate EpCAM and were able to detect this in 25% of patients with stage Ⅰ and Ⅱ pancreatic cancer prior to undergoing surgery. They detected EpCAM in 65% of patients immediately post-operatively and then in 28.6% at day 1, 23.1% at day 7 and 23.5% at 6 wk post-surgery, but they did not demonstrate an association with survival.

Zhou *et al*[32] were able to detect a range of tumour markers including CK20, CEA and C-MET in between 80 and 100% of 25 patients with pancreatic cancer. de Albuquerque *et al*[22] used a 5-marker panel and were able to detect these in 47.1% of patients with locally advanced or metastatic pancreatic cancer using immunomagnetic RT-PCR and Chausovsky *et al*[33] detected CK20 in 22 of 28 patients with pancreatic cancer using RT-PCR. These results are summarised in Table 2.

Overall, ctDNA appears to be a promising method for use as a liquid biopsy. However, the sensitivity of tests used is variable and there is no consensus as yet on which is the best marker or group of markers to use for most accurate detection.

POTENTIAL ROLES FOR LIQUID BIOPSIES IN THE MANAGEMENT OF PATIENTS WITH PANCREATIC CANCER

Trial evidence to date suggests that identification of CTCs and ctDNA could have roles at many different stages of pancreatic cancer management (Figure 2).

Biopsy

Given the challenges in obtaining suitable biopsy samples from patients with suspected pancreatic cancer, a "liquid biopsy" using a peripheral blood sample would be a highly attractive alternative.

This is a minimally invasive test and the costs are also significantly lower than those of an EUS-FNA: US$371.99 for a CellSearch analysis[34] *vs* US$1405 for EUS-FNA[35].

To be truly useful as a diagnostic tool, a liquid biopsy in patients with pancreas cancer would need to have proven utility in both localised as well as metastatic disease. Much of the evidence in favour of CTCs comes from studies including patients with metastatic tumours[13], and indeed the CellSearch system gained FDA approval following use in a trial which included patients with metastatic breast cancer[36]. There is evidence for their use in early breast[24,25,34] and prostate[37] cancer detection, though

Table 2 Studies reporting detection of circulating tumour DNA in patients with pancreatic cancer

Study	CtDNA platform	Markers	Stage of disease	Time of sampling	Number of patients, *n*	Markers detected, *n*
Zhou *et al*[32] 2011	Nested RT-PCR	H-tert, C-MET, CK20, CEA mRNAs	All stages	Pre-treatment	25	H-TERT 25/25 (100%) CMET 20/25(80%), CK20 21/25 (84%), CEA 20/25 (80%)
de Albuquerque *et al*[22] 2012	Immunomagnetic RT-PCR	KRT19, MUC1, EpCAM, CEACAM5, BIRC5	Stage Ⅲ and Ⅳ	Pre-treatment	34	16/34 (47%)
Chausovsky *et al*[33] 1999	RT-PCR	CK20	Metastatic	Not stated	28	22/28 (79%)
Sausen *et al*[28] 2015	Digital PCR	KRAS	Localised	Pre-treatment	51	22/51 (43%)
Earl *et al*[27] 2015	Digital PCR	KRAS	All stages	Pre-treatment (NB 7 patients had received chemotherapy)	31	8/31 (26%)
Sergeant *et al*[31] 2011	RT-PCR	EpCAM	Stage Ⅰ, Ⅱ and 4	Pre and post treatment	40	10/40 (25%) preoperatively, 10/35(28.6%) D1, 9/39 (23.1%) at D7, 8/34 (23.5%) at 6 wk
Bettegowda *et al*[26] 2014	Digital PCR	KRAS, NRAS, PIK3CA, BRAF	Localised and metastatic	Not stated	155	48% of those with localised and > 80% metastatic disease
Zill *et al*[30] 2015	NGS	KRAS, TP53, APC, SMAD4, FBXW7	Localised and metastatic	Post-treatment	17	17/17 (100%)
Kinugasa *et al*[29] 2015	Digital PCR	KRAS	All stages	Pre-treatment	75	47/75 (62%)

CtDNA: Circulating tumour DNA; RT-PCR: Reverse transcription polymerase chain reaction.

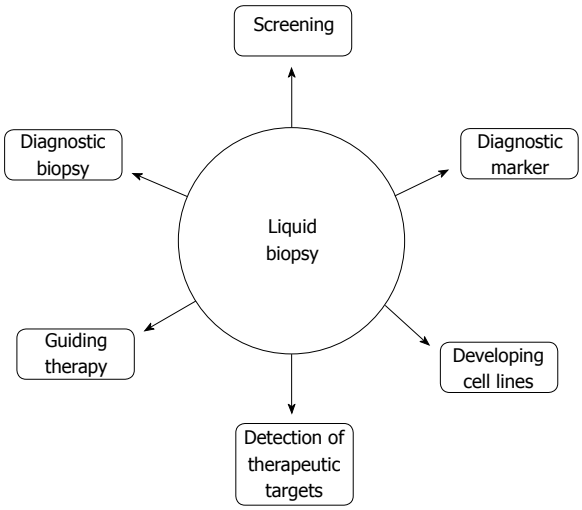

Figure 2 Potential roles of liquid biopsy. As a screening tool: As a non-invasive biopsy in patients not fit for invasive procedures or as an adjunct test where an FNA biopsy has proven inconclusive; As a biomarker used after surgical treatment to identify patients at high risk of local recurrence or distant metastasis; As a marker of prognosis in all patients prior to therapy; As a marker of chemotherapy efficacy; In the development of cell lines from circulating tumour cells; and In the detection of therapeutic targets or resistance mechanisms.

the evidence in other disease sites is less clear[13]. Widespread clinical application is not prevalent. However, multiple studies have used the system of other methods of ctDNA detection in patients with localised, curative-intent pancreatic cancer[12,19,38] and two meta-analyses discussed below incorporated data from studies including patients with resectable and non-resectable disease.

Allard *et al*[15] commented that patients with pancreatic cancer had relatively low levels of CTCs detected compared to other malignancies, even in the presence of widespread metastatic disease. Whether patients with pancreatic cancer produce fewer peripheral CTCs than patients with other malignancies remains unclear but this could account for the relatively low detection rates seen in the studies discussed above and may represent a significant challenge in the use of liquid biopsies.

Furthermore, high levels of heterogeneity have been demonstrated in CTCs[39-43] from patients with a range of malignancies. This appears to be both spatial and temporal as detectable CTCs may change in appearance over time and with treatment[11]. For example, expression of EpCAM or other tumour markers appears to be variable from cell to cell both in different patients and within the same patient, but also the expression may vary over time within the same patient. This provides a further challenge in the use of CTCs and ctDNA as a biopsy or screening tool as ctDNA might be detected in a serum sample that may not then be found in the tumour sample. This could lead to confusion around the diagnosis and perhaps necessitate more tests and delays to treatment.

Heitzer *et al*[40] demonstrated that mutations were found in CTCs from the peripheral circulation that weren't found in the primary tumour of patients with colorectal cancer. On further NGS of the primary tumour, it was demonstrated that these mutations were present at subclonal level, potentially demonstrating that issues around heterogeneity can be overcome.

A successful liquid biopsy (for diagnosis) would therefore need to rely on a combination of CTC enumeration and genetic analysis to provide the most accurate result and better sensitivity and, from currently-available evidence, the best combination of

genetic markers is not well defined.

Screening

Some patient groups are at higher risk of developing pancreatic cancer[6]. A recent review of trials into screening high-risk individuals found that screening these patients resulted in a higher curative resection rate and longer median survival time[44]. For example, a recent study by Vasen *et al*[45] demonstrates a screening benefit for patients with the CDKN2A mutation. However other studies have not found screening using a combination of clinical examination, blood tests, EUS and magnetic resonance cholangio-pancreatography (MRCP) in high risk individuals useful[46] and in current guidance it is not recommended[8]. The only existing biomarker for pancreatic cancer: CA19-9, has not been shown to be a reliable screening marker[8].

Evidence showing high levels of specificity combined with a relatively low cost would favour the use of CTCs as a screening tool. The variable sensitivity of this test means that based on current evidence, CTCs would not be suitable for screening and there is no current clinical trial evidence demonstrating the benefit of CTCs as a screening tool in any malignancy.

Prognosis following surgery

Another potential use would be as a biomarker of prognosis either before or at the time of curative-intent surgery. Sausen *et al*[28] detected CTCs in 22 patients (43% of cohort) with localised pancreatic cancer at the time of diagnosis and found that presence of CTCs did predict relapse after resection and worse outcome. They also observed that ctDNA could be detected 6 mo before a radiological confirmation of recurrence, suggesting that CTCs could play an important role in detection of residual disease post-operatively or in the detection of early recurrence. Bissolati *et al*[47] performed intra-operative collection of blood samples from both the systemic circulation and portal vein. They found that CTC positivity in the portal circulation predicted liver metastases but not any significant difference in Disease Free Survival (DFS) or Overall Survival (OS). Sergeant *et al*[31] detected EpCAM from peripheral blood pre- and post-operatively in 40 patients undergoing pancreatectomy and also assessed peritoneal lavage fluid for EpCAM. Although detectable in 65% of patients post-operatively, EpCAM positivity was not associated with a worse prognosis. These findings indicate that a significant majority of CTCs will not survive, and as few as 0.01% may go on to form metastases[48,49].

Marker of chemotherapy efficacy

A liquid biopsy could theoretically be a useful marker of disease response to chemotherapy.

Many studies have shown that CTCs can be used to predict treatment outcomes in a range of malignancies including breast, bladder, prostate and bowel[50,51] but not necessarily as a form of monitoring. Obermayr *et al*[52] revealed that the ongoing presence of CTCs during follow-up in patients with ovarian cancer was more common in those with platinum-resistant disease. The South-West Oncology Group[53] investigated the use of CTCs to guide treatment change in patients with metastatic breast cancer. Patients were treated with one cycle of standard chemotherapy and those who continued to test positive for CTCs after one cycle of chemotherapy were switched to an alternative regimen. However, a benefit in overall survival was not demonstrated. In a recent study by Tie *et al*[54] Patients with colorectal cancer had serum samples tested for ctDNA and compared with mutations within the primary tumour sample in the post-operative period. Six patients out of a cohort of 52 who went on to have adjuvant chemotherapy had detectable ctDNA. Samples were tested again every 3 mo; all patients who were ctDNA positive became ctDNA negative during chemotherapy. However two patients later became ctDNA positive again and both these patients relapsed.

There is little evidence for the use of liquid biopsies in monitoring of chemotherapy response in patients with pancreatic cancer. Ren *et al*[20] measured CTCs in patients with pancreatic cancer before and after their first cycle of 5-fluorouracil chemotherapy. The CTCs were detected in 80.5% of patients prior to chemotherapy and in 29.3%, one week after the first cycle of chemotherapy. Gall *et al*[19] detected CTCs in patients with locally advanced pancreatic cancer before, and then after 2 mo of chemotherapy. They reported that 4 of 75 (5%) patients had detectable CTCs before commencing chemotherapy and 5 of 59 (9%) had detectable CTCs after two months of treatment, however there was no crossover between the two groups. The trial was too small to demonstrate a significant difference in survival in these patients.

Overall prognosis

Two meta-analyses have reviewed the potential roles of CTCs as a prognostic biomarker: Ma *et al*[55] analysed 9 papers with a total of 603 patients included, with a range of different stages of pancreatic cancer. Four of the included papers examined prognosis after commencement of systemic treatment. The hazard ratio (HR) for DFS and OS in patients before treatment were 1.82 and 1.93 respectively ($P < 0.003$) and post treatment were 8.36 and 2.20, suggesting that the presence of CTCs after completing treatment has better predictive value for disease relapse and worse OS compared to pre-treatment.

The estimated pooled HR for OS across all 9 papers was 1.64 (95%CI: 1.39-1.94, $P < 0.00001$), showing that CTC positivity was associated with worse OS. This meta-analysis did find evidence of publication bias, however[55].

Han *et al*[56] explored 9 papers, 7 of which were

included in the Ma *et al*[55] meta-analysis, but did not report any publication bias. Overall, the study included 623 patients with a range of stages of pancreatic cancer, having either surgery or chemotherapy. The HR for progression free survival (PFS) was 1.89 (95%CI: 1.25-4.00, $P < 0.001$) and the HR for OS was 1.23 (95%CI: 0.88-2.08, $P < 0.001$), suggesting that CTC positivity did predict poorer outcomes. This seems to be consistent whether sampling was performed before or after treatment. On sub-group analysis, no variations between ethnicity or between different methods (CellSearch *vs* RT-PCR) of CTC detection were reported.

Development of cell lines

Cell lines cultured from tumour biopsy samples have been used in cancer research for many years. A number of small studies have developed cell cultures from CTCs of patients with metastatic breast, lung and prostate cancer[57-59] and a pilot study by Cayrefourcq *et al*[60] demonstrated the development of permanent cell lines from CTC samples of patients with colorectal cancer. This had not previously been reported, perhaps in part because patients with colon cancer, similar to those with pancreatic cancer, seem to have relatively low levels of CTCs in the peripheral circulation.

This technology is at an early stage of development and has not yet been reported for patients with pancreatic cancer. However, these studies indicate that liquid biopsy could form the basis of cell lines upon which investigation of genetic mutations and targets for therapies can take place for patients with pancreatic cancer. A potential issue with this technology would be temporal heterogeneity of the cancer and thus it may be possible that repeated cultures would be required.

Detection of therapeutic targets or resistance mechanisms

Studies in patients with a range of malignancies have begun to use CTCs and ctDNA to identify mechanisms of resistance and potential therapeutic targets. Murtaza *et al*[61] analysed ctDNA from patients with advanced cancer and using PCR were able to identify mutations known to be associated with acquired drug resistance. Heitzer *et al*[40] used CTC analysis and next-generation sequencing of the primary tumour in patients with colorectal cancer to identify mutations that could be of therapeutic interest. However, the issue of heterogeneity remains problematic; mutations were identified in CTCs that were present in the primary tumour, only on sub-clone analysis of the primary tumour, or were unique to that CTC. This makes the relevance of individual mutations difficult to quantify in the context of a full tumour genome.

Genetic analysis of liquid biopsy samples could, in the future, form part of a "personalised" mutation profile for a patient with pancreatic cancer and identify which targeted agents would be suitable for that patient. However, studies evaluating this approach are small and have not yet included patients with pancreatic cancer. Therefore, more studies and on a larger scale are required.

The role of microRNAs in pancreatic cancer

Although not addressed thus far in this article it is worth noting the increasing evidence supporting the potential roles for detection of micro-RNA (miRNA) in pancreatic cancer.

miRNAs are small molecules consisting of chains of RNA, typically around 20 nucleotides in length[62]. There is evidence that miRNAs play a role in modulating gene expression and thus biological processes[62].

This role seems to be highly variable as miRNAs can act as both an oncogene and a tumour suppressor gene[63]. miRNAs have been implicated in all tumour types including pancreatic cancer[64] and have been isolated in the bloodstream of patients with pancreatic cancer[65].

Small studies have shown that miRNA isolated from serum or biopsy samples can differentiate between pancreatic cancer and chronic pancreatitis[66] and IPMN[67] and small studies have shown they may be prognostic markers[62,65].

Technology for the detection of miRNAs is early in development and there is currently not sufficient evidence to make recommendations but they may in the future work concurrently with ctDNA and CTCs in the management of pancreatic cancer.

DISCUSSION

Pancreatic cancer is a disease with a prognosis that remains poor in contrast to the improvements in survival noted in other cancers over recent years[1]. The challenges faced in obtaining a diagnosis and the poor survival following treatment mean that new biomarkers and treatment strategies are necessary.

Current clinical and retrospective trial evidence indicates that CTCs are detectable in patients with pancreatic cancer, at both limited and advanced stages. Many of the studies available are hampered by their small sample size, however meta-analyses have performed statistical analysis on over 600 patients and demonstrated a clear correlation between CTC or ctDNA positivity and poorer outcome[55,56].

There are many potential applications of liquid biopsies in the care pathway of patients with pancreatic cancer. Their strength lies in being a relatively non-invasive test that can be repeated at any time. Therefore, one role could include diagnosis, particularly for patients too unwell to undergo invasive tests, or where these have proven inconclusive. A significant limitation to the use of CTCs and ctDNA as a liquid biopsy is their relatively low sensitivity and a lack of clarity on which is the most effective method of detection in this disease group.

Very few studies have directly compared CTCs to ctDNA. Dawson *et al*[68] analysed ctDNA and CTCs in patients with metastatic breast cancer and reported that ctDNA levels were more closely related to disease burden. In contrast, Maheswaran *et al*[69] detected CTCs and ctDNA in patients with non-small cell lung cancer and reported that detection of CTCs was more sensitive. Apart from Khoja *et al*[12], few trials have directly compared methods of CTC or ctDNA analysis and therefore the most sensitive method is not clear. It seems likely that the most effective test would use both CTCs and ctDNA, but data is lacking in this area both around efficacy and feasibility on a larger scale.

Another challenge is the high levels of heterogeneity found in CTCs, that has been shown to be both temporal and spatial[11]. This could impact on the sensitivity and reliability of liquid biopsies. Heterogeneity in CTCs has been found in many malignancies. Indeed it has been reported that between 63% and 69% of mutations are not present at every disease site in patients with metastatic renal cell cancer[70]. This could potentially be overcome using NGS of tumour samples in addition to CTC samples to identify more mutations. However, this has only been demonstrated in a small study[40] and may prove too costly and time-consuming to be feasible in routine practice. More work is required to evaluate this approach in pancreatic cancer.

Although only a small trial, the fact that ctDNA became detectable months before a radiologically-detectable relapse in patients who had undergone a resection[28] suggests that in future, liquid biopsies could potentially form an important role in the monitoring of patients after surgery. A larger trial would be needed to validate these results and then a future area of research might look at whether instituting chemotherapy at the point of "liquid biopsy relapse" could alter the outlook for patients with recurrent disease after surgery. Similarly, liquid biopsy could play a potential role as a non-invasive marker of treatment response to chemotherapy or as a marker of disease progression following chemotherapy in advanced disease. However, none of the trials that have detected CTCs following chemotherapy in patients with pancreatic cancer have proven that patients with detectable CTCs after chemotherapy have a worse survival[31], although trials studying this question only included small numbers of patients. Furthermore, as yet there is no evidence to show that moving directly onto second-line chemotherapy in patients with advanced pancreatic cancer with CTC-positivity following first-line chemotherapy would result in a survival benefit. A trial looking at switching chemotherapy in response to presumed resistance (based on on-going detection of CTCs following one cycle of chemotherapy) has not proven to have an impact on survival in patients with metastatic breast cancer[53]. It may well be that sufficient time for a cytotoxic effect to eliminate all CTCs from peripheral circulation requires more than one cycle of chemotherapy and there is no comparative data exploring this. A useful trial might measure CTCs following each cycle of chemotherapy and correlate on-going positivity to PFS and OS in an effort to identify a cut-off point for switching regimen.

Ultimately, liquid biopsies can only be of limited utility whilst there is still a lack of more effective treatments for pancreatic cancer. There is little purpose in demonstrating that a patient with pancreatic cancer is not responding to chemotherapy if there are limited alternatives available, therefore, on-going prospective studies developing novel therapeutic strategies are imperative.

Disappointingly, the addition of targeted therapies to conventional chemotherapies to date has failed to result in survival benefits in patients with pancreatic cancer. Despite the fact that the majority of pancreatic adenocarcinomas are KRAS positive, and a significant proportion have *EGFR* mutations, agents targeting this mutation have yet to demonstrate a clinically meaningful benefit.

Although Moore *et al*[71] reported a benefit to the use of the tyrosine kinase inhibitor, erlotinib, in addition to gemcitabine in patients with advanced pancreatic cancer for both OS and PFS, this has not been replicated in other trials[72-74]. Nor has efficacy been demonstrated with other agents; for example, mitogen-activated protein kinase inhibitors[75] or VEGF inhibitors either alone or in combination with other targeted therapies or standard chemotherapy[76,77]. In the study by Infante *et al*[75], outcomes were independent of KRAS mutations determined by circulating free DNA and archival tumour tissue. Therefore, the potential of CTC utilisation in the identification of resistance mechanisms to these and similar agents may have clinical utility.

In summary, despite offering great promise to alter the outlook of this challenging disease, a significant amount of further data, in many different areas of the management pathway, is needed before liquid biopsies are ready for prime-time.

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REVIEW

Modulation of microbiota as treatment for intestinal inflammatory disorders: An uptodate

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Abstract

Alterations of intestinal microflora may significantly contribute to the pathogenesis of different inflammatory and autoimmune disorders. There is emerging interest on the role of selective modulation of microflora in inducing benefits in inflammatory intestinal disorders, by as probiotics, prebiotics, synbiotics, antibiotics, and fecal microbiota transplantation (FMT). To summarize recent evidences on microflora modulation in main intestinal inflammatory disorders, PubMed was searched using terms microbiota, intestinal flora, probiotics, prebiotics, fecal transplantation. More than three hundred articles published up to 2015 were selected and reviewed. Randomized placebo-controlled trials and meta-analysis were firstly included, mainly for probiotics. A meta-analysis was not performed because of the heterogeneity of these studies. Most of relevant data derived from studies on probiotics, reporting some efficacy in ulcerative colitis and in pouchitis, while disappointing results are available for Crohn's disease. Probiotic supplementation may significantly reduce rates of rotavirus diarrhea. Efficacy of probiotics in NSAID enteropathy and irritable bowel syndrome is still controversial. Finally, FMT has been recently recognized as an efficacious treatment for recurrent *Clostridium difficile* infection. Modulation of intestinal flora represents a very interesting therapeutic target, although it still deserves some doubts and limitations. Future studies should be encouraged to provide new understanding about its therapeutical role.

Key words: Microbiota; Inflammation; Gut; Probiotic; Prebiotic

Core tip: Alterations of intestinal microflora may signi-

ficantly contribute to the pathogenesis of different inflammatory and autoimmune disorders. It is conceivable that selective modulation of intestinal microflora may induce benefits. In this article we tried to summarize recent evidences on microflora modulation in main intestinal inflammatory disorders, providing practical perspectives on its therapeutical role in these conditions.

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INTRODUCTION

Gut microbiota

Definition: The human intestinal microflora, known as "microbiota", includes 100 trillion (10^{14}) bacteria, quadrillion viruses, fungi, parasites, and archaea for a total weight of about 1 kg[1]. The stomach and small intestine are colonized only by a few species of bacteria, mainly because of the acid environment, the presence of bile and pancreatic secretions, and the peristaltic activity limiting bacteria stable colonization[2]. On the contrary, the colon harbours about 10^{12} microorganisms[3]. Also several yeasts ("gut mycome") are included in the gut microbiota, but their role is still not well established[1,4,5].

Composition: Colonization of human gut starts few days after birth. Pattern of intestinal microbiota may be firstly influenced by type of delivery and type of diet[2] and, later, by immune stimulaton and environmental factors, becoming more stable after the first two years[6], even though it can be modified under some circumstances, such as diarrhea, antibiotic treatment, or dietary interventions. Gut microflora may be characterized by conventional culturing techniques which fail to detect more than 30% of total bacteria in the gut, therefore molecular approaches, such as metagenomic analysis and 16S ribosomal RNA gene sequencing, are now commonly used[7].

Bacteria in stomach, duodenum, and jejunum are mostly represented by oropharyngeal origin aerobic gram-positive ones, whereas in the ileum coliforms are the predominant species. Post ileocecal valve, there is a growing of bacterial anaerobic species, mainly Bacteroides, Bifidobacteria, Clostridi and Lactobacilli[8].

Functions: The main metabolic function is represented by the fermentation of large polysaccharides and some oligosaccharides, unabsorbed sugars, and host-derived carbohydrates from mucus glycoproteins[9]. The major products of carbohydrate metabolism are gasses (hydrogen and carbon dioxide), ethanol and short chain fatty acids (SCFAs)[2,9]. These latter, represented by butyric, propionic, and acetic acid, are important energy sources for colonocytes, and may inflence glucose metabolism[10]. Colonic bacteria are also involved in vitamin synthesis, absorption of calcium, magnesium, and iron[11,12].

Differentiation and proliferation of epithelial cells is greatly influenced by interaction with resident microflora. This effect is mainly mediated by the SCFAs which promote the development of intestinal microvilli[13].

Intestinal microbiota plays also a pivotal role in development of the gut immune system, especially during early life, establishing an efficacious "cross-talk" with the host[14,15]. Germ-free animals are characterized by a different and less complex gut immune system than normal animals, showing defects in gut-associated lymphoid tissue and antibody production[15]. On the contrary, immediately after exposure to luminal microbes, development of a complete and helpful immune system is stimulated by increase in the number of intraepithelial lymphocytes and production of both mucosal immunoglobulin in germinal centers than in serum[2,15]. It is known that cells of innate immunity are able to discriminate between pathogenic and harmless microbial components by "pattern recognition receptors". Among them, toll-like receptors, mainly present on macrophages, neutrophils, dendritic cells (DCs), intestinal epithelial cells, enable these cells to recognize typical molecules present on microorganism, like lipopolysaccharides, peptidoglycans, flagellins, described as pathogen-associated molecular patterns, or better, microbe-associated molecular patterns[16-19]. It is probably that different populations of DCs are responsible for induction of tolerance for commensal while stimulating immune response for pathogens, by differentiation of naïve T cells into either regulatory T cells or effectors cells indispensable for clearing infections[15,19-22]. Components of normal microflora thus induce in the gut a state of "physiological" inflammatory response, maintained by balanced and controlled responses[19].

Non-pathogenic bacteria can also avoid access of pathogen bacteria into intestinal lumen by attachment to the epithelial cells[2]. Microbiota can regulate the production of the mucins from intestinal goblet cells, thus limiting access to pathogens. Moreover, commensal bacteria compete for nutrient availability in ecological niches[15].

RATIONAL AND APPROACHES OF MODULATION OF INTESTINAL MICROBIOTA

It is now generally accepted that the intestinal flora plays a key role in maintaining the host's health status, while its alteration or a dysregulation of the intestinal

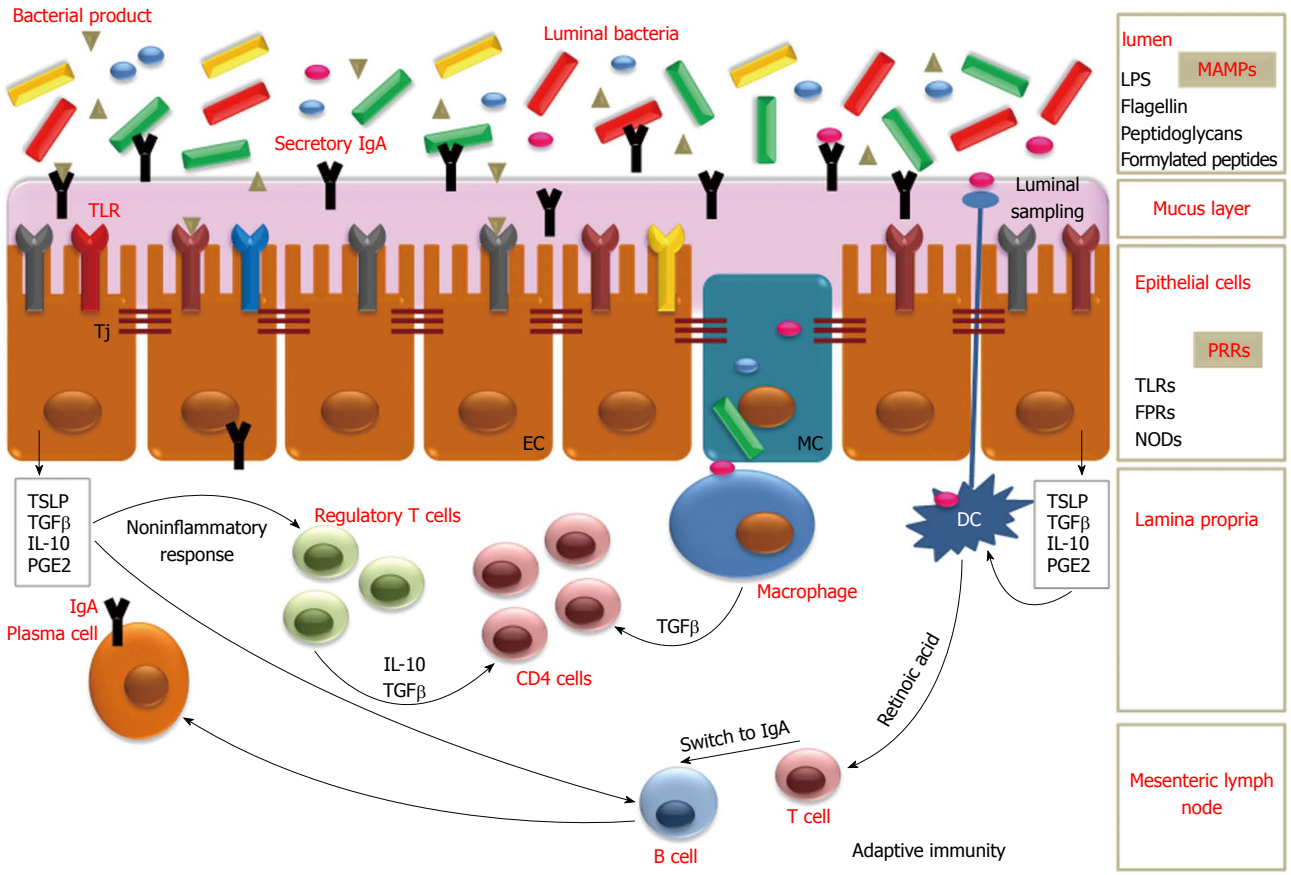

Figure 1 Interactions between luminal bacteria and intestine. The interaction between luminal bacteria and EC causes the production of cytokines, such as TGF-β, TSLP, IL-10 that may induce inflammatory cytokines synthesis from DC and mononuclear cells. These substances, together with retinoic acid produced by DC, can also promote the IgA switch in B cells. In Peyer's patches DC and macrophages uptake bacterial antigens. The TSLP produced by TLR signaling, enhance APRIL and BAFF production from DC. Anti-inflammatory response is mainly mediated by TGF-β production by epithelial cells and IL-10 from mononuclear cells. Moreover, the interaction with commensal bacteria can result in T-cell expansion and regulation of Th-cells. Probiotics may modulate intestinal function, and in particular mucosal immunity, by secreted factors and by direct interactions with immune cells and the intestinal epithelium. EC: Enterocytes; MC: M cells; Tj: Tight junctions; TGF: Transforming growth factor; TNF: Tumor necrosis factor; TSLP: Thymic stromal lymphopoietin; DC: Dendritic cells; TLR: Toll-like receptors; APRIL: A proliferation-inducing factor; BAFF: B-cell activating factor; IL-10: Interleukin-10; MAMPs: Microbial Associated Molecular Patterns; LPS: Lipopolysaccharide; PRRs: Pattern Recognition Receptors; FPRs: Formylated peptide receptors; NODs: Nucleotide-binding oligomerization domain-like receptors.

immune response to normal bacterial environment may significantly contribute to the pathogenesis of different inflammatory and autoimmune disorders[23,24]. As for example, broad-spectrum antibiotics may suppress components of normal microbiota, leading other pathogenic organisms to grow up and cause disease. Otherwise, normal cross-talk host-microbiota may be altered and the constituents of the normal intestinal flora may be recognized not as friends, thus triggering an inappropriate inflammatory response. Moreover, when gut mucosa is injured, the "leaky gut" lets bacteria to pass from the gastrointestinal tract through the epithelial layer to the submucosa and even to the circulation, potentially disseminating throughout the body and causing sepsis, shock and multisystem organ failure[2,23].

For all these reasons, in the last years, interest on the eventual role of selective modulation of microflora in inducing benefits in inflammatory intestinal disorders has been growing.

At this regards, different approaches have been investigated, mainly represented by probiotics, but also prebiotics, synbiotics, antibiotics, and, lastly, fecal transplantation.

Probiotics

Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the consumer"[25]. Probiotics include both bacteria and yeast. Most of probiotics include *Lactobacillus* species, *Bifidobacterium* species, *Escherichia coli* (*E. coli*), *Streptococcus* species, *Lactococcus lactis* and some *Enterococcus* species[26]. The most commonly used yeast is *Saccharomyces boulardii.* Probiotics must survive gastric acid and bile, so they can exert their effect in the small and large intestine[27,28]. Probiotics colonize the gut temporarily and act modifying colonic environment according to the fecal persistence of the ingested strains[28]. Different mechanisms are involved in the protective role of probiotics and are represented both by direct interaction with the host and by indirect modulation of

Table 1 Mechanisms of probiotics

Increase in barrier function
Maintenance of epithelial tight junctions integrity
Increase mucin production (Globet cells)
Increase in trefoil factors and defensins production (Paneth Cells)
Modulation of immune response
Increase secretory IgA
Production of anti-inflammatory cytokines and inhibition of pro-inflammatory cytokines
Promotion tolerogenic dendritic cells and regulatory T cells
Enhance of natural killer activity
Antagonism of pathogens
Direct killing of bacteria
Reduction of pathogen adherence
Inhibition of pathogenic bacteria growth by antimicrobial and antitoxin compound production (*i.e.*, SCFA, bacteriocins and microcins)
Production of substances
Production of enzymatic activities and/or beneficial metabolites to the host
Promotion pain relief in visceral hyperalgesia

SCFA: Short chain fatty acids.

the intestinal microbiota[29] (Figure 1 and Table 1).

These mechanisms include: (1) Enhancement of the natural gastrointestinal barrier function providing a physical barrier, also know as "colonization resistance"; in particular at level of: (a) tight junctions: probiotics can induce structural changes in epithelial tight junctions, mainly by upregulating the expression of zona-occludens 1, a tight junction protein, or by preventing redistribution of the other proteins, so stabilizing the intercellular integrity[27,30,31]; (b) mucus barrier: probiotics can increase mucin expression and secretion by goblet cells, thereby creating a mucus barrier for bacterial passage toward intestinal surface[32] and by trefoil factors and defensins produced by intestinal Paneth cells; (2) Modulation of the local and systemic immune responses; in particular by production of: (a) secretory IgA: probiotics can stimulate production of IgA in the lamina propria and secretion of IgA into the luminal mucous layer, thus limiting bacterial colonization by binding with their antigens[28]; (b) anti-inflammatory cytokines: probiotics may have many other immunomodulatory effects in the gut, helping to keep intestinal homeostasis. They can modulate the immune response, including promoting tolerogenic DCs and regulatory T cell phenotypes, improving activities of macrophages and NK cells, regulating the nuclear factor-κB (NF-κB) pathway, inducing the apoptosis of T cells, and reduce the secretion of proinflammatory factors[32-34]; (3) Antagonism of pathogens: some probiotics can directly antagonize pathogenic bacteria or their growth *via* expression of antimicrobial factors such as SCFA and "bacteriocins" or "microcins"[35]. SCFA can disrupt the outer membranes of gram-negative pathogens such as *Enterohemorrhagic E. coli*, *P. aeruginosa*, and *S. typhimurium*, causing inhibition of pathogen growth[34]. Bacteriocins can either permeabilize the inner membrane of gram-negative bacteria, leading to disruption, or interfere with cell wall synthesis and cause the formation of pores[34]. Finally, probiotics may decrease luminal pH, thus creating an inhospitable environment for pathogens[26-29]; and (4) production of helpful substances: some probiotics may produce enzymatic activities and/or beneficial metabolites for the host, may activate receptors in the enteric nervous system as to alter pain responses and promote pain relief in the setting of visceral hyperalgesia[28,29].

There is great variation in the number and combination (single or multiple strains) of probiotic organisms provided in various supplements. The effects of various probiotics may reflect species-specific properties; however, whether a probiotic mixture may yield more beneficial effects because of a synergistic action among the individual organisms, in respect to a single strain, is still matter of debate[27].

Prebiotics

Prebiotic are currently defined as "selectively fermented ingredients that result in specific changes in the composition and/or activity of the GI microbiota, thus conferring benefits upon host health"[36].

Some examples of prebiotics are dietary fiber (as for example arabinoxylan, a non-starch polysaccharide found in many cereal grains[37]) and some types of oligosaccharides, although only inulin-type fructans and galacto-oligosaccharides fulfill all the criteria for prebiotic classification[23,38]. Some polysaccharides can also be found in seaweeds and microalgae[36]. These food ingredients may modify the gut microbiota, mainly at the level of individual strain, selectively stimulating growth of health-promoting species already residing in the colon[23,39-41].

Because of their chemical structure and consequent lack of the host's capability to digest them, prebiotics are directly fermented in the colon by endogenous bacteria to SCFA[42], with consequent decrease of pH. By this process, they can exert antinflammatory effects, stimulating for example increase of T-regulatory cells (Treg) and reduction of IFN-γ[43,44]. Animal models also showed that some heteropolysaccharide (for example isolated from the fruit body of *L. edodes*) can restore the age-attenuated immune responses by increasing cytokine levels in peripheral blood[45]. Prebiotics can also inhibit the adherence of pathogens to gut epithelium, preventing them from translocating across the epithelial GI cells[36,37].

Finally, animals studies reported that prebiotics trigger an increase in the mucosa layer, with elongation of the microvilli, and an increase in the number of epithelial cells[36].

Synbiotics

About 20 years ago, the term "synbiotic" was firstly introduced to describe "a mixture of probiotics and prebiotics that beneficially affects the host by improving

the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare"[28,46]. Commonly combinations include *Bifidobacteria* and fructooligosaccharides (FOS), *Lactobacillus rhamnosus* GG (LGG) and inulin, and *Bifidobacteria* and *Lactobacilli* with FOS or inulin[28,46,47].

Antibiotics

Manipulation of the enteric microbial flora by means of antibiotics represents a possible therapeutic option[48]. The most used systemic antibiotics in intestinal disorders are represented by metronidazole and ciprofloxacin, efficacious respectively against anaerobic bacteria and some parasites, and Gram-positive e Gram-negative bacteria such as *E. coli* and *Enterobacteriacee*[49]. Nevertheless, use of antibiotics may have some disadvantages, since the occurrence of side effects, antibiotic resistance and *Clostridium difficile* (*C. difficile*) superinfection. Metronidazole is known to be associated with various untoward effects (peripheral neuropathy, metallic taste, gastrointestinal disturbances *etc*.) with a reported incidence of 50% or more[48,50]. Ciprofloxacin is better tolerated, but more expensive, and can induce nausea, diarrhea, skin rashes, with an increasing evidence of tendinitis and tendon rupture[48,51]. In the last years, growing interest has been reserved to rifaximin, a minimally absorbed (< 0.4%) antibiotic, associated with a more favourable safety/tolerability profile than systemic antibiotics[52]. Rifaximin has also demonstrated a broad spectrum antibiotic efficacy, in particular against both Gram-positive and Gram-negative aerobic and anaerobic intestinal bacteria, with minimal potential for development of bacterial resistance[52].

Fecal transplantation

Fecal microbiota transplantation (FMT) consists of the infusion of a fecal suspension from a healthy individual into gut of another one[53].

FMT can be performed by various way, that are nasogastric or nasoduodenal tube, colonoscope, enema, or capsule[54]. The suggested mechanisms of action include the competition for nutrients, the direct inhibition of growth of the pathogen, the modulation the immune system of the host by interaction with normal flora[52-55].

It has been speculated that FMT may be more effective than probiotics in the restoration of altered gut microbiota, since fecal infusion overcomes the intrinsic quantitative gap of probiotics by a durable alteration of the recipient's gut microbiota while probiotics are able to colonize the gut lumen only for a temporary period[56]. Molecular techniques demonstrated that intestinal flora of the host and the donor closely resembled about 2 wk after FMT and persisted for more than a month, with a predominance of *Bacteroides* spp.[57].

MODULATION OF MICROFLORA IN INTESTINAL INFLAMMATORY DISORDERS

Inflammatory bowel disease

The involvement of an aberrant immune response to intestinal flora in the pathogenesis of the inflammatory bowel disease (IBD), mainly in susceptible individuals, has been widely suggested[58]. Moreover, IBD is not seen in germ-free animals[26] and different studies reported a decline in bacterial diversity microbial in IBD subjects[58-60]. Nevertheless, disease-specific bacterial or fungal communities have not been identified, although *Mycobacterium paratuberculosis* and invasive *E. coli,* have been etiologically linked to Crohn's disease (CD)[60]. Pathogens may trigger intestinal inflammation in genetically predisposed individuals by disruption of the mucosal barrier and consequent increase of translocation of luminal antigens, mimicry of self-antigens, and activation of the mucosal immune system by modulation of transcription factors such as NF-κB[61].

An extensive literature supports the impact of various probiotics in experimental models of IBD[26,61-64]. Up to now, most of studies in humans were focused on induction of remission and prevention of relapse[65-90]. The strongest evidences derive from clinical trials conducted with VSL#3, a probiotic mixture containing 900 billion viable lyophilized bacteria represented by four strains of Lactobacilli (*L. casei, L. Plantarum*, *L. acidophilus, L. bulgaricus*), three of Bifidobacteria (*B. longuum, B. breve* and *B. infantis*), and one of *Streptococcus salivaris* subspecie *thermophilus*[69-71].

As regarding ulcerative colitis (UC), previous meta-analysis reported insufficient evidence about the efficacy of probiotics both for induction[86] or maintenance of remission[87], while the more recent data support their role[88-90] (Table 2). In particular VSL#3 was effective in maintaining remission in mild to moderately active disease, conversely than for moderate-severe disease[88-90]. Probiotics also showed similarity of efficacy when compared with mesalazine in preventing the relapse of disease[88-90].

Nevertheless, there are many limitations in the different meta-analysis linked to the heterogeneity of the cited studies, including variations in the selection of subjects, dosage, type and concentrations of the probiotics, duration of therapy, concomitant medications.

As for example, Kruis *et al*[75] found equivalent percentages of efficacy and safety in maintaining remission in 162 UC patients giving probiotic Escherichia coli Nissle 1917 (200 mg once daily) in respect to 165 patients giving mesalazine (500 mg three times daily) for 12 mo[75]. Twenty-nine newly diagnosed UC

Table 2 Role of probiotics in inflammatory bowel diseases

Ref.	No. (intervention/control)	Probiotic	Control	Results
Remission induction in UC				
Kato *et al*[68]	20 (10/10)	*B. breve*, *B. bifidum*, *L. acidophilus* + 5ASA/SASP	Placebo	slight ↑
Tursi *et al*[69]	90 (30/60)	VSL#3 + balsalazide	Balsalazide alone 5ASA	↑
Miele *et al*[70]	29 (14/15)	VSL#3	Placebo	↑
Sood *et al*[71]	147 (77/70)	VSL#3	Placebo	↑
Matthes *et al*[72]	57 (46/11)	*E. coli* Nissle 1917	Placebo	↑
Tursi *et al*[73]	144 (71/73)	VSL#3	Placebo	↑
Maintenance of remission				
Kruis *et al*[74]	103 (50/53)	*E. coli* Nissle 1917	5ASA	Similar efficacy
Kruis *et al*[75]	327 (162/165)	*E. coli* Nissle 1917	5ASA	Similar efficacy
Zocco *et al*[76]	187 (127/60)	*Lactobacillus* GG *Lactobacillus* GG + 5ASA	5ASA	Similar efficacy (LGG more effective than 5ASA alone in prolonging relapse free time)
Miele *et al*[70]	29 (14/15)	VSL#3	Placebo	↑
Wildt *et al*[77]	32 (20/12)	*L. acidophilus* La5, *B. animalis* subs *P. lactis* BB-12	Placebo	Similar effect
Maintenance of remission in Pouchitis				
Gionchetti *et al*[78]	40 (crossover)	VSL#3	Placebo	↑
Gionchetti *et al*[79]	40 (20/20)	VSL#3	Placebo	↑
Mimura *et al*[80]	36 (20/16)	VSL#3	Placebo	↑
Maintenance of remission in CD				
Prantera *et al*[81]	45 (23/22)	*Lactobacillus* GG	Placebo	Similar effect
Bousvaros *et al*[82]	75 (39/36)	*Lactobacillus* GG	placebo	Similar effect
Raju *et al*[83]	90 (43/47)	*L. johnsonii*	placebo	Similar effect
Van Gossum *et al*[84]	70 (34/36)	*L. johnsonii*	placebo	Similar effect
Induction of remission in CD				
Steed *et al*[85]	24 (13/11)	*B. longum*, Synergy 1 + conventional therapy	Placebo + conventional therapy	Similar effect

All article are randomized controlled trials. 5ASA: Mesalazine; UC: Ulcerative colitis; CD: Crohn's disease.

children were randomized by Miele *et al*[70] to receive either VSL#3 (weight-based dose, range: 450-1800 billion bacteria/day) or placebo together with steroid (induction period) or mesalamine (remission period). Results showed achievement of remission in 13 patients (92.8%) treated with VSL#3 *vs* 4 patients (36.4%) treated with placebo[70]. Relapse within 1 year of follow-up occurred in 3 of 14 (21.4%) patients on probiotic *vs* 11 of 15 (73.3%) patients on placebo[70]. Sood *et al*[71] enrolled adult patients with mild-to-moderate UC who were randomly given 3.6×10^{12} CFU VSL#3 (n = 77) or placebo (n = 70), twice daily for 12 wk, showing that both at week 6 than week 12, improvement in clinical score was significantly higher in the probiotic group (25; 32.5%) than in the placebo group (32.5% *vs* 10% and 42.9% *vs* 15.7%, respectively)[71]. In conclusion, these data are interesting and encouraging, but well-designed randomized controlled trials are still limited about this topic and further works are still warranted to support these promising results

Patients undergoing ileo pouch-anal anastomosis for UCs may develop pouchitis up to 50% of cases[91]. Only a few studies have been published about the use of probiotics in the treatment of active pouchitis. Instead many studies are available on the assessment of the potential of probiotics in the prevention of relapses. For this indication the most studied is the multispecies preparation VSL#3[26,75-79] (Table 2).

Gionchetti *et al*[79] studied 40 patients with ileal pouch-anal anastomosis receiving VSL#3 (n.20) or placebo (n.20), reporting occurrence of pouchitis in 10% of patients giving probiotic *vs* 14% patients receiving placebo. In the study by Mimura *et al*[80] on 36 patients with pouchitis, randomly assigned to receive VSL#3 6 g (20 patients) or placebo, maintenance of remission after one year was achieved in 17 patients (85%) on VSL#3 and in one patient (6%) on placebo[80]. Protective role of probiotics *vs* placebo in the management of pouchitis was also confirmed in a meta-analysis by Elahi *et al*[92] and Nikfar *et al*[93]. Based on these results, also confirmed by European guidelines[94], use of VSL#3 is suggested for the prevention and the maintenance of remission of pouchitis[94].

In contrast to the encouraging findings in UC, data on probiotics in CD patients are still disappointing[22,58,81-85,89,90] (Table 2). Butterworth *et al*[95] in the Cochrane Collaboration review concluded for not sufficient evidences supporting role of probiotics in inducing remission in CD[95]. These findings were confirmed in meta-analysis by Rahimi *et al*[96], as well as, later,

by Fujiya *et al*[89] and Shen *et al*[90]. The most of the analyzed studies showed no significant effects on either the induction or maintenance of remission in CD. In their meta-analysis, Shen *et al*[90] proposed that the different efficacy of probiotics between UC and CD patients may be explained by the dissimilar inflammatory pattern in the two conditions. For example an impaired production of the regulatory cytokine interleukin (IL)-10 (not influcenced by probiotics) has been showed in CD4+ T cells of CD patients, as well as presence of circulating antibodies against bacterial antigens and deficiency in human defenses[90].

In conclusion, to date, probiotics have some efficacy in UC and in pouchitis, while disappointing results are available for CD.

Evidences regarding the use of prebiotics for IBD therapy are less stronger than for probiotics[58]. The efficacy of prebiotics in IBD, showed in *in vitro* and animal models[97,98], was investigated only in few human studies involving a small number of patients[99,100]. In 2002, Bamba *et al*[100] reported that germinated barley foodstuff (at doses of 20-30 g of Germinated Barley Foodstuff daily) may induce remission in 11 patients with mild to moderate active UC in respect to 7 patients receiving standard therapy[100]. However, sample study was too small to draw any final conclusion. In a more recent study[101], 103 CD patients were treated with 15 g/d FOS or placebo for 4 wk. Results showed no clinical improvement in patients on prebiotics, although differences in inflammatory pattern of lamina propria DCs, with reduced of levels of IL-6-in and increased IL-10 were described[101].

As regarding patients with pouchitis, Preidis *et al*[101] demonstrated that inulin ingestion may induce increased level of butyrate, lower concentration of *Bacteroides fragilis* and reduced pH and bile acids.

As for prebiotics, also for role of synbiotics in IBD, there are only few available and inconclusive studies[67,101,102]. It has been speculated that enteral nutrition may influence composition and activity of the gut microbial flora, but it deserves further exploration[103,104]. Leach *et al*[103] found in six CD children that enteral nutrition may significantly change intestinal bacterial composition, when compared with controls. Tjellström *et al*[104] found that 79% of the 13 children with small bowel/colonic CD responded clinically positively to enteral nutrition treatment, by a modulation of gut microflora activity with decreased levels of pro-inflammatory acetic acid as well as increased concentrations of anti-inflammatory butyric acids and also of valeric acids.

In patients with IBD, antimicrobial therapy, mainly by ciprofloxacin and metronidazole, is usually reserved for those with septic complications[105]. As regarding human studies, data on antibacterial agents, such as oral vancomycin and intravenous metronidazole in UC subjects, are limited and results are still controversial[64].

Also for pouchitis, available data are not conclusive[64,93,106]. As for example, a single study by Shen *et al*[107] showed that therapy with either metronidazole (1000 mg/d) or ciprofloxacin (20 mg/kg per day) for 2 wk induced a significant improvement in clinical and endoscopic scores of pouchitis, while a meta-analysis by Nikfar *et al*[93] showed that antibiotic were not significantly more effective than placebo in managment of pouchitis.

The role of antibiotics in uncomplicated CD has not been clearly demonstrated[49]. Different randomized trials have shown that metronidazole is efficacious in inducing remission in patients with active CD with colic and ileo-colic localization[49,108,109], while results on ciprofloxacin, alone or in association with metronidazole, in patients with active CD, remain uncertain[49,110-115]. A recent meta-analysis by Khan *et al*[116] suggested role of antibiotcs as primary therapy of CD, although the 2010 "Consensus on the diagnosis and management of Crohn's disease", did not recommend their use except for the treatment of septic complications, symptoms linked to bacterial overgrowth, or perianal disease[117].

Only few data are available for FMT in IBD[57]. Patients with refractory UC may benefit from FMT, although multiple infusions seem to be required. Nevertheless, only few cases have been reported yet and large clinical trials are lacking[57]. Moayyedi *et al*[118] in the first randomized trial demonstrate efficacy of FMT in UC. FMT induces remission in a significantly greater percentage of patients with active UC than placebo, with no difference in adverse events. Fecal donor and time of UC appear to affect outcomes. Wei *et al*[119] in an uncontrolled pilot clinical trial in fourteen IBD patients (11 UC and 3 CD) demonstrate that FT improves quality of life in patients with IBD. In conclusion, available data are too scanty and further and well-designed study are necessary.

Infectious diarrhea

Different probiotics were tested in regard to their efficacy for preventing nosocomial diarrhea and, in particular, rotavirus-associated diarrhea[101]. Most of data derived from pediatric studies. A systematic review of 23 RCTs, involving a total of 1917 participants[120], reported beneficial effects of probiotics in reducing the duration of acute gastroenteritis compared with placebo. Three RCTs (including 1043 children) tested LGG supplementation showing a significant lower incidence of nosocomial rotavirus diarrhea, with a concomitant shortening of the duration of the illness[121-123]. Moreover, a recent meta-analysis including 15 RCTs (2963 participants) showed that LGG significantly reduced the duration of diarrhoea when compared with placebo or no treatment, mainly when used at a daily dose $\geqslant 10^{10}$ CFU than at a daily dose $< 10^{10}$ CFU[124]. Also a meta-analysis by Salari *et al*[125] showed that probiotics decrease the duration of diarrhea and fever significantly in children, while these results were not confirmed in adults' diarrhea, amoebiasis, *C. difficile*-associated diarrhea, diarrhea in HIV positive patients, radiation-induced diarrhea, and chemotherapy-induced diarrhea[125]. On the other hand, few other trials[126,127] did not show any benefit. Taken together, these findings

may suggest that that not all probiotics are equally effective for preventing nosocomial diarrhea.

Therefore, up to now, probiotic use may be advised in acute gastroenteritis, mainly rotavirus-associated diarrhea, while it is not recommended for critically ill hospitalized patients in prevention of nosocomial infections.

Clinical studies of both prebiotic and synbiotic preparations in the treatment of specific acute gastroenteritis are limited. One prospective RCT enrolling 496 children in day care centres tested a synbiotic blend of *Bifidobacterium lactis* oligofructose, and acacia gum, showing a 20% reduction in the number of days with diarrhea[128].

Further studies are necessary to confirm these preliminary results.

Antibiotic treatment strategies are limited, can worsen clinical course of disease, exacerbating toxin-mediated effects. For treatment of travelers' diarrhea (TD), the Infectious Diseases Society of America (IDSA) guidelines recommended three antibiotics, that are the fluoroquinolones, rifaximin, and azythromicyn[129]. Nevertheless, there is a growing concern about using of systemic antibiotics since the occurrence of side effects; therefore, increasing evidences support the use of rifaximin, which is currently recommended for the empiric treatment of afebrile, non-dysenteric forms of TD[101,129].

Antibiotic-associated diarrhea

Antibiotic-associated diarrhea (AAD) and *C. difficile*-associated diarrhea (CDAD) are common complication linked to antibiotic use, mainly cephalosporins, clindamycin, broad-spectrum penicillins and fluoroquinolones[130].

A large systematic review and meta-analysis on role of probiotics in preventing AAD, including 63 randomized clinical trials and involving almost 12000 participants, showed a significant reduction in AAD in the probiotic groups compared with the control group[131]. However, heterogeneity in effectiveness among the patients, differences in the antibiotics and probiotic strains should be considered.

In more recent meta-analysis, Szajewska *et al*[132] evaluated the efficacy of LGG in preventing AAD in children and adults. They showed significant results only in children and in the subgroup of adult patients receiving antibiotics for *Helicobacter pylori* eradication. Also in this work, some questions remained answered, as the best treatment regimens, duration of LGG therapy and the optimal dose of LGG for preventing AAD[132].

Moreover, since AAD does not occur in the majority of patients and when it occurs, it is usually self-limiting, identifying populations most likely to benefit from adjunct probiotics should be desirable.

At this regards, only a small number of RCTs were performed on elderly participants. In a double-blind RCT of 135 hospital elderly patients, administration of *L. casei*, *L. bulgaricus*, and *S. thermophilus*, in combination with antibiotics and for one week after cessation, significantly reduced the risk of developing antibiotic-associated diarrhea[133].

Otherwise, Ehrhardt *et al*[134] in a multicenter, randomized, placebo-controlled trial, showed no beneficial effect of *S. boulardii* in preventing AAD or CDAD in a population of hospitalized patients giving systemic antibiotics. As the same, the large PLACIDE trial did not find a lower incidence of AAD or CDAD in elderly inpatients receiving supplementation with Lactobacilli and Bifidobacteria[135].

Finally, a recent meta-analysis by Xie *et al*[136], showed that probiotics may not reduce the risk of AAD and *C. difficile*-associated diarrhea in older patients, while Szajewska *et al*[137] showed that *S. boulardii* significantly reduces the risk of diarrhoea in patients treated with antibiotics in general, also in those of extreme ages. However, since *S. boulardii* can cause fungaemia, particular concern is required for more susceptible populations[137].

In conclusion, although available data on probiotics are encouraging, a more prudent and selected use of antibiotics represents the best strategy of preventing AAD.

Antibiotic use can lead to dysbiosis (microbial imbalance), and this allows *C. difficile* to flourish[138]. Up to now, studies documenting a reduction in *C. difficile*-associated diarrhea (CDAD) by probiotics are far fewer and remain the subject of controversy[65]. A series of systematic reviews showed promising but no definite results[125,139]. In particular, Johnston *et al*[139], in a meta-analysis including twenty trials and 3818 participants, concluded that moderate-quality evidence suggests probiotic prophylaxis as strategy in reducing CDAD without an increase in clinically important adverse events.

Waiting for more consistent data, actual clinical practice guidelines do not recommend the use of probiotics for the primary prevention of *C. difficile* infection (CDI)[27,65,101].

As regarding prebiotics and synbiotics in preventing CDAD, only few and RCT are available and data are not consistent to support this strategy[140,141].

In the last years, FMT has been recognized as an efficacious treatment for recurrent *C. difficile* infection (rCDI) by remodulation of intestinal microflora *via* donor feces. Patients with rCDI showed reduced levels fecal of *Bacteroidetes* and *Firmicutes* as compared to patients with just one episode of CDI or antibiotic-associated diarrhoea[57]. There has been an average 87%-90% cure rate (defined by resolution of diarrhea) for the over 500 cases that have been reported in the literature[142,143].

A systematic review on 36 studies evaluating a total of 536 patients receiving with FMT for CDI, showed that 467 (87%) experienced resolution of diarrhea following the first FMT procedure. The higher

Table 3 Role of probiotics in irritable bowel syndrome

Ref.	Diagnostic criteria	No. participant	Probiotic	Time (wk)	Results (*vs* placebo)
O'Sullivan *et al*[147]	Rome	25	*Lactobacillus* GG	20	No difference
Nobaek *et al*[148]	Rome	60	*L. plantarum*	4	↓ pain and flatulence
Kim *et al*[149]	Rome Ⅱ	25	VSL#3	8	↓ bloating
O'Mahony *et al*[150]	Rome Ⅱ	80	*L. salivarius* UCC4331, *B. infantis* 35624	8	↓ symptoms and pro-inflammatory cytokines (only for *B. infantis*)
Kajarder *et al*[151]	Rome/Rome Ⅱ	103	Mixture of *L. rhamnosus* GG, *L. rhamnosus* LC705, *B. breve* Bb99, *P. freudenreichii*	24	↓ symptoms
Kim *et al*[152]	Rome Ⅱ	48	VSL#3	8	↓ flatulence
Niv *et al*[153]	Rome Ⅱ	54	*L. reuteri* ATCC55730	24	No difference
Whorwell *et al*[154]	Rome Ⅱ	362	*B. infantis* 35624	4	↓ symptoms (only at dose of 1×10^8 CFU)
Guyonnet *et al*[155]	Rome Ⅱ (C-IBS)	274	*B. animalis* DN173010	4	↑ QoL and bloating score
Kajander *et al*[156]	Rome Ⅱ	86	*L. rhamnosus* GG, *L. rhamnosus* Lc705, *P. freudenreichii* sp *P. Shermanii* JS, *B. animalis* sp *P. lactis* Bb-12	20	↓ bloating and abdominal pain
Zeng *et al*[157]	Rome Ⅱ (D-IBS)	29	*S. thermophilus*, *L. bulgaricus*, *B. longum*	4	↓intestinal permeability and symptoms
Drouault-Holowazcz *et al*[158]	Rome Ⅱ	100	*B. longum* LA101, *L. acidophilus* LA102, *Lactocuccus lactis* LA103, *S. thermophilus* LA104	4	↑ QoL ↓ flatulence, pain and bloating
Sinn *et al*[159]	Roma Ⅲ	40	*L. acidophilus* SDC 2012, 2013	4	↓ symptoms
Simrén *et al*[160]	Rome Ⅱ	74	*L. paracasei* F19, *L. acidophilus* La5, *B. lactis* Bb-12	8	No difference
Søndergaard *et al*[161]	Rome Ⅱ	52	*L. paracasei* F19, *L. acidophilus* La5, *B. lactis* Bb-12	8	No difference
Guglielmetti *et al*[162]	Rome Ⅲ	122	*B. bifidum* MIMBb75	4	↓ symptoms ↑ QoL
Ducrotté *et al*[163]	Rome Ⅲ (D-IBS)	214	*L. plantarum* 299v	4	↓ pain and bloating
Kruis *et al*[164]	Rome Ⅱ (D-IBS)	120	*E. coli* Nissle 1917	12	No difference
Ki Cha *et al*[165]	Roma Ⅲ (D-IBS)	50	*L. acidophilus*, *L. plantarum*, *L. rhamnosus*, *B. breve*, *B. lactis*, *B. longum*, *S.thermophilus*	8	↑ QoL
Dapoigny *et al*[166]	Rome Ⅲ	50	*L. caseirhamnosus*	4	↓ symptoms
Roberts *et al*[167]	Rome Ⅲ	179	*B. lactis* CNCMI-2494	12	No difference
Shavatehi *et al*[168]	Rome Ⅱ	160	*Lactobacillus*, *Bifidobacterium strains* and *Streptococcus thermophiles*	2	No difference
Yoon *et al*[169]	Rome Ⅲ	80	*L. acidophilus*, *L. rhamnosus*, *B. breve*, *B. actis*, *B. longum*, *S.thermophilus*	4	↓ symptoms
Pineton de Chambrun *et al*[170]	Rome Ⅲ	179	*S. cerevisiae*	8	↓ pain and discomfort

All the studies are randomized controlled trials *vs* placebo. QoL: Quality of life.

response rate (93%) was achieved by fecal infusion *via* colonoscopy[56]. The literature to date supports FMT for use in CDI as a safe, well-tolerated, effective treatment with few adverse events[144].

Irritable bowel syndrome

Evidences suggest alterations in gut microbiota in patients with irritable bowel syndrome (IBS)[132] with a dysbiosis of the luminal and mucosal colonic microbiota that is frequently characterised by a reduction in species of Bifidobacteria[145].

This is also supported by the hypothesis that IBS may be induced by bacterial gastroenteritis (postinfectious IBS)[23]. Therefore, targeting the intestinal microbiota for the treatment of this condition has been hypothesized as a valid approach.

Recent pre-clinical data on mouse reported beneficial effects of probiotics on visceral nociceptive reflexes[23,146]. As regarding human studies, different probiotic species have been shown to improve individual symptoms in IBS, such as bloating, flatulence, and constipation, but only a few products affect pain and global symptoms[147-170]. Results of more relevant studies were summarized in Table 3.

Results of meta-analyses were still controversial, mainly since the clinical and statistical heterogeneity. Demographic features of the study populations, length of follow up and dosage and type of probiotics used were very different among the reviewed studies. Moreover, clinical score was evaluated by different scales.

In a meta-analysis by Nikfar *et al*[171] including 8 randomized placebo controlled clinical trials, probiotics were reported to significantly increase clinical improvement compared to placebo[171]. One large systematic review including 19 studies on IBS patients concluded that probiotics significantly improved clinical outcomes[172]. This finding was confirmed by Moayyedi *et al*[173], who reviewed 19 RCT performed in 1650 patients with IBS, and Didari *et al*[174], who analyzed 1793 IBS patients.

On the other hand, two trials and a meta-analysis

by Brenner *et al*[175] in adults did not show clinical benefit above placebo[160,176]. Nevertheless, it is possible that a subgroup of IBS subjects, such as those who developed the disease following gastroenteritis or an antibiotic course, show better beneficial effects by probiotics, as reported in a recent study involving 120 IBS patients[164], thus supporting the hypothesis of a greater efficacy of probiotics in patients with a dysbiosis as main plausible cause of their IBS[177].

In conclusion, probiotics seem to have a beneficial therapeutic role in IBS patients if administered accurately, although these data need to be confirmed by further well-designed trials. Recent United Kingdom guidelines formulated by the British Dietetic Association have made strain specific recommendations; in particular *Bifidobacteria lactis* DN 173010 showed limited weak evidence in improving overall symptoms, abdominal pain and urgency in constipation-predominant IBS, while VSL#3 showed limited weak evidence in reducing flatulence[178].

Few studies evaluated role of prebiotics for IBS. Few available data suggest that higher doses of prebiotics may have a negative impact on symptoms, maybe because increase of luminal gas production following fermentation and consequent worsening of intestinal symptoms[179]. Otherwise, some recent studies suggest that prebiotics may have some benefits in IBS[180,181]. Nevertheless, there are insufficient prospective and adequately powered studies to permit definitive conclusions to be drawn[23].

Also for synbiotics there are promising but too scanty results in IBS management[182].

Based on the hypothesis that alteration of intestinal microbiota could represent a cause of IBS, rifaximin, a nonabsorbable antibiotic, has been studied in IBS subjects and now represents the only antibiotic with a proven long-term benefit in these subjects[101,183]. Two double-blind, placebo-controlled trials (TARGET 1 and TARGET 2), involving more than 1200 patients with IBS (without constipation), showed that treatment with rifaximin for 2 wk provides significant relief of IBS symptoms[183]. It is conceivable that rifaximin exerts its effect by reducing bacterial products that negatively influence the host, or by reducing local immune responses of the host, or both of them[183].

FMT used in the treatment of IBS has been reported in some clinical studies and mainly in case series. Conclusions on the effectiveness cannot be made because the available data are limited and subject to bias[142].

Other conditions

Intestinal bacteria could play a role in initiation of colorectal cancer (CRC) through production of carcinogens, cocarcinogens, or procarcinogens[2,184]. Up to now, only a human study showed reduction of recurrence of adenoma atypia after 4 years of *Lactobacillus casei* administration[185]. Also for prebiotic and synbiotics, studies in CRC patients are not only insufficient but, in several aspects, inconclusive[186]. Therefore, available data are too scanty to draw any final conclusion.

Radiotherapy and chemotherapy kill replicating cells in small and large intestine. Probiotics can reduce side effects of these therapies, with some successful results in mice and animals[187-191] and also some trials on patients undergoing radiation and chemo therapy showed a reduction of radiation induced diarrhea and bowel toxicity at the end of the treatment[188-191]. Also in allergic disorders alterations of gut microbiota have been reported, with an imbalance between "beneficial" and potentially harmful bacteria, dysbiosis is not only a consequence but also a cause of allergy[192]. It has been reported that children with food allergies are found to exhibit, *i.e.*, decreased *Lactobacilli*, *Bifidobacteria* and *Enterococcus* species and increased coliforms, *Staphylococcus aureus* and *Clostridium* species, suggesting that microbial inhabitants of the human body, may play either a pathogenic or protective role in allergies[192].

Probiotics have been studied as possible dietary interventions for allergic disorders, although the majority of studies have evaluated eczema as the main outcome rather than food or other allergies[192-194]. However, recently, the World Allergy Organization stated that "probiotics do not have an established role in the prevention or treatment of pediatric allergy. No single probiotic supplement or class of supplements has been demonstrated to efficiently influence the course of any allergic manifestation or long-term disease or to be sufficient to do so"[195].

For prebiotics and synbiotics, very few and not definitive results are available.

Intestinal bacteria are essential for the development of NSAID-induced small bowel lesions, since "germ-free" animals were found to be resistant to indomethacin injuries[196]. Moreover, NSAID ingestion may modify composition of intestinal flora, inducing the overgrowth of Gram-negative and anaerobic bacterial species, responsible for lower mucosal defense and an increase susceptibility to damage[197,198].

Therefore, modulation of intestinal flora could provide protection against NSAID-induced enteropathy, as already reported *in vitro* studies[199,200]. So far, only few studies have been performed in humans and results are not all concordant[201-204]. Gotteland *et al*[202] found in 16 healthy volunteers that the ingestion of live LGG may preserve the integrity of the gastric mucosal barrier against indomethacin, but has no effect at intestinal level. More recently, in a double-blind, cross-over trial on twenty healthy volunteers receiving indomethacin, the concomitant ingestion of a probiotic mixture (VSL#3) reduced small bowel inflammation, evaluated by fecal calprotectin concentrations[203]. Later, in a randomized, controlled trial on 35 chronic users of low-dose enteric-coated aspirin (100 mg daily) plus omeprazole (20 mg daily), the Authors reported a

significant decrease in the number of mucosal breaks evaluated by videocapsule endoscopy in the *L. casei* group than in the control group[204]. Nevertheless, as stated in a recent review on this topic, current evidences supporting the role of probiotics in NSAID enteropathy are promising, but still weak e further and larger studies are necessary[14]. No significant data are available as regarding prebiotics, synbiotics and other therapeutic approaches targeting intestinal microflora in NSAID enteropathy.

CONCLUSION

Gut microbiota has a pivotal role in inducing a state of "physiological", balanced and controlled, inflammatory response. Alterations of intestinal bacterial flora, or a dysregulation of the intestinal immune response to normal bacterial environment, may significantly contribute to the pathogenesis of different inflammatory and autoimmune disorders. For all these reasons interest is rising on the eventual role of selective modulation of microflora in inducing benefits in inflammatory intestinal disorders, mainly by probiotics, prebiotics, synbiotics, antibiotics, and, lastly, by fecal transplantation.

Most of larger and well-conducted studies, including review and meta-analysis, evaluated the role of probiotics in different gastrointestinal disease. However, several limitations are important to consider in interpreting the results, since the great heterogeneity in probiotic strains, dosage, age groups, treatment lengths, and outcomes.

Probiotics have been demonstrated of some efficacy in induction and maintaining of remission in UC and in particular in pouchitis, while disappointing results are available for CD. Moreover a definite role of antibiotics has been established for treatment of pouchitis. It is possible that probiotic supplementation may significantly reduce rates of rotavirus diarrhea. Efficacy of probiotic in IBS is still controversial, while a significant benefit has been reported for rifaximin in IBS patients without constipation. For food allergic disorders, modulation of intestinal microflora is not convincing and use of probiotics and prebiotics is not recommended. The role of probiotics in NSAID enteropathy is promising but still weak. Finally, FMT has been recently recognized as an efficacious treatment for recurrent *C. difficile* infection but these finding need to be confirmed by larger studies.

In conclusion, while representing a very interesting therapeutic target, modulation of intestinal flora still deserves some doubts and limitations. Firstly, the actual gut microbiota composition in healthy individuals is still unclear, since high cost for more specific methods of characterization and the lack of a standardization. Then, despite increasing evidences for probiotics, intestinal bioavailability of bacterial strains, the optimal dose, and treatment time are still matter of debate.

It is conceivable that future studies will provide a better gut microbiota characterization, leading to a more selective modulation by the above described means, that could improve its composition and, consequently, the host's health status.

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REVIEW

Changes in cellular mechanical properties during onset or progression of colorectal cancer

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Abstract

Colorectal cancer (CRC) development represents a multistep process starting with specific mutations that affect proto-oncogenes and tumour suppressor genes. These mutations confer a selective growth advantage to colonic epithelial cells that form first dysplastic crypts, and then malignant tumours and metastases. All these steps are accompanied by deep mechanical changes at the cellular and the tissue level. A growing consensus is emerging that such modifications are not merely a by-product of the malignant progression, but they could play a relevant role in the cancer onset and accelerate its progression. In this review, we focus on recent studies investigating the role of the biomechanical signals in the initiation and the development of CRC. We show that mechanical cues might contribute to early phases of the tumour initiation by controlling the Wnt pathway, one of most important regulators of cell proliferation in various systems. We highlight how physical stimuli may be involved in the differentiation of non-invasive cells into metastatic variants and how metastatic cells modify their mechanical properties, both stiffness and adhesion, to survive the mechanical stress associated with intravasation, circulation and extravasation. A deep comprehension of these mechanical modifications may help scientist to define novel molecular targets for the cure of CRC.

Key words: Colorectal cancer; Biomechanics; Pressure; Mechanical signalling; Atomic force microscopy; Wnt

Core tip: Physical forces, either within tissues or externally applied, affect all tissues of the body. Cell mechanotransduction converts such forces into cellular responses that affect gene expression, protein synthesis, proliferation and morphogenesis. Here, we focused on recent studies covering the impact of physical stimuli such as compression, shear stress, adhesion and stiffness, in the development of colorectal cancer. We highlight that such stimuli play a major role in the tumor progression, affecting the Wnt pathway, being involved in the differentiation of non-invasive

cells into metastatic variants and helping metastatic cells to survive the mechanical stress associated with intravasation, circulation and extravasation.

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INTRODUCTION

Colorectal cancer (CRC) is the 3th most commonly diagnosed malignancy and the 4th cause of cancer death in the world, with approximately 1.4 million new cases and almost 700000 deaths in 2012. Its burden is expected to increase by 60% by 2030[1].

CRC development is a multistep process that results from genetic alterations that underlie the transformation of normal cells into malignant cells, conferring them growth advantages such as anomalous multiplication, self-sufficiency with respect to growth signals, insensitivity to growth-inhibitor signals and evasion of apoptosis[2].

The earliest mutations that occur in CRC are usually in components of the Wnt pathway that regulates colon cell homeostasis, being involved in the control of cell proliferation, differentiation and adhesion (Figure 1). A recently published genetic study performed on 224 colorectal tumours indeed confirmed that in 94% of cases a mutation in one or more members of the Wnt signalling pathway is detected[3]. Subsequent mutations occur at the level of the RAS-MAPK, P13K, TGF-β, p53 and DNA mismatch-repairs pathways[4].

These genetic mutations are accompanied by changes in the behaviour of cells which result in deep structural and biomechanical alterations that may occur at the tissue level, such as crypt buckling[5-19] or to more subtle modifications occurring at the cellular level[5,6,20-27] and in the extracellular matrix (ECM)[2,28]. Such modifications are not only a mere consequence of genetic alterations. In fact, there is a growing consensus that an evolving balance between mechanical and genetic cues exists and plays a key role in the genesis and the development of malignancies[2,29-41]. Indeed while the malignant potential is mainly dictated by the intrinsic genetic state of the cells, the tumour phenotype is regulated by a complex interplay between the biomechanical and biochemical properties of the cellular constituents and the ECM, which synergistically alters cellular behaviour stimulating migration, invasion, proliferation and survival[42].

During colorectal cancer development, cells within tissues are exposed to a highly heterogeneous and continuously evolving mechanical landscape. To provide a more in-depth understanding of this complex mechanical behaviour, a large number of studies have focused on isolated cell lines cultured in well-defined *in vitro* systems where each biomechanical cue, such as compression[6,20,21,24,43,44], ECM stiffness[24,25,45-48], flow conditions could be precisely controlled[26,27,49-51]. These *in vitro* studies opened the way to more advanced *in vivo* studies showing how biomechanical cues contribute to the malignant behaviour of colon epithelium by activating detrimental biochemical and genetic signalling pathways[5,42].

In this review, we focus on the most recent studies investigating the role of the biomechanical signals in the development of colorectal cancer. A particular attention is paid to highlight how the modifications of the tumour microenvironment and the extracellular matrix actively contribute to this process. A deep comprehension of the mechanism by which the mechanical cues modulate the onset and the development of the pathology may help to define novel molecular targets for the cure of colorectal cancer.

MECHANICAL SIGNALS CONTRIBUTE TO SHAPE HEALTHY COLON CRYPTS THROUGH A STRESS-RELAXATION MECHANISM

The epithelial layer of the human colon consists of a single sheet of columnar epithelial cells, which are arranged into finger-like invaginations in the underlying connective tissue of the lamina propria forming crypts, the basic functional unit of the intestine[52]. Three different types of cells are found in the epithelium, the goblet cells (secreting mucin into the crypt and intestinal lumen), the enterocytes and the neuroendocrine cells. The base of the crypts contains stem cells, which proliferate continuously producing transit cells, which divided several times before differentiating into the different type of cells that constitute the epithelium[53,54].

Crypt development occurs approximately seven days after birth in mice; before to this, the intestinal wall is smooth[53]. However, the mechanism through which these structures are formed is still not fully understood. It has been hypothesized that crypt growth could be regarded as a stress-relaxation phenomenon. Similarly to what happens with solid inorganic materials, where a tensile layer is coupled with a compressive one[55,56], the epithelial layer coating the intestinal wall might induce compressive residual stress in a tissue that can in turn be relaxed *via* a buckling instability, which can triggers the formation of crypts[18,57].

The above-described phenomenon has been investigated by using continuous mechanics. Edwards and Chapman[18] modelled a cross-section of an unfolded (smooth) colorectal crypt as a beam connected to the underlying tissue by a series of viscoelastic springs. This model was able to predict that an increase in the cellular proliferation rate can initiate buckling.

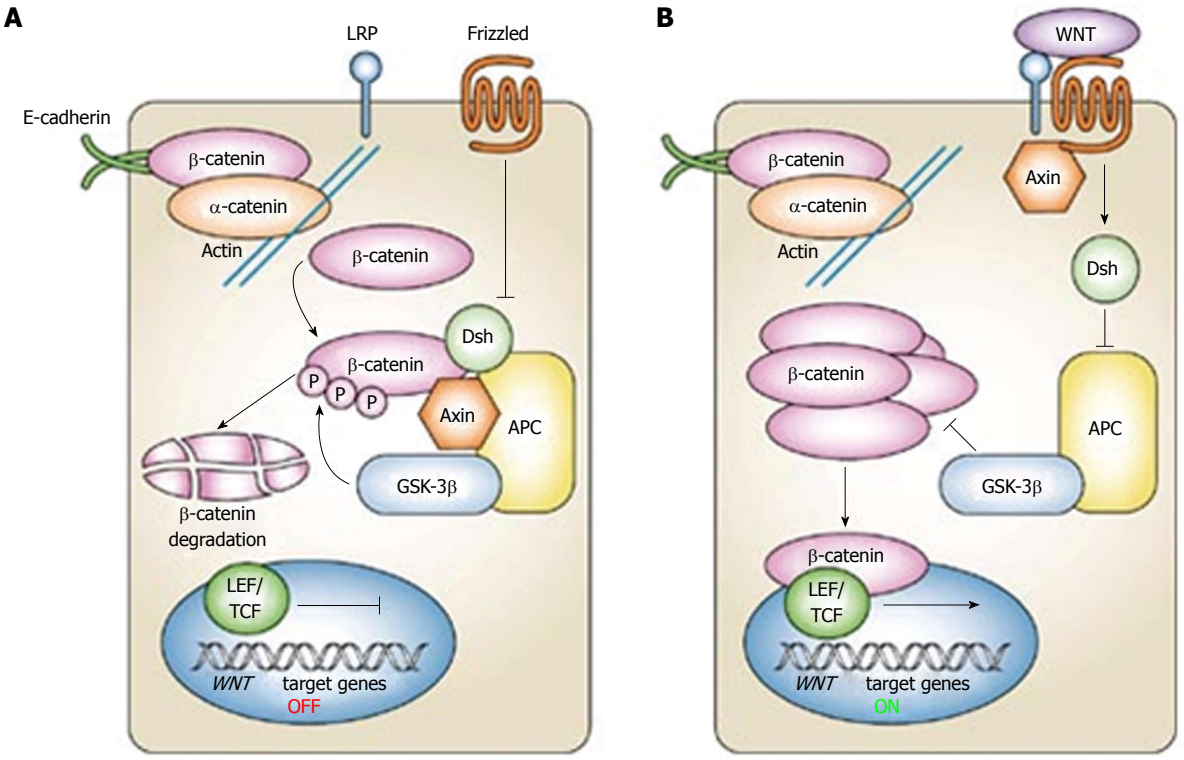

Figure 1 Canonical Wnt signaling pathway (reproduced with permission from[95]). the WNT pathway consist of two states, one referred to as "off" state where the small lipid modified Wnt protein does not bind to frizzled receptors (A), the other refereed as to "on state" when Wnt binds to frizzled (B). In the off state a large destruction complex is formed by the APC, Axin and GSk3β proteins. This complex binds to free β-catenin, phosphorylates it, thus triggering its degradation and preventing it from entering the nucleus. In the on-state dishevelled is activated, inhibits the formation of the destruction complex and leads to an abundance of cytoplasmic β-catenin, some of which enters the nucleus, binds with TCF, leading to cell proliferation.

A similar method was used by Nelson *et al*[58] that modelled the unfolded crypt as a bilayer in which a growing cell layer adheres to a thin compressible elastic beam. Authors confirmed that the buckling instability could be induced as a consequence of the stress relaxation driven by the epithelial cells proliferation. Moreover, it was pointed out that non-uniformities in cell growth and variations in cell-substrate adhesion are predicted to have minimal effect on the shape of resulting buckled states. Interestingly the authors provided also an experimental verification of their theoretical model, by culturing a monolayer of epithelial cells on a flexible PDMS-based surface and showing by optical microscopy that cell growth could cause out-of-plane substrate deflection. These results provide another piece of understanding on how mechanical signals has a key role, both, in physiological and pathological processes.

For the sake of completeness, we deem appropriate to mention other mathematical models, such as cell-based methods or lattice-based models[13-17], that characterize the position and behaviour of individual cells within the crypt, lattice-free models[7-12], that allow for a more realistic approach considering interaction between adjacent cells, and kinetic continuum models that take into account stem cells proliferation[19]. These models are deeply described in the comprehensive review from van Leeuwen *et al*[59].

MECHANICAL CUES COULD HAVE A ROLE IN THE ONSET OF COLORECTAL CANCER THROUGH THE CONTROL OF THE WNT SIGNALING PATHWAY

An altered tissue mechanics is one of the key hallmark of cancer. A large body of evidence is emerging that a modified mechanical landscape might be not merely a by-product of the malignant progression, but it could contribute to cancer onset and/or accelerate its progression[29-41].

This is particularly interesting for colon cancer, because gastrointestinal (GI) tract is naturally submitted to significant endogenous mechanical stress as a consequence of intestinal transit[60]. The high-amplitude propagating contractions that periodically move luminal contents from the ascending colon toward the sigmoid, for instance, generate luminal pressures in excess of 80 mmHg (approximately 10 kPa). In pathological conditions, the increase of cell mass due to the deregulated cell proliferation, apoptosis resistance and neoangiogenesis, exerts a considerable stress on adjacent healthy tissues. Moreover, cancer cells of the primary neoplasm are embedded in the tumour "reactive stroma" that is associated with an increased number of fibroblasts, enhanced capillary density and anomalous ECM-molecules deposition, rich in collagen-I

Table 1 List of experimental studies investigating the effect of external pressure on colon cancer cells proliferation

Ref.	Applied load	Pressure loading system and experimental conditions	Results
Hirokawa *et al*[44], 1997	40-120 mmHg (5-16 kPa)	The pressure-loading apparatus consists of a flask of which cap was pierced and connected to a tubing by which compressed He gas was introduced to raise internal pressure.	Pressurization from 40 to 120 mmHg for 48 h significantly increased cell (IEC18) number with peak proliferation at 80 mmHg. Pressure-induced DNA synthesis was further enhanced by the addition of interleukin-2, suggesting the regulation of intestinal epithelial growth by pressure could be dependent on cytokines.
Hirokawa *et al*[43], 2001			Applied pressure for 48 h induced proliferation of IEC18 cell, with a significantly peak at 80 mmHg. The pattern of F-actin distribution was not significantly altered. The pressure-induced increase in phosphorylation of Elk-1 fusion protein corresponding to the activation of MAPK.
Basson *et al*[63], 2000	15 mmHg (2 kPa)	Cell plates was positioned in an airtight acrylic box, in which pressurized gas was introduced by a tubing to increase pressure.	Increasing ambient pressure stimulated the adhesion of human Caco-2, SW1116, SW620, and HT-29 cells to Matrigel, type I collagen, laminin, and fibronectin.
Whitehead *et al*[6], 2008	0.8 kPa	A controlled mechanical strain was applied on short segments of colon explants obtained from normal and APC1638N/+ mice. Tissues were placed into a mechanical deformation box and compressed in the z-direction of approximately half of their relaxed thickness for 20 min.	APC1638N/+ mice showed the expression of the two oncogenes Myc and Twist1, not observed in wild-type colon explants. Myc and Twist1 activation was found to be correlated with an increased presence of nuclear β-catenin . Almost no nuclear β-catenin was detected in the wild-type colon epithelium. The mechanical stimulation of APC1638N/+ tissue leads to the phosphorylation of β-catenin at tyrosine 654, the site of interaction with E-cadherin, affecting cell adhesions properties.
Fernández-Sánchez *et al*[5], 2015	1.2 kPa	A controlled pressure was applied *in vivo* in APC1638N/+ and control mice by subcutaneously inserting a magnet close to the mouse colon. The magnet generates a magnetic force on ultra-magnetic liposomes, stabilized in the mesenchymal cells of the connective tissue surrounding colonic crypts.	The magnetically induced load led to a rapid Ret activation and the phosphorylation of β-catenin on Tyr654, impairing its interaction with E-cadherin. β-catenin nuclear translocation was observed after 15 days with a consequent increased expression of β-catenin-target genes at 1 month, together with crypt enlargement accompanying the formation of early tumorous aberrant crypt foci. Such malignant behavior was induced in, both, APC1638N/+ and control mice, irrespective of the presence of prior genetic abnormalities.
Avvisato *et al*[27], 2007	1.5 kPa	Cells were plated on 38 mm × 76 mm slides and subjected to a laminar shear stress in a rectangular flow channel for 12 h.	β-catenin signalling of SW480 cells decreased to 22% of control values. The β-catenin signalling were measured for 0-24 h during shear stress exposure, it decreased significantly following 12 h of flow, reaching a minimum after 24 h.

and fibrin[61]. This "reactive stroma", together with the uncontrolled cells proliferation, modifies tissue topography, density and stiffness, exerting a mechanical stress of a few kPa on the tumour itself and the adjacent normal tissues[5]. High abdominal pressure are also common during insufflations for laparoscopy and after surgery, as a result of tissue edemas, whereas pressure during surgical manipulations can be as high as 1500 mmHg or more[62].

Many experimental findings suggested that repetitively applied physical forces, such as those related to GI transit, or constantly applied forces might contribute to initiate intracellular signals capable of altering intestinal epithelial proliferation[5,6,27,43,44,60,63-65]. Some of these studies are summarized in Table 1.

Hirokawa *et al*[43,44] investigated the effect of intraluminal pressure on cultured intestinal epithelial cells (IEC18 cell line). Pressure was applied to cells by helium gas in a culture flask, up to reach a load of 80 mmHg (approximately 10 kPa). Authors showed that such an external pressure induces cell proliferation, probably *via* the activation of Myc expression, a β-catenin related oncogene[43,44]. Similarly, a pressure of 15 mmHg applied to colon 26 cells implanted in rat model increases liver metastasis suggesting that even a low pressure increase might influence malignant cell proliferation[66].

Other than an altered cellular proliferation, extracellular pressure can influence cancer growth by promoting cell adhesion[60,63-65]. In this regard, Basson and co-workers showed that the exposure of non-adherent primary human colon cancer and SW620 cells to 15 mmHg of extracellular pressure increases cell adhesion *via src*-mediated or cytoskeleton-mediated FAK activation. Both mechanisms promote FAK association with integrin, altering its binding

affinity and facilitating colon cancer cell adhesion[64,65].

As stated above, loss of APC function triggers the chain of molecular and histological changes leading to colorectal tumours. In this context, Whitehead *et al*[6] applied a controlled mechanical strain on short segments of colon explants from normal and APC deficient mice ($APC^{1638N/+}$). Differently from humans, where GI tumours are found primarily in colon, mice develop cancer predominantly in the small intestine. Therefore $APC^{1638N/+}$ mice colon tissues are both, morphologically normal and APC deficient, thus providing an ideal model system to study the earliest event in colorectal tumorigenesis[6]. Both control and APC deficient tissues were placed into a mechanical deformation box and compressed in the z-direction of approximately half of their relaxed thickness for 20 min with an applied load of approximately 800 Pa. Compressed tissues showed elongated crypt openings hinting at some shape changes at the cellular level. Such modifications were accompanied by the expression of the two oncogenes Myc and Twist1 in APC deficient colon tissue explants, but not in wild-type colon explants. Authors showed that Myc and Twist1 activation is strongly dependent on the presence of nuclear β-catenin, in agreement with[43,44]. In response to mechanical strain, the APC deficient colon tissues showed an increased number of β-catenin positive nuclei per crypt, whereas almost no nuclear β-catenin was detected in the wild-type colon epithelium. The mechanical stimulation of $APC^{1638N/+}$ tissues was found to induce a phosphorylation of β-catenin at tyrosine 654, the site of interaction with E-cadherin, thereby dramatically affecting cell adhesions properties. These data demonstrate that, when APC is down expressed, mechanical strain, such as that associated with intestinal transit, presence of polyps or tumour growth, can be interpreted by cells of pre-neoplastic colon tissue as a signal to initiate a β-catenin dependent transcriptional program characteristics of cancer[6].

Even though the above mentioned *in vitro* experiments provided convincing data that establish a clear correlation between endogenous mechanical pressure and tumorigenesis, they cannot take properly into account all factors contributing to the mechanical environment *in vivo.* To overcome this limitation, Fernández-Sánchez *et al*[5] developed a novel and effective method that allows the delivery of a defined mechanical pressure *in vivo* by subcutaneously inserting a magnet close to the mouse colon. The implanted magnet generates a magnetic force on ultra-magnetic liposomes, stabilized in the mesenchymal cells of the connective tissue surrounding colonic crypts after intravenous injection (Figure 2).

Such method appears to be a significant breakthrough in the field of cancer biomechanics as it permits to control mechanical stimuli *in vivo* with same precision that can be achieved *in vitro*[42]. As pointed out in the recent review by Ou and Weaver, this novel technique has the potential to boost a new era in tissue biomechanics, providing a direct link between mechanical perturbation occurring *in vivo* and tumorigenic cell modifications[42].

The authors used this revolutionary method to induce a controlled pressure of approximately 1.2 kPa, mimicking the endogenous stress produced by the early tumour growth on healthy tissues. The applied strain led to a rapid Ret activation and downstream phosphorylation of β-catenin on Tyr^{654}, impairing its interaction with the E-cadherin in adherents junction and promoting β-catenin nuclear translocation. Consequently, authors observed an increased expression of β-catenin-target genes, together with the formation of aberrant crypt foci. Interestingly the authors showed that the mechanical induction of a malignant behaviour in normal tissues adjacent to the tumour does not depend on the presence of prior genetic abnormalities, adding another piece of understanding to the growing consensus that the mechanical environment intrinsic to cancerous tissues has the potential to directly modify cells behaviour even in absence of genetic mutations.

Taken together, these results show how mechanical signals can contribute to the onset of a malignancy by affecting the Wnt pathway and triggering the consequent disruption of the physiological crypt dynamics. Interestingly, this behaviour may be propagated by a positive feedback loop in which mechanical pressure from the primary tumour and the stroma induce a breakdown of the normal Wnt signalling pathway in non-transformed adjacent cells. This event, in turn, can trigger an abnormal cell growth that generates further mechanical stress.

For the sake of completeness we want to stress that the Wnt/β-catenin pathway - being one of most important regulators of proliferation in various systems - can be modulated by a wide range of factors other than mechanical stimuli. To give an example, recent experimental findings showed that α6A(β4) integrin regulates cell proliferation and the Wnt/β-catenin pathway through the control of DVL2/GSK3β[67].

MECHANICAL CUES COULD PLAY AN IMPORTANT ROLE IN THE EARLY PHASES OF METASTASES BY TUNING THE TUMOUR MICROENVIRONMENT STIFFNESS

An effective identification of metastasis triggering-signals appears to be a crucial step in the fight against cancer since metastasis accounts for the most of cancer deaths. The process leading to the formation of metastasis is strongly mediated and supported by the tumour microenvironment, which consists of different structures with different mechanical responses, such as tumour-infiltrating cells, blood vessels, extracellular matrix (ECM) and other matrix-associated proteins[2,28].

A large body of evidence suggests that the tumour

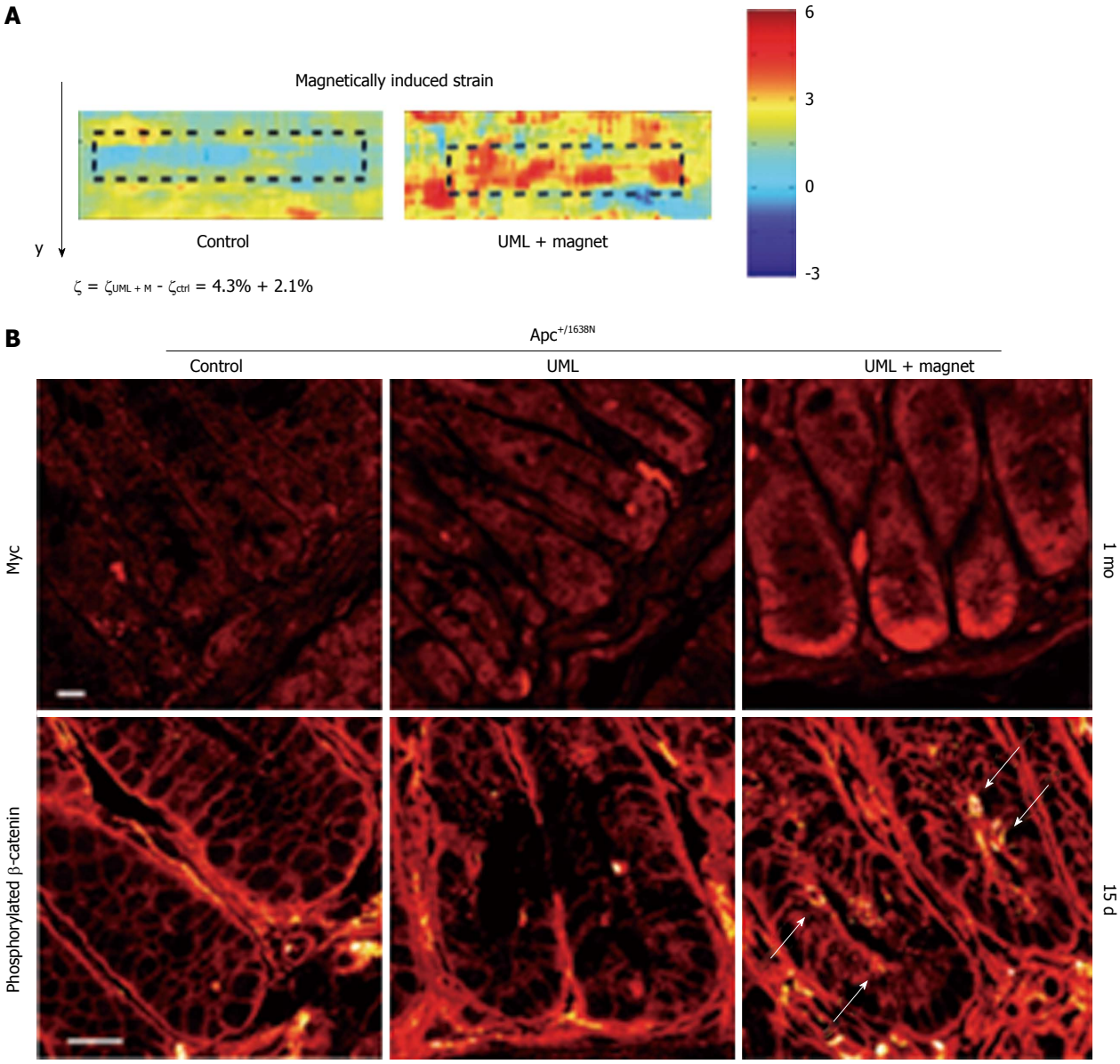

Figure 2 Novel method by Fernández-Sánchez *et al*[5] to deliver a defined mechanical pressure *in vivo* (reproduced with permission from[5]). A: Strain maps of non-magnetized (left) and magnetized (right) colon crypt injected with ultra-magnetic liposomes; B: Top: immunefluorescence of Myc expression in APC deficient crypts 1 mo after the ULM injection, in control (left), non-magnetized (middle) and magnetized (left) tissues; Bottom: β-catenin Y654 phosphorylation after 15 d under UML in control (left), non-magnetized (middle) and magnetized (left) tissues.

microenvironment with its mechanical stimuli, including stiffness, might play a major role in initiation of metastasis. For instance, a highly aggressive metastatic variant of murine B16-F1 melanoma cells is produced by culturing cells in a soft fibrin scaffold[22]. Weaver and collaborators showed indeed that ECM stiffening obtained through collagen crosslinking promotes malignant behaviour in mammary epithelial cells by modulating integrin's expression[68,69].

These results suggest that a fine tuning of the microenvironment stiffness might be involved in the differentiation of non-invasive cells into metastatic variants[70,71]. A confirmation of this hypothesis is provided by the experimental findings of Tang and collaborators[23-25], carried out mainly on HCT-8 cells, a low metastatic colon cancer cell line (Grade I), epithelial in phenotype (E-cells). Previous experimental works demonstrated that when cultured on conventional stiff plastic substrates, low grade HCT-8 E-cells adhere and proliferate, resulting in monolayers covering the entire dish with occasional mounds consisting of 2-3 layers of cells. On top of these mounds, a small number of rounded-shaped metastatic variants of these cells can be detected (1 variant per 2×10^5 epithelial-shaped cells). Due to their shape, such metastatic-like variants are called rounded cells (R-cells)[46-48,72].

By culturing E-cells on polyacrylamide (PA) substrates of well-defined stiffness, Tang *et al*[24] demonstrated that the proportion of the metastatic-like R-cells can be increased by several orders of magnitude up

to reach 70%-90% of the original cell population. To this purpose, authors cultured HCT-8 cells on several fibronectin-coated substrates with a different Young's modulus, namely a stiff 3.6 GPa polystyrene surface (PS) and a set of polyacrylamide (PA) substrates with a Young's modulus lying in the range 21-47 kPa. Such stiffness range mimics the rigidity of the tumour microenvironment, thus being suitable to reproduce the mechanical stimuli sensed by cells in pathological conditions[24]. Cells cultured on 21 kPa PA substrates form colonies in 2-4 culture days; after 7 culture days cells begin to dissociate and after 11 d the entire colony dissociates in single round shaped cells. Similarly, HCT-116 cells cultured on 10 kPa sPA fibronectin-coated gel substrates form cell colonies in 2-5 culture days and begin to dissociate from colonies on the 10th day. The metastatic variant is not observed on the 3.6 GPa stiff PS substrate. Similar results were obtained on laminin coated substrates and by using other human colon cancer (SW480) and prostate cancer cell lines[23]. Interestingly, the stiffness-mediated E-to-R transition cannot be reversed by plating the dissociated cells on stiff substrates[24]. The irreversible nature of the transition is likely due to the fact that dissociated cells loose mechano-sensitivity to the substrate[25].

The shape modifications occurring in the E-to-R transition hint at a complex cellular remodeling at the cytoskeleton level. Not-dissociated cells cultured on hard PS substrates indeed show a well-organized cytoskeleton network made of aligned actin stress bundles. Such network is also associated to the presence of large intracellular tension forces that induces significant nuclear stretching[24]. Conversely, R-cells have an almost spherical-shaped nucleus and do not display intracellular stress bundles. The loss of stress bundle, in turn, is associated to a down-expression of E-cadherin along cell-cell contact borders in the metastatic variant[6].

Tang *et al*[23] studied also the invasive behavior of both cell variants, showing that HCT-8 R cells are remarkably more invasive and tumorigenic than E cells. R cells were found to express many of the molecular signatures associated with resistance to hypoxia, apoptosis, as well as genes linked to metastasis. Of particular interest is the reported down regulation of CKB gene that is linked to the epithelial-to-mesenchymal transition (EMT) in colon cancer[73]. One of the major elements that characterize EMT of carcinoma cells is the loss of E-cadherin-mediated cell-cell adhesion[74], a second characteristic that is in common with the E-to-R transition, providing further evidence that a metastasis-enhancing gene pattern is activated in R cells and this activation might be associated with the characteristics of *in vivo* EMT[23]. Some of these studies are summarized in Table 2.

COLON CANCER CELLS MODIFY THEIR MECHANICAL PROPERTIES TO RESIST INTRAVASATION, SHEAR STRESS ASSOCIATED WITH CIRCULATION AND EXTRAVASATION

Following the detachment from the primary tumour, cancer cells intravasate into blood vessels to disseminate. The process of intravasation is still not fully understood in the case of colon cancer. Several molecular steps involving matrix metalloproteinases and interaction between cancer cells and endothelial adhesion molecules have been described. Such processes are discussed in detail in the comprehensive review from Gout and Hout[2] and involve also the tumor-infiltrated macrophages (TAM) that are stimulated by cancer cells to secrete matrix metalloproteinase MMP-7, MMP-12 and vascular endothelial growth factor (VEGF)[28,75-77].

After ECM degradation, cancer cells can gain access to the blood vessels. At this step, the vessel diameter - often smaller then cell sizes - play a crucial role being a key parameter underling colon cancer intravasation *via* passive entry[78,79].

Moreover, once entered in the blood stream circulating cancer cells are usually not able to generate metastasis. In the most of the cases, indeed, they undergo disruption because of the mechanical stress imposed by circulation, which appears one of the major defence mechanism in the host microenvironment[80,81].

Metastatic cancer cells have developed several strategies to survive the mechanical stress related to both, the migration within the degraded ECM and the shear stress in the blood stream. Such strategies include the occurrence of major modifications at the cytoskeleton level deeply altering the cells viscoelastic properties.

In this context, recent *in vitro* studies compared the viscoelastic properties of different colon cancer cell lines[20,21,26,27]. To this purpose, two main techniques are used: micropipette aspiration (MA) and atomic force microscopy (AFM). The former permit to investigate the mechanical properties of the whole cell[82,83], whereas the latter provides information on the morphological and mechanical properties at the cellular and sub-cellular level[20,21,30-33,84-90]. Both methods can be coupled to advanced finite element simulation methods[26,91,92].

Pachenari *et al*[26] recently studied the viscoelastic properties of grade Ⅰ (HT29) and grade Ⅳ (SW480) cancer cells trough micropipette aspiration (MA) method, showing that SW480 are significantly more deformable

Table 2 List of cell properties that depends on substrate stiffness

Substrate-related mechanical properties	Substrate stiffness	Substrate type and composition	Outcomes	Related-biochemical and genetic pathway	Ref.
E-to-R transition	1 kPa	Laminin or fibronectin coated PA gel	The E to R transition is not observed.	Not applicable.	Tang *et al*[24], 2015
	21 kPa	Laminin coated PA gel	Approximately 70%-90% of E cells start transiting to R cells after culturing for 7 d. Transition takes approximately 5-10 h.	E-Cadherin decreases in dissociated R cell by a factor 4.73 ± 1.4.	
		Fibronectin coated PA gel	Approximately 70%-90% of E cells start transiting to R cells after culturing for 15 d. Transition takes approximately 5-10 h.	Replanted cells retain their dissociated phenotype irrespective of the substrate stiffness.	
	3.6 GPa	Fibronectin coated PA gel	Not observed.	Not applicable.	
	20 kPa	E-cadherin coated PA gel	E cells transit to R cells in 6 h.	Vinculin in mainly located at the cell-cell junction.	
		Fibronectin coated PA gel	E cells transit to R cells in 6 h.	Vinculin in mainly located at the cell- substrate junction.	Ali *et al*[45], 2014
	~70 GPa	E-cadherin coated stiff glass substrates	Transition is not observed.	Not applicable.	
	Extremely stiff[1]	Plastic/glass stiff substrate	Occasionally E cells transit to R cell (1 cell over 2×10^5).	R cells are deficient in αE-catenin (protein linking the cell-cell adhesion molecule E-cadherin to the action cytoskeleton).	Vermeulen *et al*[46-48], 1995, 1998, 1999
Cell colony sizes	1-20 kPa	Gradient stiffness fibronectin coated PA gel	E type: colony size positively correlated with substrate stiffness R type: colony size (smaller than E-colony size) positively correlated with substrate stiffness.	Not applicable.	Tang *et al*[25], 2012
	Soft[1]	Agar gel	Equal numbers of E and R cells were plated and examined after 10 d, 75% of the E cells plated formed colonies while R cells formed no colonies.		Rosenthal *et al*[72], 1977
Adhesion	1-20 kPa	Gradient stiffness fibronectin coated PA gel	E type: cells show a strong cell-cell adhesion and cell-substrate adhesion evaluated through the measurement of the cell-substrate contact area (188.1 ± 80.7 μm^2) by confocal microscopy. Moreover, a strong aspecific adhesion of ~250 nN is detected trough a novel MEMS system. R type: cells show a weak cell-cell adhesion on very soft substrate (1 kPa). No cell-cell contact is observed on stiffer substrate (5-10-15-20 kPa). A weak cell/substrate adhesion is demonstrated through the measurement of the cell/surface contact area (49.5 ± 20.9 μm^2). A week aspecific adhesion of ~2.5 nN is measured through a MEMS system.	Reduced E-cadherin expression on R cells.	Tang *et al*[25], 2012

[1]Young's modulus not provide.

than HT29. The former are indeed characterized by instantaneous and an equilibrium Young's modulus of E_0 = 331.67 Pa and E_∞ = 123.47 Pa, respectively, the latter by E_0 = 574.72 Pa and E_∞ = 84.76 Pa. The higher compliance of the metastatic cells is accompanied to deep modifications occurring in the cytoskeleton organization, mainly at the level of actin filaments. Authors indeed unveiled a significant decrease in the ratio of actin filaments to microtubules by western blot analysis and fluorescence measurements. Taken together these results confirm that cancer invasiveness is related with an increased cell deformability that, in turn, is instrumental to squeeze through slim capillaries with diameters less than cell sizes as well as to tolerate frictional forces arising between their outer surface and vessel walls.

Avvisato *et al*[27] recently investigated the behaviour of metastatic SW480 cell lines under shear

stress. In particular, cells were cultured on glass and on fibronectin and laminin coated substrates, placed in a rectangular flow channel and exposed to a laminar shear stress lying in the range 0.4 Pa to 3.5 Pa, comparable to human blood shear stress[93]. After 12 h exposure, authors observed a decrease in β-catenin, showing that Wnt signalling pathway is also shear stress dependent (Table 1). Interestingly, such a decrease is greater on laminin-coated substrates, suggesting that the effect of shear stress could be mediated by integrin cell adhesion receptors that in turn have a key role in the intra- and extravasation processes. One way to escape the shear stress associated with circulation is the overexpression of integrin and E-cadherin that allow cells to adhere on the blood vessel wall and epithelial tissues, respectively, favouring extravasation[40,68].

Palmieri *et al*[20] recently compared the biomechanical properties of SW480 and SW620 colon carcinoma cell lines, derived from primary tumour and lymph-node metastasis of the same patient, respectively. The limited genetic variability of these cells makes them an ideal system to analyse phenotypic variations associated with the metastatic process. Authors studied by confocal microscopy the actin organization of both cell lines, demonstrating that SW620 cells show a decreased cytoskeleton organization with respect to SW480, as quantitatively evaluated by measuring the actin filament-junction density and coherency[20]. Such loss of structure affects also the overall mechanical properties of SW620 cells that appear to be significantly more compliant (480 Pa) than SW480 (1.06 kPa) as demonstrated by atomic force spectroscopy measurements. These results point out that cells extracted from metastases undergo a further destructuration process with the respect to those extracted from the primary tumour that might be related to the cell's ability to escape from primary tumour mass, to resist to blood shear stress and to extravasate. Moreover, authors unveiled that cells from lymph-node metastasis (SW620) exhibit a higher non-specific adhesion force (95 pN) than SW480 (50 pN), suggesting that the non-specific adhesion forces could participate, together with the high specific one (receptor-ligand binding), in the attachment to the blood vessel walls, in the consequent extravasation and in the metastasis formation. Interestingly, two morphologically different sub-populations of SW480 cells having an elongated (E-type) and a rounded (R-type) shape were reported[20,21]. Similarly to HT29, E-type SW480 cells are significantly stiffer ($E \sim 1$ kPa) that R-type cells ($E \sim 0.5$ kPa), indicating a less-organized cytoskeleton in the latter case. At variance with the R-type HT29 cells, SW480 E-type cells do not show impaired adhesion properties with the respect to E-type cells[21] and consistently do not metastasize when injected in nude mice[94].

CONCLUSION

Physical forces either within tissues or externally applied, affect all tissues of the body. Cell mechano-transduction indeed converts biophysical forces into cellular responses that may influence gene expression, protein synthesis, proliferation and morphogenesis. In this review, we focused on recent studies covering the impact of physical stimuli such as compression, shear stress, adhesion and stiffness, in the development of colorectal cancer, showing that such stimuli may have a role in each step of the tumour progression.

An anomalous tissue compression due to a modified microenvironment or an altered abdominal pressure can indeed affect cell proliferation and adhesion properties. A large body of experimental evidence show that mechanical strain can activate a β-catenin dependent pathway, characteristic of cancer, that is able to disrupt the physiological crypt dynamics, leading to the formation of aberrant crypt foci. The mechanism behind this process was recently unveiled by a pioneering *in vivo* study. The application of a controlled strain *in vivo* was demonstrated to foster the phosphorylation of β-catenin on Tyr654, leading to an impaired interaction with E-cadherin and promoting β-catenin nuclear translocation with the consequent overexpression of β-catenin targeted oncogenes.

Mechanical cues have also the potential to affect the early phases of metastasis. Tumour progression is accompanied by deep modifications in the tumour microenvironment, which is characterized by a rapidly evolving mechanical landscape. In this context, microenvironment stiffness modifications was indicated as one of the signalling-pathways involved in the initiation of metastasis. This hypothesis was confirmed by recent *in vitro* studies carried out on a wide range of primary colon cancer cell lines cultured on artificial substrates of a given stiffness. Such studies showed that, in these experimental conditions, substrate stiffness is the main responsible of the differentiation of non-invasive cells into metastatic variants, irrespective of the surface chemistry.

We highlighted also how physical stimuli can support metastatic cells dissemination. Metastatic cells undergo deep structural and mechanical modifications occurring mainly at the cytoskeleton level, that allow them to resist the stress related to migration within the degraded ECM, to intravasate and to survive at the shear stress associated with circulation. To this purpose, actin molecules and microtubules are rearranged within the cell cytoskeleton to make the metastatic cell more compliant than the primary tumour.

Taken together, the experimental findings here reviewed show that mechanical forces are an important player in the development of colon cancer. Therefore, a deep comprehension of the role of physical forces may help scientist to develop both novel diagnostic tools

and innovative pharmacological approaches.

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REVIEW

Multisciplinary management of patients with liver metastasis from colorectal cancer

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Abstract

Colorectal cancer (CRC) is one of the leading causes of cancer-related death. Surgery, radiotherapy and chemotherapy have been till now the main therapeutic strategies for disease control and improvement of the overall survival. Twenty-five per cent (25%) of CRC patients have clinically detectable liver metastases at the initial diagnosis and approximately 50% develop liver metastases during their disease course. Twenty-thirty per cent (20%-30%) are CRC patients with metastases confined to the liver. Some years ago various studies showed a curative potential for liver metastases resection. For this reason some authors proposed the conversion of unresectable liver metastases to resectable to achieve cure. Since those results were published, a lot of regimens have been studied for resectability potential. Better results could be obtained by the combination of chemotherapy with targeted drugs, such as anti-VEGF and anti-EGFR monoclonal antibodies. However an accurate selection for patients to treat with these regimens and to operate for liver metastases is mandatory to reduce the risk of complications. A multidisciplinary team approach represents the best way for a proper patient

management. The team needs to include surgeons, oncologists, diagnostic and interventional radiologists with expertise in hepatobiliary disease, molecular pathologists, and clinical nurse specialists. This review summarizes the most important findings on surgery and systemic treatment of CRC-related liver metastases.

Key words: Liver metastases; Colorectal cancer; Liver resection; Multidisciplinary team; Chemotherapy

Core tip: Approximately 25% of colorectal cancer patients have liver metastases at the initial diagnosis and almost half develop liver metastases later. Although unresectable liver metastases can be converted into resectable disease with the help of combination chemotherapy with targeted therapy, patients should be accurately selected. Multidisciplinary teams including health professional with expertise in hepatobiliary disease is needed to decide the best way to manage these patients' treatment.

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INTRODUCTION

Globally, colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females[1]. Moreover, CRC is the second leading cause of cancer mortality in the United States, accounting for 9% of cancer deaths[2]. In Europe, it caused nearly 204000 deaths in 2004[3]. The liver is the most common metastatic site[4], probably due to tumor spread *via* the portal system[5]. Twenty to twenty-five percent of patients have clinically detectable colorectal liver metastases (CLM) at the initial diagnosis and approximately 50% of the patients develop CLM during their disease course[6]. Resection of the CLM, sometimes in combination with other local treatment modalities such as radiofrequency ablation (RFA), has become the standard of care, despite lack of evidence from randomized controlled trials, and offers the only potential for cure[7,8]. The natural history of metastatic colorectal cancer (mCRC) is variable, however, untreated CLM have a poor prognosis with median survival rates of less than 8 mo[6,9]. Only 20%-30% patients with mCRC have disease that is confined to the liver[6]. Patients presenting with CLM can generally be divided into three groups: those with initially resectable disease; those with metastases that may become resectable following treatment ("conversion" therapy); and patients whose liver metastasis never will be resectable[10]. Unfortunaly, only a minority of patients (10%-20%) with CLM are considered eligible for resection, while about 85% of them have liver disease considered unresectable at presentation[11,12]. Recent data suggest that of those undergoing resection of CLM, around one out of three patients will be still alive after 5 years from diagnosis. A single center 5-years survival now approaches 60% following hepatectomy, with 10 years survival in excess of 25%; about half of them will be alive after 10 years, so considered as cured[13]. A systematic review and meta-analysis of 142 studies published in 1999-2010 has also confirmed these data, showing 5-year survival rates of 16%-71%, for patients with CRC, after surgical resection of liver metastases[14]. Even more, long-term survival rates for those patients with initially unresectable metastases treated with chemotherapy prior to surgery are similar to those of patients whose metastases were considered to be resectable[15-21]. Indeed, since there is a strong correlation between tumor response and resection rate[3,22], this has led to an increased use of chemotherapeutic and biological agents as "conversion therapies" in patients with mCRC. Indeed, these strategies can facilitate downsizing of CLM and convert initial unresectable metastases to resectable. Hence, the percentage of patients potentially eligible for curative liver resection is increasing. This has been due to advances in surgical and perioperative management, the use of more effective chemotherapies and combination therapies, the incorporation of targeted therapies and new local treatment approaches (*e.g.,* hepatic intra-arterial chemotherapy, RFA, stereotactic radiotherapy)[23]. Nonetheless, difficulties remain in deciding who is a candidate for resection, and often underestimated since many patients with liver metastasis never were referred to a hepatobiliary surgeon[24].

Therefore, the goal for patients with metastatic colorectal disease is a multidisciplinary treatment approach, in order to decrease peri-operative morbidity and mortality, as well as long-time survival by increasing the number of patients undergoing potential curative liver resections.

RESECTION OF CLM - CURRENT EVIDENCES

Surgical treatment

Hepatectomy remains the standard of care for CLM. In the past, post-operative mortality was high but nowadays it has decreased to around 1%[25-27] allowing more extended hepatic resections by more advanced surgical techniques. Nevertheless, liver failure after hepatectomy remains the major concern for the hepatobiliary surgeon. Resection, even partial, can

Table 1 Response and resection rates from trials with first-line chemo/biologic therapy

Study	Selection	Treatment	Response	Resection
Van Cutsem *et al*[88] 2009	Unselected	Bevacizumab + chemotherapy (FOLFOX/FOLFIRI)	NA	11.8% 6% (R0)
Okines *et al*[89] 2009	Unselected	Bevacizumab + Oxaliplatin-based chemotherapy	38%	6.3%
Wong *et al*[90] 2011	Unselected	Bevacizumab + XELOX	68%	48.0%
Loupakis *et al*[91] 2013	Unselected	Bevacizumab + FOLFOXIRI	64%	15.0%
Martin *et al*[92] 2014	Unselected	Bevacizumab + FOLFOX + DEBIRI	78%	35.0%
Bokemeyer *et al*[94] 2009	*KRAS* WT	Cetuximab + FOLFOX	57%	7.3% 4.7 (R0)
Van Cutsem *et al*[95] 2011	*KRAS* WT	Cetuximab + FOLFIRI	57%	16.0% 7% (R0)
Folprecht *et al*[22] 2010	*KRAS* WT	Cetuximab + chemotherapy (FOLFOX/FOLFIRI)	68%	43.0% 34% (R0)
Garufi *et al*[96] 2010	*KRAS* WT	Cetuximab + FOLFOXIRI	79%	60.0%
Ye *et al*[97] 2013	*KRAS* WT	Cetuximab + FOLFOXIRI	57%	25% (R0)
Köhne *et al*[100] 2012	*KRAS* WT	Panitumumab + FOLFIRI	56%	15.0%
Douillard *et al*[101] 2010	*KRAS* WT	Panitumumab + FOLFOX	57%	27.0% 5.2% (R0)
	NRAS WT	Panitumumab + FOLFOX		31.0%

NA: Not available.

result in a small postoperative remnant liver function, hence increasing the risk of postoperative liver failure and subsequent very high mortality. In 2006, a national multicenter study by the group of Schroeder *et al*[28] showed an overall mortality rate after hepatectomy of 8.5% in the perioperative period. This mortality rate increases up to 16% when performing an hepatectomy of 3 segments or more.

Below a critical liver volume, the remnant liver cannot sustain metabolic, synthetic, and detoxifying functions[29]. However liver volume is not the best surrogate for liver function, in particular for patients with concomitant liver disease[30,31]. Based on data from the transplantation literature, it has been postulated empirically that each percent increase in fat content, either microvesicular or macrovesicular, decreases the functional mass of a donor liver by 1%[32]. In patients with cirrhosis, with non-alcoholic steatohepatitis, with obstructing jaundice due to a tumor or livers after chemotherapy regeneration capacity may be impaired (Table 1).

Major partial hepatectomy in combination with underlying parenchymal disease correlate well with increased morbidity and mortality rates[33-37]. In several series, the overall liver failure rate leading to death ranges from 25% to 100% following hepatic resection for hepatocellular carcinoma (HCC)[38-42]. However patients with HCC mostly have underlying cirrhosis as an etiologic factor for their tumors. Instead mortality rates after resection for CRC have a wide range, with up to 50% of deaths from liver failure[43,44]. Prolonged recovery and also mortality can also occur for the same reason as for patients with HCC, further indicating the importance of liver reserve in recovery from hepatic surgery in patients who received chemotherapy[45]. Most chemotherapeutic agents, even 5-fluorouracil, can cause liver damage[15]. Some studies suggest that patients who receive chemotherapy develop steatosis[35,46-48] whereas others show no correlation between any chemotherapy regimen and severe steatosis[49]. Others found that irinotecan is associated with the development of steatohepatitis in some patients[49,50]. Therefore, successful liver resection implicates correct recognition of remnant liver function. The group of Van Gulik could nicely show that pre-operative measurement of 99mTc-mebrofenin uptake in the future remnant liver on functional hepatobiliary scintigraphy proved more valuable than measurement of the volume of the future remnant in the assessment of the post-hepatectomy risk of liver failure and liver failure-related mortality[30,31,51,52].

In addition, sparing residual liver parenchyma should be of considerable importance in patients who received neo-adjuvant chemotherapy for CLM. The definition of what are resectable lesions is extremely variable and it depends on the experience and aggressiveness of the surgical team. A study of Lalmahomed *et al*[53], showed that patients treated with liver sparing resections, had to undergo more interventions for local recurrence than patients undergoing anatomical resections. Another population-based study in England showed that of 115 patients undergoing surgery for CRC between 1998 and 2004, 2.7% had minimum 1 hepatic resection. Another disadvantage of liver sparing resections was reported in the literature by DeMatteo *et al*[54] finding higher incidence of positive resection margins when performing liver sparing resections. Indeed, 50% to 75% of patients develop disease recurrence after initially curative resection of CLM. Anatomical resections may not offer the same advantage for these lesions as for HCC, which arise within a segment of the liver and might benefit from the removal of the complete functional liver unit. Indeed, several studies in patients with CLM have been reported in which no significant difference in morbidity, mortality, recurrence rate, or survival according to resection type and liver

sparing resections has been observed[55-57]. Moreover, the cure rate by initial hepatectomy is only 20% to 30% of cases[58,59]. Several studies in patients with CLM reported no significant difference in morbidity, mortality, recurrence rate, or survival according to resection type[60-62]. Karanjia *et al*[63] showed that patients who underwent right and extended right hepatectomy had a poorer short-term outcome, with a higher incidence of operative morbidity and mortality, compared to patients, undergoing other types of surgical treatment for the same disease. The degree of hepatic resection seems to influence tumor growth. Indeed, growth factors such as hepatocyte growth factor, epidermal growth factor, and insulin-like growth factor are generally upregulated early in liver regeneration, producing a mitogenic response and resulting in rapid hepatocyte cell proliferation[64,65]. Some other studies with less than 50% hepatectomies showed no tumor growth stimulation[66,67]. A larger resection causes the liver to express higher levels of growth factors and cytokines to restore the liver to its functional size in approximately the same time as for a smaller hepatectomy[68-71]. A number of studies have found that the larger the percentage of resection, the higher the incidence and volume of recurrence[72,73]. In addition, as mentioned before, although neoadjuvant chemotherapy increases resectability for CLM, it is associated with hepatic changes, such as hepatic sinusoidal obstruction, periportal inflammation, and steatohepatitis, which can affect patient outcome[74] and which might increase the risk of progressive hepatic failure and death after major liver resection. An extensive resection can be tolerated with virtually no risk related if the underlying liver is normal. In contrast, even a minor hepatectomy can be dangerous in patients with severely compromised livers[46]. Actually, the assessment of the underlying liver function is critical for the type of surgery.

Given the implications of these recent advances that have extended the indications for hepatectomy in the treatment for CRC metastases, as well as the positive and negative effects of an extended liver resection, there is need of a multimodality approach to treat patients with metastatic liver disease.

Chemotherapy treatment

In the last years the role of chemotherapy in the management of CLM is considerably increased. Nowadays, it may be considered for both unresectable and resectable CLM. For patients with unresectable CLM, "conversion chemotherapy" aims to convert unresectable, to resectable disease, often representing the initial treatment choice. Standard regimens comprising 5-FU/LV plus either irinotecan (FOLFIRI) or oxaliplatin (FOLFOX) can facilitate resection in 7%-40% of patients[24]. In 1999 the group of Giacchetti reported that 5-FU/LV plus oxaliplatin treatment could reduce the size of liver metastases by > 50% in 59% of the patients with unresectable CLM and complete resection was achieved in 38% of patients[75]. Treatment with 5-FU/LV, oxaliplatin and irinotecan (FOLFOXIRI) regimens permitted R0, curative-intent resections, in 15% of patients, and 36% of patients with liver metastases only[76]. Recently, a randomized, phase Ⅱ trial, comparing intensified chemotherapy regimens (high-dose FOLFIRI, FOLFOX7, FOLFIRINOX) with standard chemotherapy regimens (FOLFOX4, FOLFIRI), for initially unresectable mCRC, has shown that FOLFIRINOX appears more active than other regimens (conversion rate to resectability: 67%; mOS > 48 mo; all others < 30 mo). Furthermore, this trial has confirmed that patients who undergo R0/R1 resections do much better than non-operated, or R2 (R0/R1: mOS > 65.2 mo; not-operated/R2:mOS: 18.3 mo, $P < 0.001$)[77].

For patients with resectable disease, "perioperative chemotherapy" has become an attractive option, in order to reduce the incidence of cancer relapse, occurring in up to 50%-70% of them after resection, through the eradication of occult disease[78,79]. Recently, the randomized, phase Ⅲ trial, EORTC 40983, comparing peri-operative (both neoadjuvant and adjuvant) FOLFOX4 chemotherapy with surgery alone in 364 patients with resectable CLM, has shown a significant increment of PFS, in favour of perioperative treatment, but no significant differences in long term OS between the two treatment arms[80]. In addiction, the risk of post-operative complications has been shown to be significantly more frequent in the chemotherapy arm compared with surgery alone, and also to correlate with the duration of perioperative treatment. Several trials currently ongoing, such as the EORTC trial 40091 (NCT01508000) are investigating the combination of targeted agents such as Bevacizumab and Panitumumab with FOLFOX-chemotherapy regimen in perioperative treatment of patients candidate for resection of CLM, but results are not available yet.

Targeted biological treatment

Our increased understanding of the biology of CRC has led to the development of biologic therapies targeting two different mechanisms, angiogenesis (bevacizumab) and epidermal growth factor receptors (EGFRs) (cetuximab and pantumumab)[81]. One strategy to further increase the number of candidates eligible for surgery is the addition of active targeted agents to standard chemotherapy. In general, response rates appear to be highest with the EGFRIs, therefore these agents may potentially also lead to greater resection rates.

Resection rates with anti-angiogenesis agents: Bevacizumab

Addition of bevacizumab to first and second line chemotherapy for mCRC improves progression-free survival[82-85] and in some studies overall survival[83,84]. However, data on the role of bevacizumab added to chemotherapy in the perioperative setting are limited,

perhaps as a result of concerns about potential wound healing complications[86,87]. The Bevacizumab Expanded Access Trial showed that resection of hepatic metastasis after first-line bevacizumab plus chemotherapy was feasible and curative-intent hepatic resection of metastasis was performed in (11.8%) of patients overall (R0 in 6%)[88]. However, resection rates were higher in patients treated with bevacizumab plus Oxaliplatin chemotherapy (16.1%), than in those treated with bevacizumab plus Irinotecan chemotherapy (9.7%).

In a further first-line trial comparing oxaliplatin based chemotherapy plus bevacizumab or placebo, 6.3% of patients with bevacizumab and 4.9% of those treated with placebo underwent R0 resection of the metastasis (P = 0.24)[89]. Another study of neoadjuvant CAPOX plus bevacizumab allowed 12 out of 30 (40%) patients with initially unresectable CLM to be converted to resectable[90]. Loupakis *et al*[91] have recently reported a RR of 64% and a resection rate of 15%, in patients treated with FOLFOXIRI plus bevacizumab, as compared with respectively 53% and 12%, of those treated with FOLFIRI plus bevacizumab. Finally, the combination of intra-arterial infusion of irinotecan-loaded drug-eluting beads (DEBIRI), with the FOLFOX plus Bevacizumab regimen, led to a 78% RR, and 35% of downsizing to resection, in patients with unresectable, liver-limited CRC, representing a new, promising, treatment strategies in this subset of patients[92].

Resection rates with anti-EGFR agents: Cetuximab and panitumumab

Anti-EFGR agents, cetuximab and panitumumab, are active both as single agents as well as in combination with chemotherapy in mCRC, with activity is confined to patients with RAS (both *KRAS* and *NRAS*) wild type tumors[93]. Five key randomized trials have evaluated the effects of cetuximab in patients with unresectable liver metastasis: (1) OPUS (Oxaliplatin and cetuximab in first-line treatment of MCRC)[94]. Addition of cetuximab to FOLFOX-4 almost doubled the R0 resection rate from 2.4% (FOLFOX-4 alone) to 4.7% (cetuximab plus FOLFOX-4); (2) CRYSTAL (cetuximab combined with irinotecan in first-line therapy for MCRC)[95]. Addition of cetuximab to FOLFIRI led an increase in the R0 resection rate from 3.7% to 7.0%; (3) colorectal Liver Metastases (CELIM)[22]. Patients received neoadjuvant treatment with cetuximab plus either FOLFIRI or FOLFOX6 and resections were performed in 43% of patients overall; 34% had RO resections; (4) POCHER (Cetuximab plus chronomodulated irinotecan, 5-fluorouracil, leucovorin and oxaliplatin as neoadjuvant chemotherapy in CLM)[96]; and (5) a Randomized Controlled Trial of Cetuximab Plus Chemotherapy for Patients With *KRAS* Wild-Type, Unresectable, Colorectal Liver-Limited Metastases, has recently shown that the addiction of Cetuximab to chemotherapy, significantly improved the R0 resection rate (25.7% *vs* 7.4%, P = 0.01)[97].

We need to discuss the reasons for the discrepancies in secondary liver resection rates in KRAS WT liver limited disease between these five studies following CT + Cetuximab. Overall RR was 60%-79% across these 5 studies but hepatectomy rates after CT + Erbitux was 9% in OPUS; 16% in CRYSTAL, 43% (33%R0) in CELIM, 60% in POCHER, and 25% in the recent Chinese trial. In the latter studies, resectability was detected by a multidisciplinary team, including a liver surgeons, while in CRYSTAL and OPUS, it was detected by non-specialist oncologists.

Two other randomized trials (COIN[98] and NORDIC Ⅶ[99]), have recently shown that Cetuximab adds no benefit to the Oxaliplatin chemotherapy regimen, in first-line treatment of mCRC, irrespectively of K-RAS status, even if in the COIN study cetuximab resulted in a higher response rate in patients with wild-type *KRAS* tumors[98].

Resection rates have also been reported in first-line panitumumab trials in patients with mCRC. Indeed, in a phase Ⅱ single-arm study, panitumumab plus FOLFIRI treatment resulted in resection rates of 15% *vs* 7% in the *KRAS* WT and mutant (MT) groups, respectively[100]. In a large, randomized phase Ⅲ study, of the 16% of patients with liver-limited disease, R0 resections were achieved in 32% of patients receiving FOLFOX4 plus panitumumab *vs* 28% of those receiving FOLFOX4 alone[101]. Baseline resectability was not recorded and so conversion rates could not be assessed. However, in a subsequent post-hoc analysis of the PRIME study, including RAS WT (both *KRAS* and *NRAS*) patients with liver metastasis only, Panitumumab plus FOLFOX resulted in conversion of about one-third of initially unresectable patients, enabling metastasectomy in 31% and complete resection in 29%, compared with 22% metastasectomy and 17% complete resection in the chemotherapy arm[102]. Recently, a retrospective-prospective analysis of PRIME study has shown that NRAS mutations predicted a lack of response to anti-EGFR Panitumumab. Infact, the subgroup of patients reporting NRAS mutations, representing 17% of non-mutated KRAS population, reported inferior outcomes, which were consistent with the outcomes of the KRAS mutated patients[93]. This highlights the importance for detecting other RAS mutations to better select a subgroup of patients most likely to benefit from anti-EGFR Mabs.

USE OF RESECTION IN CLINICAL PRACTICE

Patient selection

Difficulties remain in deciding who is resectable in clinical practice. Most liver surgeons accept the current AHPBA consensus on definition of resectability[103]. Until recently, CLM resection was mostly offered to those patients with liver-only disease that was (ideally)

detected metachronously after curative resection of the primary tumor, confined to one lobe of the liver, had less than 3 metastases, the largest of which was smaller than 5 cm in diameter. These patients need to have a margin of healthy liver tissue of more than 1 cm[104,105]. This would restrict CLM resection to < 10% of patients with liver-limited disease. Although the definition of resectable disease is broadening, patient selection guidelines for resection of CLM remain controversial, with an increase in aggressive management approach being used in recent years[8]. The criteria for CLM resectability are not standardized and are related to the experience of the surgeon and of the multidisciplinary team (MDT). Different teams and surgeons might approach the same patient differently. Current guidelines state that resection should be considered for solitary or confined liver metastases[24]. The remaining liver also needs to be healthy (viable vascular inflow and biliary and vascular outflow) and represent 20%-25% of liver volume at presentation[106]. Extra-hepatic disease is no longer an absolute contraindication for CLM resection[27]. This means that at least 20% of patients with liver-only disease are now considered candidates for resection. Multiple resections can also be safely performed if there is sufficient healthy remnant liver[12] and the risks of surgery are not too great. Survival benefit following repeat resection appears similar to that following the first liver resection[107,108]. General factors that influence safe liver resection include patient age, performans status, and concurrent parenchymal liver disease. Contraindications include unresectable extrahepatic disease, significant parenchymal liver disease, or patient unfit to undergo the procedure[12]. As difficulties remain in deciding who is resectable, many studies have examined potential prognostic factors for outcome following resection, with the aim of developing preoperative criteria for the selection of patients who may benefit from resection of CLM. Many clinical and pathological factors have been evaluated as potential prognostic determinants of survival after surgical resection of CLM. Such as: age, sex, primary tumor stage, synchronous or metachronous hepatic metastases, extrahepatic distant metastases, surgical margin, tumor size, number and distribution of CLM; carcinoembryonic antigen level, type of hepatectomy, and adjuvant chemotherapy. In Japan, Fong et al evaluated clinical, pathologic and outcome data for 1001 patients with mCRC undergoing resection[109]. Seven criteria were identified that predicted for worse prognosis after resection. Five of these were subsequently chosen for a preoperative scoring system (the Clinical Risk Score). These were: node-positive primary, disease-free interval from primary to metastases < 12 mo, number of hepatic tumors > 1, largest hepatic tumor > 5 cm, and carcinoembryonic antigen level > 200 ng/mL. Patients with a score less than 2 had favorable prognostic characteristic after resection, scores of 3-4 were considered candidates for resection followed by adjuvant therapy. Prognosis was poor for those with scores of 5. This Clinical Risk Score has subsequently been validated and found to be highly predictive of patient outcome and survival[110]. More recently another scoring system was developed in Japan[111,112]. This, included six variables which showed overlap with those used in the Clinical Score (multiple tumors, the largest tumor > 5 cm in diameter, resectable extrahepatic metastases, serosa invasion, local lymph node metastases of primary cancers, and postoperative disease free interval of less than 1 year including synchronous hepatic metastasis). In line with the criteria mentioned above, a recent population-based study of patients with isolated CLM, increasing age, poor performance status, and high initial tumor burden were all associated with a decreased rate of referral to a hepatobiliary surgeon[8]. Novel qualitative morphologic criteria by CT evaluation have also been identified to predict the response to bevacizumab-containing chemotherapy in patients with CLM[113]. Moreover, the optimal response to preoperative treatment according to these morphologic criteria translated into a survival benefit following hepatic resection. Finally, a recent study by Karagkounis *et al*[114], consistently with the findings of other 3 studies[115-117], has shown that both RAS and BRAF mutations are associated with a worse prognosis after resection of CLM. These interesting evidences support the introduction of new treatment decision models in the management of CRC patients with liver metastatic disease, taking into account the new molecular factors as indicators of "biological resectability", together with the other clinical-pathological factors, in order to predict the outcomes of patients undergoing resection of CLM, and select good candidates for surgery.

MDT APPROACH TO PATIENT MANAGEMENT

Patients with cancer have complex needs and so their care cannot be addressed optimally by a single specialty or discipline. To ensure the optimal management and treatment of patients with mCRC throughout their treatment history, a MDT approach is now the norm in most European countries. Colorectal MDTs should also identify/establish a specialised hepatobiliary MDT that can provide the required additional expertise and facilities for patients with CLM[6]. Some studies in patients with liver-only metastases have showed improved survival among patients undergoing resection who are managed by a MDT including a liver surgeon[118,119]. The MDT would normally comprise two or more specialist surgeons with a high level of skills and training in liver resection surgery. Other team members may include an oncologist, diagnostic and interventional radiologists with expertise in hepatobiliary disease, a histopathologist, and clinical nurse specialist[6]. There should be regular interaction

and discussion within the MDT to ensure that resection is utilized where appropriate and to ensure that patients not initially considered resectable are brought into the resectable category wherever possible. For example the MDT should be consulted regarding choice of combination chemotherapy and targeted agents, duration of chemotherapy break before/after surgery, care choices and follow-up screening *etc*.

Thus, patients with mCRC may see a colorectal surgeon, a liver surgeon, and a medical oncologist to define optimal therapy. Medical oncologists should use the most active treatment for the shortest time by combination of chemotherapeutic regimens and targeted drugs to achieve tumor shrinkage without harming the normal liver. Defining the acceptable residual functioning liver volume may require assessment by a radiologist working with a liver surgeon[6]. Resection can be useful even at later lines of therapy and so it is important that the MDT is consulted at each stage of a patient's treatment. Repeat resection can be safely and effectively performed with survival races similar to those following initial resection[107,108,120]. Throughout the patient's disease course, the clinical nurse specialist/nurse practitioner is key to providing them with advice, support and information.

CONCLUSION

Patients with pretreated mCRC have few treatment options available, resection of metastatic disease is the only potentially curative strategy. Criteria for resectability have changed in recent years leading to an increased use of resection in patients with mCRC. No OS differences between simultaneous resection and staged resection of the primary tumour and resectable synchronous liver metastases. Increasing data suggest that biological agents (alone of combined with chemotherapy)-especially those targeting the EGFR-may be particularly useful in facilitating resection of liver metastases. Molecular biomarkers (first KRAS, and more recently NRAS), influences dramatically the anti-EGFR Mab activity and their identification have become mandatory for proper treatment planning in oncology. No OS benefit to adding perioperative chemotherapy to surgery for resectable liver metastases. Patients with mCRC should be managed by a MDT to ensure optimal treatment choices are made over their disease course, including optimizing opportunities for potentially curative resection of metastatic disease.

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REVIEW

Integrated approach to colorectal anastomotic leakage: Communication, infection and healing disturbances

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Abstract

Colorectal anastomotic leakage (CAL) remains a major complication after colorectal surgery. Despite all efforts during the last decades, the incidence of CAL has not decreased. In this review, we summarize the available strategies regarding prevention, prediction and intervention of CAL and categorize them into three categories: communication, infection and healing disturbances. These three major factors actively interact during the onset of CAL. We aim to provide an integrated approach to CAL based on its etiology. The intraoperative air leak test, intraoperative endoscopy, radiological examinations and stoma construction mainly aim to detect and to prevent communication between the intra- and extra-luminal content. Other strategies including postoperative drainage, antibiotics, and infectious-parameter evaluation are intended to detect and prevent anastomotic or peritoneal infection. Most currently available interventions for CAL focus on the control of communication and infection, while strategies targeting the healing disturbances such as lifestyle changes, oxygen therapy and evaluation of metabolic biomarkers still lack wide clinical application. This simplified categorization may contribute to an integrated understanding of CAL. We strongly believe that this integrated approach should be taken into consideration during clinical practice. An integrated approach to CAL could contribute to a better understanding of the etiology of CAL and eventually better patient outcome.

Key words: Colorectal anastomotic leakage; Integrated approach; Prevention; Prediction; Intervention

Core tip: Colorectal anastomotic leakage (CAL) remains

the most dangerous complication after colorectal surgery. In this review, we propose an integrated approach for CAL, consisting of three major parts, communication, infection, and healing disturbances. This simplified categorization is based on the etiology of leakage and may contribute to our integrated understanding of CAL, and eventually facilitate an integrated approach to CAL and eventually better patient outcome.

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INTRODUCTION

Colorectal anastomotic leakage (CAL) still remains a frequent and dangerous complication after gastrointestinal surgery, occurring in 4%-33% of patients and contributing to one third of postoperative mortality[1]. An anastomotic defect causes leakage of colonic content into the abdominal or pelvic cavity leading to peritonitis, abscess formation or sepsis[2]. CAL substantially prolongs hospital stay - by one to two weeks - and increases medical costs by as much as $24000 within the first period of hospitalization, thereby approximately tripling the expenditure relative to that of normal recovery[3,4]. Moreover, CAL is identified as a risk factor for local recurrence of colorectal cancer and is reported to reduce long-term cancer specific survival[5]. The need for more effective strategies to prevent and detect CAL is undoubtedly urgent. Many previous studies have explored techniques targeting the prevention, detection and intervention of CAL, but little attention has been paid to the systematic categorization of these strategies. To this end, we aim to provide surgeons with an integrated understanding of these strategies by a categorization based on CAL etiology.

INTEGRATED ETIOLOGY

In research, many efforts have been devoted to identifying risk factors of CAL such as being male[6], smoking[7], alcohol abuse[7], obesity[8], a high American Society of Anesthesiologists (ASA) score[9], low level (*e.g.,* rectal) anastomosis[10], late tumor stage[6], urgent operation[9], increased blood loss[11], after-hours surgery[12], corticosteroids administration[13], and prolonged duration of surgery[14]. However, these risk factors seem to cover most patients, and thus do not contribute to the understanding of the etiology of CAL.

Doctors and researchers still do not understand the detailed etiology of CAL. In many previous studies, CAL was attributed to technical failure or ischemia[15,16], but neither of these seem to explain the whole mechanism[17]. This emphasizes the need for an integrated approach regarding the etiology of CAL.

Based on previous literature and our investigations, we categorized the etiology of CAL into three major components: communication, infection, and healing disturbances (Figure 1).

Communication represents the classic definition of CAL: "communication between the intra- and extra-luminal compartments of the anastomotic bowel"[2]. Infection indicates bacterial infection at the anastomotic site, which is usually shown as anastomotic abscess or peritonitis. Healing disturbances represent pathological factors that may cause delay in wound healing.

We propose these three major components mainly due to two reasons. First, based on our observations and previous studies, evidence regarding these three aspects was always observed in patients with leakage such as lower anastomotic bursting pressure, anastomotic abscess, peritonitis, ischemia or anastomotic hypoxia[18-21]. Second, we also found that at least one of these factors can be identified as the main cause in CAL cases, which may also cause the other two as these factors actively interact with each other. For instance, it is known that severe infection significantly reduces organ perfusion[22], which may further worsen the healing process of the anastomosis, resulting in CAL. Furthermore, bacterial endotoxins activate the inflammatory response and cause infiltration of inflammatory cells, including subtype-I macrophages, which produce nitric oxide by inducible nitric oxide synthase (iNOS)[20,23]. This overexpression of iNOS is associated with a decrease in collagen deposition[24,25], which eventually causes a delay in wound healing and subsequent communication between intra- and extra-luminal bowel compartments.

PREVENTION

Nowadays, several techniques are available which could contribute to the prevention of CAL. In previous studies, surgeons and researchers have often categorized these strategies based on the time of application (*e.g.,* preoperative, intraoperative and postoperative)[26]. In addition to that, we divided these strategies into the three proposed categories, which further reveals their underlying mechanism (Figure 2).

Prevent communication

Many preventive strategies aim to prevent communication between intra- and extra-luminal compartments of the anastomosis.

The air leak test (ALT) is most frequently used as an intraoperative test in colorectal surgery to identify a technically failed anastomosis, which may cause direct communication between intra- and extra-luminal compartments. The rate of this intraoperative test varies greatly in studies evaluating the ALT[27]. Surprisingly, our

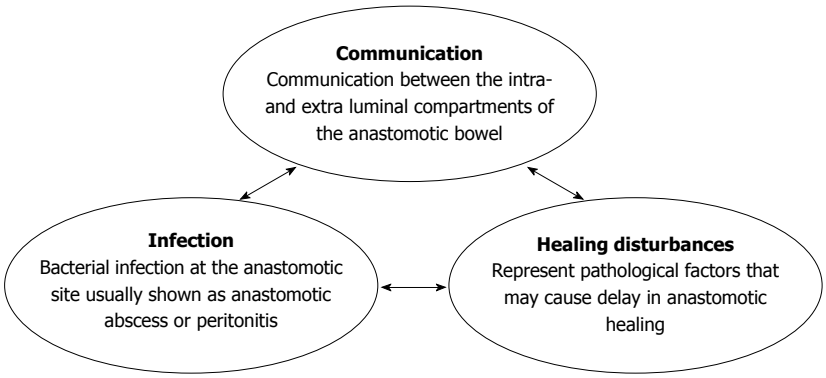

Figure 1 Integrated approach with proposed categorization based on the etiology of colorectal anastomotic leakage (communication, infection and healing disturbances).

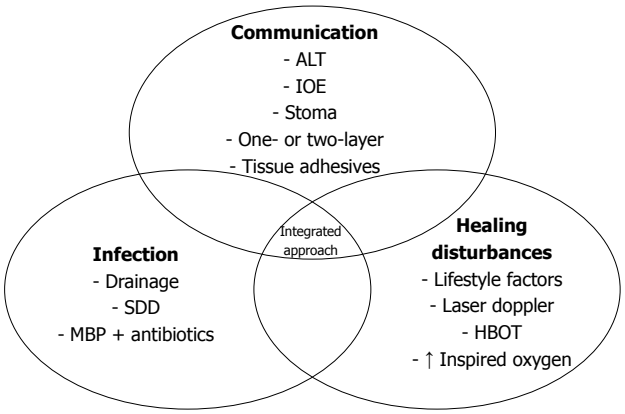

Figure 2 Preventive strategies for colorectal anastomotic leakage with regard to the proposed categorization based on the etiology of colorectal anastomotic leakage (communication, infection and healing disturbances). ALT: Air leaking test; IOE: Intraoperative endoscopy; SDD: Selective decontamination of the digestive tract; MBP: Mechanical bowel preparation; HBOT: Hyperbaric oxygen therapy.

on-going study shows that meta-analysis of previous studies did not find a significant decrease in the rate of CAL in patients who underwent the ALT[28]. This may partly be due to marked variation in ALT methodology. However, we also found a much higher CAL rate in patients who had a leak during the test[28], thus the ALT is still necessary in our daily practice.

Similar to the ALT, intraoperative endoscopy (IOE) is another intraoperative test which, ideally, could allow immediate diagnostic and therapeutic interventions. However, relevant studies on this topic are very limited and show a low level of evidence. Several authors suggest the selective use of IOE in patients during surgery based on their retrospective data. However, there are at least two studies which show that routine IOE does not reduce the CAL rate compared to selective use[29,30]. Since performing IOE requires certain facilities and equipment, it still seems too early to draw conclusions regarding this technique, especially for routine use[31]. Further research on this topic is required.

Another way to prevent communication is to reinforce the anastomosis. One conventional strategy is to perform a second layer anastomosis. This technique has been used for decades, if not centuries, and was once considered the standard technique for colorectal anastomosis. However, studies have shown that the one-layer anastomosis does not result in a higher CAL rate, hence it is as safe as the double-layer technique[32,33]. Due to these non-inferior results, both the one-layer and the double-layer techniques have their own followers and are being used by different surgeons.

Reinforcing an anastomosis with tissue adhesives is used as another strategy and may serve as a sealant and prevent possible microscopic leakage. The most frequently used tissue adhesive in clinical practice is fibrin glue, which is considered to both reinforce the strength of the anastomosis and facilitate wound healing due to its ingredients[34]. However, analysis of the clinical data shows no actual beneficial influence of the intraoperative application of fibrin glue[35].

Our *ex vivo* research demonstrated that fibrin glue, together with many other sealants, were very weak in mechanical tests[36]. Many animal studies have also shown that fibrin glue does not accelerate wound healing[37,38]. Nevertheless, one type of tissue adhesive, cyanoacrylates, has emerged from our series of experiments[39]. This glue is preferred over the other glues in mechanical tests, as it increases the mechanical strength of colorectal anastomosis in both normal and technically insufficient situations[40]. Although animal studies have suggested many promising applications of various tissue adhesives[20,23], clinical data are limited and inconclusive. Further clinical research on this topic is planned by our group.

A temporary stoma is also a technique which prevents communication by diverting the intra-luminal content. Although the effect of preventing CAL with diversion seems unquestionable[41], previous studies on this topic have resulted in different conclusions[42-45]. We should be careful with the unselective use of stomas to prevent CAL as stomas are associated with high complication and comorbidity rates[46]. Therefore, routine diversion with a "temporary" stoma should not

be recommended in regions with sufficient follow-up of the patients.

Prevent infection

Preventing infection is another major area in CAL prevention. One important technique is drainage placement. The purpose of drainage placement seems evident: it helps to eliminate localized toxins and thus prevents infection and its further advancement. Nowadays, drainage placement is omitted in more and more colonic surgeries especially in centers applying the ERAS (Early Recovery After Surgery) program, while in most centers it remains routine practice after anterior rectal resection. However, several contradictory meta-analyses are available regarding the effect of drainage[47-49]. The most recent meta-analysis indicates that a pelvic drain reduces the incidence of extra peritoneal CAL and the rate of re-intervention after anterior rectal resection. These findings are based on the analysis of observational studies. In contrast, the analysis of RCTs did not indicate any benefit of drainage[48].

Another strategy to prevent infection is the application of preoperative selective decontamination of the digestive tract (SDD), which aims to eradicate pathogenic microorganisms with oral antibiotics before elective resection. There is currently one on-going randomized controlled trial, the SELECT trial[50], which is investigating the use of SDD. The results of this trial are expected to further modify the current clinical regimen.

Bowel preparation also follows the concept of preventing infection by eliminating intraluminal pathogens. However, the conventional "mechanical bowel preparation" has been greatly challenged by accumulating evidence which suggests that it may not reduce the risk of CAL, but only substantially delays the return of bowel function[51]. However, evidence for or against the use of oral mechanical bowel preparation is still too weak to change this worldwide clinical practice. Whether bowel preparation should be included into routine preparation for colorectal surgery still requires data from future investigations.

Prevent healing disturbances

Many healing disturbances have been identified as preoperative risk factors of CAL such as diabetes mellitus and smoking. Therefore, a preoperative assessment of the patient's condition is important in the prevention of CAL. Many life-style changes and medical interventions should be arranged before admission. However, the clinical influence of many of these strategies remains unclear and is yet to be determined.

Of course, not all healing disturbances are reversible before surgery. Bowel ischemia contributes to the occurrence of CAL[16,52,53], and therefore intraoperative measurement of the cutting edges may help to detect ischemic edges and may theoretically assist surgeons in the alternative management of the anastomosis (reconstruction or diversion)[54]. However, it is important to note that there is no solid (*i.e.*, high level) evidence supporting such an application. Although observational studies have demonstrated the safety of such a device, it remains unclear whether those detected "ischemic" edges would eventually cause any clinical side effects. Further studies on this topic are necessary before further wide application.

Perioperative tissue oxygen tension measurement could also provide information on anastomotic perfusion[55]. In 1985, it was demonstrated in rabbits that lower tissue oxygen tension was associated with CAL[56]. Therefore, several animal experiments were performed to establish whether Hyperbaric Oxygen Therapy (HBOT) could prevent CAL[57-59]. All studies demonstrated that HBOT increases tissue oxygen tension and improves anastomotic healing. In addition, it is known that high intraoperative inspired oxygen fraction reduces surgical site infections[60,61]. A double-blinded RCT indicated that perioperative supplemental oxygen administration reduced postoperative anastomotic dehiscence after total gastrectomy[62]. The same study group performed a RCT on major rectal cancer surgery and found similar results[63]. With these data, the perioperative application of oxygen therapy seems promising; however, its application is still limited in current clinical practice.

PREDICTION AND EARLY DETECTION

CAL is usually detected between day 5 and day 8 postoperatively, or even later after surgery[64], with more than 50% of cases requiring a reoperation[2,65]. This suggests that with the current strategy many early stages of leakage are not detected until they progress to a severe status. Early diagnosis is necessary as delayed diagnosis of CAL increases postoperative mortality[66]. Figure 3 provides an overview of the methods of prediction and early detection, which have been assessed during the last decades.

In most cases, conventional radiological examinations are still required to confirm the occurrence of CAL. However, decision-making on radiological examinations depends on the surgeon's awareness, which is based on clinical manifestations and laboratory tests. Fever, abdominal pain and prolonged ileus are considered clinical manifestations of CAL but are common after colorectal surgery[67,68]. Based on risk factor analysis and expert opinions, several scoring systems have been developed to predict the individual risk of developing CAL after surgery[69-71]. Dekker *et al*[69] proposed the Colorectal Leakage Score based on the literature and expert opinions. In 2013 den Dulk *et al*[70] suggested the modified DULK score, which evaluated postoperative factors to estimate the risk of CAL. These scores may help the surgeon make an individualized decision, but prospective evaluation of

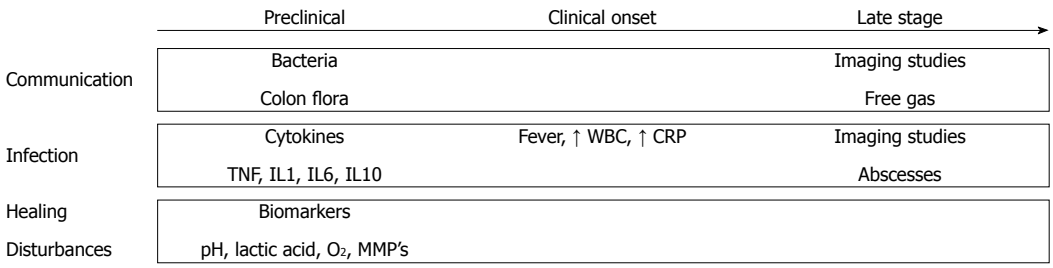

Figure 3 Overview of the methods of prediction and early detection of colorectal anastomotic leakage with regard to the proposed categorization based on the etiology of colorectal anastomotic leakage (communication, infection and healing disturbances). TNF: Tumor necrosis factor; IL: Interleukin; WBC: White blood cell; CRP: C-reactive protein; MMP: Matrix metalloproteinase.

these scores is still limited to date.

Early detection of communication

Imaging studies aim to show whether communication exists between the intra- and extra-luminal compartments of the anastomosis. Routine imaging studies may decrease the interval between diagnosis and treatment of CAL, but are not ideal due to radiation exposure, costs, patient discomfort and false positives because of subclinical CAL[66,72]. In addition, the diagnostic accuracy of imaging tests is still under debate. The sensitivity of CT-scanning for the early detection of CAL varies from 15% to 52%. The main problem for routine use of CT-scanning is the high reported rate of false negatives[73-75]. The other option for radiological evaluation of colorectal anastomoses is contrast radiography. The sensitivity and specificity of this alternative imaging test range from 20% to 52% and from 85% to 87%, respectively, when performed routinely at postoperative day 7 or 8[76,77]. When contrast radiography is performed in patients with clinical symptoms, the diagnostic accuracy is reported to be higher, with a sensitivity of 68% and a specificity of 94%[73]. Nevertheless, we should be aware that the interval between operation and the examination is often more than a week, indicating that this technique may not be adequate in detecting CAL at an early stage, but only when leakage has already progressed to a severe state in which abscesses or free gas are already present and indicated by imaging studies.

Recent studies focus on innovative strategies to detect CAL as routine radiological examinations are not preferred because leakage is detected at a relatively late stage. An early screening tool for CAL could be the detection of colon flora in drain fluid. The presence of colon flora in drain fluid is suggestive of communication between intra- and extra-luminal compartments and causes infection at the anastomotic site in patients with leakage[2]. Although promising, there are few studies which have considered the predictive value of bacterial measurement in drain fluid. Fouda *et al*[78] evaluated intraperitoneal bacterial colonization using cultures during the early postoperative period after rectal surgery. *Escherichia coli*, Bacteroides and Pseudomonas showed significant differences between leaking and non-leaking patients at postoperative day 1, 3 and 5. These results indicate that this method may decrease the period to diagnosis of CAL. However, it takes at least 48 hours before bacteria can be identified on quantitate cultures, resulting in an inevitable delay in diagnosis. Therefore, Komen *et al*[79] proposed the use of RT-PCR techniques for the detection of bacteria in drain fluid. This technique is much faster, more sensitive and less susceptible to contamination than culture. It achieved a negative predictive value of 98.7%, although its positive predictive value was unsatisfactory (31.6%).

Early detection of infection

Leukocyte count and serum C-reactive protein (CRP) levels are often abnormal after surgery both in CAL patients and in a substantial number of patients with uncomplicated recovery. Therefore, these parameters have a limited predictive value for CAL[67,80]. In 2014, a meta-analysis by Singh *et al*[81] was published which assessed the predictive value of serum CRP levels for CAL. Rather than determining the positive predictive value, this article reported a negative predictive value of 97% for CRP on postoperative day 3-5, while the corresponding positive predictive value for leakage ranged between 21% and 23%.

In addition to white blood cell count and CRP levels, other innovative inflammatory biomarkers have also been tested in several studies for the early detection of CAL. Inflammatory cytokines such as TNF-a, IL-1b, and IL-6 have been evaluated in both peritoneal drain fluid and blood samples. Cini *et al*[82] performed a meta-analysis and found that cytokine levels in drain fluid were significantly higher in CAL cases. However, Ellebæk *et al*[83] reported that serum levels of inflammatory cytokines were the same in patients with CAL and in those with normal recovery. This is because the onset of CAL is a progressive process. A localized response at the site of the anastomosis occurs before systemic changes such as fever, leukocytosis and septic symptoms become manifest[84]. Therefore, monitoring changes in cytokine levels in drain fluid could contribute to the early detection of CAL[85], while systematic changes remain latent until CAL is at an advanced stage[86]. The data from these studies seem promising; however, the main problem with the available literature

is that these reports provide a low level of evidence due to low sample sizes, poor patient selection and lack of standardization[78,87-91]. Further examination of these parameters may be an interesting topic for future studies.

Early detection of healing disturbances

As many metabolic biomarkers represent healing disturbances, the detection of metabolic parameters may be another strategy for the early detection of CAL. However, clinical data on this topic are very limited, mainly due to a lack of proper sensors[18]. Daams *et al*[92] showed promising results using the minimally invasive method of intraperitoneal microdialysis. This technique enabled measurement of real-time local ischemia and changes in metabolism by establishing dialysate levels of lactate, pyruvate, glucose and glycerol[93-95]. Due to a lack of clinical data, how to correctly interpret these metabolic data and associate them with anastomotic healing remains difficult and requires further investigation[96,97].

INTERVENTION

Once leakage has occurred, an effective intervention should be undertaken to control morbidity and mortality. The ultimate goal of prediction or early detection of CAL is to initiate timely treatment to improve patient outcome. The type of intervention strongly depends on the severity of CAL, which as discussed above, is hard to determine and therefore the choice of intervention for a suspicious leakage is quite complex with very limited evidence available at present[2].

Despite individual experience from surgeons, the best knowledge regarding intervention in CAL came from a Delphi analysis, which used an expert panel and aimed to emphasize consensus[98] and to construct evidence-based guidelines[99]. Phitayakorn *et al*[100] used this technique to develop a treatment algorithm for CAL.

Interventions for CAL can be divided into two main groups: treatment of infection and treatment of communication. Interventions which prevent communication also contribute to infection control, therefore most interventions for CAL require an integrated approach.

Administration of antibiotics is often the first intervention when CAL is suspected. Antibiotics are usually modified after the results of the susceptibility test are obtained when drainage or blood samples are cultured. A retrospective study showed that both surgical and non-surgical interventions based on the presentation of CAL are both effective and safe[101]. There are several surgical intervention options: drainage, repair of the anastomosis, deviating ileostomy or permanent colostomy. It is known that a stoma after colorectal surgery moderates quality of life. Moreover, half of patients who undergo the formation of a stoma due to CAL are left with a permanent stoma[102]. Given that routine construction of a stoma for CAL repair should not be recommended, alternative surgical strategies should be discussed and considered before reoperation[103].

If surgical re-intervention is indicated, and the surgeon decides to construct a stoma, the choice between diversion of the anastomosis with a loop ileostomy and resection of the anastomosis with end colostomy should be made. A questionnaire completed by members of the Dutch Society for Gastrointestinal Surgery showed that Dutch colorectal surgeons prefer to preserve the anastomosis in young non-septic patients, whereas the anastomosis is broken down and a colostomy is constructed in older patients or in those with abdominal sepsis[104]. Despite the surgeon's experience, this choice strongly depends on the severity of leakage and comorbidities in the patient[105]. Some data suggest that diversion with loop ileostomy is safe and is associated with less mortality and morbidity if no sepsis or fecal contamination is present[106,107], but no solid evidence or consensus is available in this regard.

Most re-interventions were initiated with an open approach until recently when two retrospective cohort studies showed that laparoscopic re-intervention for CAL was safe and feasible[108,109]. With more and more surgeons experienced in the laparoscopic approach, we may expect laparoscopy as the first choice for re-interventions in the future.

CONCLUSION

CAL remains the most dangerous complication after colorectal surgery. Surgeons still have to deal with this critical issue mainly based on their experience and limited knowledge from the literature. In this review, we proposed an integrated etiology of CAL, consisting of three major parts including communication, infection, and healing disturbances. Based on the etiology, we categorized the currently available strategies into at least one of these major factors. This simplified categorization may contribute to our integrated understanding of CAL. All three aspects should be taken into consideration during clinical practice regarding prevention, prediction, early detection and intervention of CAL, which we believe will eventually facilitate an integrated approach for CAL and result in better patient outcome.

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REVIEW

Transient elastography (FibroScan®) with controlled attenuation parameter in the assessment of liver steatosis and fibrosis in patients with nonalcoholic fatty liver disease - Where do we stand?

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease worldwide. Currently, the routinely used modalities are unable to adequately determine the levels of steatosis and fibrosis (laboratory tests and ultrasonography) or cannot be applied as a screening procedure (liver biopsy). Among the non-invasive tests, transient elastography (FibroScan®, TE) with controlled attenuation parameter (CAP) has demonstrated good accuracy in quantifying the levels of liver steatosis and fibrosis in patients with NAFLD, the factors associated with the diagnosis and NAFLD progression. The method is fast, reliable and reproducible, with good intra- and interobserver levels of agreement, thus allowing for population-wide screening and disease follow-up. The initial inability of the procedure to accurately determine fibrosis and steatosis in obese patients has been addressed with the development of the obese-specific XL probe. TE with CAP is a viable alternative to ultrasonography, both as an initial assessment and during follow-up of patients with NAFLD. Its ability to exclude patients with advanced fibrosis may be used to identify low-risk NAFLD patients in whom liver biopsy is not needed, therefore reducing the risk of complications and the financial costs.

Key words: Non-alcoholic fatty liver disease; Transient elastography; Controlled attenuation parameter

Core tip: Non-alcoholic fatty liver disease (NAFLD) patients are at risk of NAFLD-related cirrhosis and

hepatocellular carcinoma, particularly in the setting of liver fibrosis with concurrent metabolic syndrome. Transient elastography (TE) with controlled attenuation parameter (CAP) is a fast, reliable, repeatable non-invasive method for the assessment of liver steatosis and fibrosis. TE with CAP may be used to diagnose and monitor patients with NAFLD. TE with CAP is a favorable means of excluding advanced fibrosis.

Mikolasevic I, Orlic L, Franjic N, Hauser G, Stimac D, Milic S. Transient elastography (FibroScan®) with controlled attenuation parameter in the assessment of liver steatosis and fibrosis in patients with nonalcoholic fatty liver disease - Where do we stand? *World J Gastroenterol* 2016; 22(32): 7236-7251 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i32/7236.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i32.7236

INTRODUCTION

An increasingly common cause of chronic liver disease in adults and children is nonalcoholic fatty liver disease (NAFLD). In adults, the prevalence of NAFLD ranges from 17% to 33%[1], whereas in children, it ranges from 2.6% to 9.6%, and from 22.5% to 44% in children with obesity[2]. Because of the ongoing epidemics of metabolic syndrome (MetS) and its individual components, the incidence of NAFLD is increasing in both adults and children. The individual components of MetS include diabetes mellitus type 2 (T2DM), obesity, arterial hypertension and dyslipidemia. The presentation of NAFLD may vary from simple steatosis to nonalcoholic steatohepatitis (NASH), liver cirrhosis and hepatocellular carcinoma (HCC)[1-4]. It is expected that NASH related cirrhosis and NASH related HCC may soon become the most frequent indications for liver transplantation[1]. Interestingly, in patients with NAFLD, liver-related mortality is the third cause of death and malignancy, whereas cardiovascular diseases are the main cause[1]; thus, cumulative evidence indicates that NAFLD is correlated with many extrahepatic diseases[1-3]. Many authors have suggested that NAFLD is not only a marker of MetS but also a risk factor for cardiovascular diseases (CVD), chronic kidney disease (CKD) and T2DM. Moreover, it is a risk factor for malignancy (colorectal carcinoma) and other conditions (sleep apnea, osteoporosis, psoriasis, and polycystic ovary syndrome)[1,3]. The clinical implication of these findings is that NAFLD patients may benefit from more intensive monitoring and early therapeutic interventions to lower the risk of cardiovascular and kidney diseases, as well as the risk for malignancies, HCC and colorectal carcinoma[3].

NAFLD AND METS

The association of NAFLD with MetS has been described with respect to the relation of both conditions with insulin resistance (IR), the basic pathogenetic factor underlying NAFLD and MetS. The main problem in assessing MetS risk is that it is not a disease but a clinical syndrome. MetS is manifested by the clustering of acquired metabolic factors that primarily increase the likelihood of cardiovascular events (myocardial infarction, stroke, and peripheral arterial disease). Thus, it has been suggested that NAFLD should be included as a fifth risk factor in the definition of MetS (the other four factors include obesity, dyslipidemia, arterial hypertension and glucose intolerance/diabetes mellitus); however, others consider NAFLD not as a hepatic manifestation of MetS but a separate condition only associated with MetS[3,4]. Whether the former or latter is true, NAFLD is strongly associated with all components of MetS. The prevalence of NAFLD in the general population is 20%-30%, whereas the prevalence rates are approximately 50%, 50%-90%, 30%-50%, and 80%-90% in patients with hypertension, hyperlipidemia, T2DM, and obesity, respectively. Compared with MetS, one-third of patients with NAFLD have MetS, and 90% of NAFLD patients have at least one positive MetS criterion[5-7].

However, cross-sectional studies cannot define the time relationship between NAFLD and MetS components. The general belief that MetS precedes NAFLD has been questioned after a longitudinal study demonstrated an increased risk for the development of MetS in NAFLD patients (HR = 1.70, 95%CI: 1.55-1.87)[8]. The similar, yet opposing effect of MetS increasing the risk for NAFLD development has also been confirmed, with a slightly increased hazard ratio (1.94, 95%CI: 1.78-2.13) compared with that reported in the previous study[9]. This bidirectionality is not limited to the occurrence of NAFLD and MetS; it also affects MetS components and disease severity. The presence of NAFLD increases the risk of developing arterial hypertension (HR = 1.07, 95%CI: 1.00-1.15 for mild steatosis; HR = 1.14, 95%CI: 1.00-1.30 for severe steatosis) and type 2 diabetes mellitus (two-fold risk increase)[10,11]. However, the presence of MetS increases the risk of developing NAFLD, depending on the number of MetS components present, with the combination of dyslipidemia and central obesity carrying a 3.01 hazard ratio (95%CI: 2.68-3.37) of NAFLD development. NAFLD severity is also affected by the presence of MetS, which increases the risk of inflammation - NASH (OR = 3.2, 95%CI: 1.2-8.9) and severe fibrosis (OR = 3.5, 95%CI: 1.1-11.2)[12]. Of the individual components, the strongest correlation appears to occur with abdominal obesity. A 1% increase in visceral fat carries an OR of 2.4 (95%CI: 1.3-4.2) for increasing liver inflammation and an OR of 3.5 (95%CI: 1.7-7.1) for increasing fibrosis. The predictive value regarding advanced steatohepatitis and fibrosis remains after correcting for IR and hepatic steatosis, with an OR of 2.1 (95%CI: 1.1-4.2) for advanced steatohepatitis and an OR of 2.9 (95%CI: 1.1-4.2) for fibrosis[13]. From a clinical viewpoint, the presence of NAFLD increases the risk of cardiovascular

event-associated deaths by approximately two-fold, independently of other cardiovascular risk factors[14,15].

PATHOGENESIS OF NAFLD

The pathogenesis of NAFLD remains an unsolved problem with a plethora of implications and potential solutions for clinical practice. Many people are affected by NAFLD; however, most (approximately 60%-70%) remain asymptomatic, with simple liver steatosis. The main challenge faced by researchers is the identification of patients in whom the disease will progress and why. Elucidating the details of pathogenesis will provide the answer to this question, thereby allowing researchers to focus on the 30%-40% of NAFLD patients who require intensive observation, follow-up and prevention (treatment) to halt the development of cirrhosis and HCC[16,17].

The central role in the development of NAFLD is reserved for IR. IR disrupts lipid metabolism by increasing peripheral fatty acid release. The increased free fatty acid levels combined with hyperinsulinemia result in the development of hepatic IR and increased hepatic triglyceride synthesis. Hepatic triglycerides accumulate as fat droplets in hepatocytes, thus resulting in what is referred to as a fatty liver[18,19]. Whether IR may represent a consequence of NAFLD appears to have been disproved by the discovery of specific genetic mutations (*e.g.*, PNPLA gene), which result in the development of NAFLD without peripheral IR[20]. This simplistic view is mirrored by the lack of an effect of IR-reducing medications on NAFLD and the current lack of an effective, established anti-NAFLD treatment other than lifestyle modifications and increased physical activity. IR affects NAFLD; however, the interplay of multiple factors appears to affect the character of the disease[3,21].

Another important factor underlying NAFLD is oxidative stress. Multiple studies have demonstrated a close relationship among mitochondrial dysfunction, the overproduction of reactive oxygen species (ROS) and NAFLD, namely, NASH[22,23]. Imperfect fatty acid degradation results in increased free radical production, which is manifested by the production of various lipid peroxides. If sufficient mitochondria are affected, the resulting leak of mitochondrial components may induce hepatocyte apoptosis[24,25].

Extracellular signals, such as proinflammatory cytokines, also appear to affect the development of NAFLD. Studies have demonstrated an effect of free hepatocyte lipids on the induction of various, predominately proinflammatory, intracellular signaling pathways (NF-κB, c-Jun, and diacylglycerol), which in turn worsen IR and contribute to the hepatic production of various proinflammatory cytokines (*e.g.*, interleukin-6 and tumor necrosis factor α)[26].

In previous years, studies have implicated gut microbiota dysbiosis as a potential building block in NAFLD pathogenesis. The effect does not appear to be directly mediated by bacteria; instead, it may be mediated by various bacterial products that enter the portal blood stream. Moreover, the mechanisms include the effects of bacteria-produced short-chain fatty acids on the energy balance and intestinal barrier permeability of the host, the effect of bacteria on intestinal motility and the effect of various absorbed toxins (lipopolysaccharides) on the liver[27].

The main challenge with understanding NAFLD pathogenesis is correctly positioning the small pieces (risk factors) in their respective places to "see the big picture". Currently, there are three main theories regarding how the "pieces" should be positioned: the "two-hit", "multi-hit" and "distinct-hit" theories[28].

The two-hit theory was initially suggested in the late 1990s. It is based on the finding that only a portion of patients with a fatty liver develop advanced forms of NAFLD, indicating that the "first hit", IR-induced liver steatosis, is like a barrel of gasoline that requires a "second hit", ignition (*e.g.*, mitochondrial dysfunction, cytokines, or bacterial endotoxins), for activation[29].

The theory is highly contested because of findings that some patients develop hepatic inflammation without pre-existing IR, and in most patients, more than two factors are typically present. Thus, the second "multiple parallel-hits" theory was created. It is based on the premise that the "first hit" is not a single factor, but a sum of multiple distinct factors that wear down liver defenses. Again, the basic mechanism is IR and its associated metabolic disturbances. The result, a fatty liver, is prone to multiple "hits", which wear down liver defenses and eventually lead to inflammation (NASH) and fibrosis[30].

In recent years, a third "distinct-hit" theory has been proposed. It is based on the presumption that NAFLD and NASH are two separate diseases, which are associated with IR but unrelated to each other. This theory is based on epidemiological data indicating that patients with NASH have a 10%-20% chance of disease progression to cirrhosis during a 5-10-year period, whereas individuals with NAFLD typically manifest a stable disease, with a low risk of disease progression. Other data include the previously described genetic NASH predisposition, in which liver inflammation occurs without peripheral IR[31,32].

TRANSIENT ELASTOGRAPHY (FIBROSCAN®) WITH CONTROLLED ATTENUATION PARAMETER

In clinical practice, the initial diagnosis of NAFLD is typically established through laboratory findings (increased levels of aminotransferases and gamma-glutamil transferases) and radiological imaging techniques in the absence of other recognized causes of fatty liver (*e.g.*, alcohol, virus, drugs, or autoimmunity). Because of space limitations, this review will

not discuss the use of the various NAFLD diagnostic techniques[3]. In everyday clinical practice, biomarkers are needed to determine excess fat in the liver, as well as inflammation and fibrosis of the liver. However, it is less likely that specific proteins/biomarkers will be identified for the detection of liver steatosis/fibrosis. Whether currently available biomarkers for NAFLD severity are useful in monitoring NAFLD progression (or regression) in people with MetS is uncertain. In recent years, substantial attention has been focused on one dimensional transient elastography (TE). TE is a non-invasive ultrasound-based method that uses shear wave velocity to assess tissue (*e.g*., liver) stiffness. Shear (secondary or S-) waves were initially discovered in seismology as slow waves that follow the primary compressional wave, hence their name. They are the manifestation of elastic waves that travel through the body of an object, as opposed to the surface waves, which, as the name implies, travel on the surface. In contrast to sound waves, which are longitudinal, shear waves are transverse, thus the motion of the affected tissue is perpendicular to the direction of wave propagation. As a result, shear waves move slowly (< 50 m/s) and are rapidly attenuated by liver parenchyma. This effect depends on the elastic properties of the tissue, with the speed of shear waves inversely proportional to the tissue elasticity. The method was designed at the Langevin institute in 1995 and was initially implemented for quality control in the food industry; however, since 2001, it has been applied in medical practice under the name FibroScan®[33].

In practical terms, the TE device consists of a vertically oriented mobile cuboid main body and one or several cylindrical probes. Measurements are performed on patients lying supine with their right rib cage spread (which is accomplished by elevating the right hand and/or crossing the right leg over the left). After gel application, the probe is positioned perpendicular to the skin surface in one of the intercostal spaces adjacent to the right lobe of the liver (typically the 9th to 11th intercostal space, on the midaxillary line). Shear waves are affected by changes in the medium density, particularly in the presence of liquid medium; thus, the operator must avoid large vascular structures. To avoid this problem and ensure better results, the TE device is equipped with a small scale, real-time, ultrasonographic display of the tissue that underlies the probe in both A- and M-modes. After adequate positioning, a low frequency shear wave is induced by a small piston positioned on the tip of the probe that hits the skin surface. On the basis of the physical characteristics (velocity and intensity attenuation) of the shear wave, the acquired data are processed and displayed on the screen as the liver stiffness measurement (LSM) and controlled attenuation parameter (CAP). Unsuccessful measurements are automatically excluded by the device; the numerical results are not displayed, and the message *"invalid measurement"* is displayed[34,35].

The measurement of liver stiffness is based on Hook's law, which states that the velocity of shear waves that travel through an elastic object is proportional to the object's stiffness (*i.e*., inversely proportional to the object's elasticity). The law is mathematically expressed as $E = 3\phi v^2$, where E represents Young's modulus (expressed in kPa), φ represents the tissue density (expressed in kg/m^3, assumed to be the same as water) and v shear represents the wave velocity (expressed in m/s). Young's modulus clinically corresponds to the LSM and is typically referred to as *E* or *LSM*. The practical application is made possible using a probe that emits two types of waves. The probe (piston) initially causes a slow-spreading low-frequency (50 Hz) shear wave, after which the fast ultrasound waves (emitted from the same probe) in a pulse-echo fashion are used to determine the position of the shear wave front in relation to time; thus, the velocity of the shear wave and hence the LSM are determined. LSM values range from 1.5 to 75 kPa; lower values indicate a more elastic liver. The shear waves spread from the point of skin impact in a spherical manner, whereas the ultrasound waves are released in a straight line along the probe's axis, *i.e*., in one dimension. To ensure that the measurements are accurate and reproducible in the same patient and are comparable among different patients, the accompanying software modifies the shear wave characteristics by maintaining the shear wave frequency and shape while modifying the shear wave amplitude and energy output. Thus, the full name of the method is vibration-controlled 1D TE. The results are also affected by the amount of pressure applied to the probe, in which a lack of pressure results in incomplete contact with the underlying skin, whereas too much pressure modifies the shear wave by changing the stiffness of the underlying tissue. These errors are prevented by the software, which displays warning signs and disables probe activation when the applied pressure is not adequate for measurement[36,37]. The applied technical solutions have resulted in high intra- and interobserver levels of agreement, 98% in both cases, according to clinical data[38]. The resulting LSM is translated into an estimate of the level of liver fibrosis in a simple and straightforward manner. However, this is estimation is possible only under the assumption that the liver is homogeneous and non-viscous, and its elasticity is predominantly affected by the level of fibrosis. This feature is true for liver parenchyma; however, a problem arises with regard to the capsule of Glisson. The capsule is a sturdy tissue that provides the liver with its form and protects it from mechanical injuries. It adapts over time to changes in the liver size; however, it does not respond well to abrupt changes. Consequently, a rapidly developing mass effect inside the liver will increase the intrahepatic pressure and thereby reduce the liver elasticity. These conditions include right-sided (global) congestive heart failure, acute inflammation and/or edema of the hepatic

tissue, and extrahepatic cholestasis[39-41]. Therefore, in everyday practice, LSM is not an absolute measure of liver fibrosis but is instead a component of liver assessment and cannot be interpreted independently of other clinical results (*e.g*., anamnesis, physical examination, laboratory tests, and imaging methods). Interestingly, food intake and alcohol consumption have also been demonstrated to affect LSM. Regarding food intake, different studies have reported varying results; however, a minimum two-hour fast is currently recommended prior to the exam[42,43]. Active alcohol consumption appears to lead to an overestimation of the LSM because one study has found that patients who were actively drinking at the first TE exam and subsequently stopped had significantly lower LSM values at the control TE exam several months later[44].

The basis for TE development was the measurement of liver stiffness; thus, LSM has been present in TE devices from its inception. However, conventional ultrasonography has demonstrated that liver steatosis, another important liver parameter, affects ultrasound waves by strongly attenuating their intensity. The changes in signal attenuation are followed by an increased reflection of incoming ultrasound waves, which results in the liver appearing bright (hyperechoic). The main problems with conventional ultrasonography are its subjective operator-dependent nature and multiple uncontrolled variables included in the examination, which decrease the sensitivity of the examination in the detection of liver steatosis. The effect is more pronounced when small amounts of fat are observed, and the sensitivity becomes substantially lower (12% in patients with a 5%-9% fat content in contrast to 80% in patients with a $\geqslant$ 30% fat content)[45]. The theoretical background consists of the formula for intensity attenuation: $I_z = I_0 e^{-\alpha_f z}$, where I_z represents the ultrasound intensity (expressed in W/m^2) at depth z (expressed in m), I_0 represents the initial intensity (expressed in W/m^2) and α_f represents the ultrasound attenuation coefficient (expressed in dB/m). The α_f coefficient is primarily affected by two parameters, including the frequency of the emitted ultrasound wave and the properties of the conducing object (liver). With a fixed and known frequency (3.5 MHz), α_f is directly affected and proportional to the level of steatosis; thus, it is typically referred to as the CAP. CAP values range from 100 to 400 dB/m, and higher numbers indicate more pronounced steatosis. The advantage of CAP is that it is simultaneously calculated with the LSM and from the same region of interest. The clinical application of CAP began in 2011, ten years after the introduction of LSM[46].

The benefit of TE compared with liver biopsy is that it measures a larger region of interest, namely, a cylindrical liver segment 1 cm wide and 4 cm long at a medium depth of 4.5 cm. This region amounts to a volume of 3 cm^3, which is approximately 100 times larger than the volume of the liver cylinder obtained by liver biopsy. The drawback is that the information (LSM and CAP) cannot be obtained by a single measurement. The final result is obtained as a median of at least 10 measurements. The procedure is deemed a failure if 10 valid measurements cannot be obtained, the percentage of valid measurements compared with the total number of measurements is less than 60% and/or the interquartile range exceeds 30% of the median[47]. Boursier *et al*[48] have investigated a group of 1165 chronic liver disease patients and have determined that an interquartile median ratio $\leqslant$ 10% is the best predictor of accuracy. In addition to the previously described factors that are controlled by the device, two important factors that increase the measurement failure and may be only partially offset by the device include the body size and intercostal space width. Similarly to conventional ultrasonography, the body mass index (BMI) negatively affects TE measurements, resulting in falsely increased LSM values in obese individuals and rendering the standard probe unreliable in patients with a BMI $\geqslant$ 28 (30) kg/m^2. A study by Castéra *et al*[49] has reported a 3.1% failure rate for obtaining valid results, which was associated with a BMI $\geqslant$ 30 kg/m^2 (OR = 7.5) and operator inexperience (defined as having performed fewer than 500 examinations, OR = 2.5). The number of unreliable results was higher and affected 15.8% of the examined patients; again, unreliability was related to a BMI $\geqslant$ 30 kg/m^2 (OR = 3.3) and operator inexperience (OR = 3.1). Of the obesity measures, LSM failure and LSM unreliability were predominantly related to waist circumference (> 80 cm in women, > 94 cm in men; OR = 3.0). To solve this problem, a new probe was developed with a more sensitive ultrasound transducer using a lower shear wave frequency, increased vibration amplitude, deeper focal length (mean depth 5.5 cm) and a greater depth of measurement. The probes were renamed after clothing sizes, and the standard probe represents the M probe, whereas the new probe represents the XL probe. Similar problems regarding narrow intercostal spaces have been identified in children and asthenic adults, thus necessitating the development of the S probe. Similarly to the M probe, the new probes could initially measure only the LSM; however, this issue has been resolved with the adjustment of CAP measurements for the new probes[50-53]. The advantages and disadvantages of TE are summarized in Tables 1 and 2.

The main initial clinical focus of TE was to assess the level of liver fibrosis (LSM) in patients with chronic viral hepatitis and to reduce the need for invasive procedures (liver biopsy). To date, the studies performed have demonstrated a good correlation of LSM with liver biopsy in the identification of significant liver fibrosis (F $\geqslant$ 2) and cirrhosis (F4). The AUROC for the identification of significant fibrosis in hepatitis B patients (cut-off values from 5.2 to 8.0 kPa) ranges from 0.86 to 0.97, with a sensitivity range of 70%-94% and a specificity range of 38%-99%. The AUROC for the identification

Table 1 Limitations of transient elastography with controlled attenuation parameter

Limitations	Explanation
Ascites	Elastic waves do not travel through liquids
Obesity	BMI > 30 kg/m^2 is associated with TE failure. With the development of the XL probe, the failure rate in obese patients has decreased
Acute hepatitis	Tissue changes in acute hepatitis may increase LSM
Chronic hepatitis with transaminases flare	At ALT levels greater than 5 × the upper normal limit, there is a risk of overestimating the fibrosis stage. LSM interpretations in patients with high ALT levels must be made with caution
Extrahepatic cholestasis	Increases LSM independently of fibrosis stage
Congestive heart failure	May lead to increased LSM because of an increased blood volume in the liver
Narrow intercostal spaces	Associated with a lower success rate or failed acquisition of LSM. Reduced failure rate with the development of the S probe

BMI: Body mass index; LSM: Liver stiffness measurement; ALT: Alanine-aminotransferase.

Table 2 Advantages of transient elastography with controlled attenuation parameter

Most widely used and validated non-invasive technique
High range of values
Well defined quality criteria
Good reproducibility
Detects liver stiffness and steatosis from the same region of interest
Excellent for the exclusion of cirrhosis
Prognostic value in cirrhosis
User-friendly
Short duration, painless
Applicable as a screening method in large populations

of significant fibrosis in hepatitis C patients (cut-off values from 5.2 to 9.5 kPa) ranges from 0.73 to 0.91, with a sensitivity range of 56%-97% and a specificity range of 32%-91%. Regarding cirrhosis, the AUROC for identification in hepatitis B patients (cut-off values from 9.7 to 14.0 kPa) ranges from 0.80 to 0.97, with a sensitivity range of 52%-98% and a specificity range of 59%-99%. The AUROC for the identification of cirrhosis in hepatitis C patients (cut-off values from 11.9 to 14.8 kPa) ranges from 0.87 to 0.98, with a sensitivity range of 72%-94% and a specificity range of 85%-98%[39,54-69]. In summary, TE is better at the identification of liver cirrhosis compared with significant fibrosis (mean AUROC 94% *vs* 84%, respectively), and among hepatitis patients, it is better at excluding than confirming liver cirrhosis (negative predictive value 96%, positive predictive value 74%)[70,71]. The main drawback is the lack of clear-cut cut-off values for different stages of liver fibrosis because the ranges for different fibrosis levels often overlap, particularly with lower levels of liver fibrosis. One recently published meta-analysis including 4386 chronic hepatitis B patients has confirmed these statements. The meta-analysis has indicated cut-off values for significant fibrosis (F $\geqslant$ 2), a fibrosis level $\geqslant$ 3 and cirrhosis in the following ranges: 5.85-8.8 kPa, 7.0-13.5 kPa and 9.0-16.9 kPa, respectively. The respective mean AUROCs for the cut-off values are 0.88, 0.91 and 0.93, respectively. Thus, the increasing accuracy of TE in the diagnosis of higher levels of fibrosis should be noted, as well as the substantial range in the cut-off values used in different studies. The latter finding may be explained by the differences in the cirrhosis prevalence in the studies, which affects the interpretation of the results, as well as the significance of the cut-off values[72].

Despite the shortcomings, the role of TE in the assessment of the level of fibrosis in viral hepatitis patients has been recognized by the most recent EASL guidelines[54,73,74]. TE is currently considered to be the non-invasive standard for the measurement of liver stiffness, and it is the most accurate non-invasive method for the identification of liver cirrhosis in patients with chronic viral hepatitis[55]. Consequently, initial hepatitis C (HCV) staging includes the performance of TE to exclude liver cirrhosis. The gold standard for the non-invasive assessment of the degree of fibrosis includes performing TE with serum biomarkers because of the superior accuracy in comparison with that of either test alone. However, the use of two tests also results in increased costs, as well as the need to perform a liver biopsy when the tests are not in agreement[59,75,76]. In the case of hepatitis B (HBV), values less than 5-6 kPa indicate absent or minimal liver fibrosis, whereas values greater than 12-14 kPa are highly suggestive of cirrhosis. TE is also recommended in the initial assessment for HBV.

The use of TE in HCV patients to monitor the therapeutic response (reversal of cirrhosis) is discouraged because of a lack of clinical data. Even more disappointing, a single study has demonstrated a low sensitivity of 61% with 95% specificity in determining the reversal of cirrhosis[77]. Regarding HBV, the disease activity is a primary concern because inflammation and increased ALT levels are correlated with the overestimation of liver stiffness. The recommendations prompt the use of TE at least several months after ALT normalization to reduce the number of false positive results and to obtain a realistic value of the liver stiffness. However, the use of TE has been demonstrated to have a good prognostic value regarding the development of HCC. This association was initially identified in cross-sectional studies; however, because of the study design, the prognostic value could not be established[78,79]. Proof has come from subsequent prospective longitudinal studies that have demonstrated a progressive increase in the risk of HCC development with increased initial LSM values (Table 3)[80,81].

Table 3 Hazard ratio of hepatocellular carcinoma development in relation to liver stiffness measurement (according to Masuzaki *et al*[80] and Jung *et al*[81])

HCV		HBV	
LSM (kPa)	HR	LSM (kPa)	HR
10.1-15	16.7	13.1-18	4.68
15.1-20	20.9	18.1-23	5.55
20.1-25	25.6	> 23	6.60
> 25	45.5		

HCV: Hepatitis C virus infection; HBV: Hepatitis B virus infection; LSM: Liver stiffness measurement.

LSMS FOR THE PREDICTION OF FIBROSIS STAGE IN NAFLD

In daily clinical practice, specific biomarkers are needed that will demonstrate the amount of excess fat present in the liver, the level of fibrosis and the level of inflammation. In patients with NAFLD, the most important factor is the assessment of fibrosis severity and monitoring fibrosis progression. Most patients remain asymptomatic until their liver function is compromised; thus, the identification of the presence and severity of liver fibrosis remains a clinical challenge. This issue is important because efficient treatment for NAFLD has not yet been established. Therefore, the identification of risk factors for HCC and liver cirrhosis, such as liver fibrosis, should facilitate the implementation of risk-reduction mechanisms in NAFLD patients[82,83]. For the evaluation of fibrosis severity, a liver biopsy represents the "gold standard" in various liver diseases. Nevertheless, it is restricted by its complications and costs[84]. It is unrealistic to perform a liver biopsy for the diagnosis or monitoring of disease progression on all patients because 15%-40% of adults have NAFLD.

Despite the potential presence of high risk factors for fibrosis in NAFLD patients, such as diabetes, the population remains too large to implement an invasive method to exclude fibrosis[84,85]. Thus, noninvasive methods have been intensively investigated[84]. The various approaches include standard biochemical and hematological tests, surrogate fibrosis markers in the blood and their algorithms and, the most recently investigated approach, TE[85]. To date, TE assessment of liver fibrosis has predominantly been implemented in patients with chronic viral hepatitis, as well as patients with other chronic liver diseases of different etiologies[86]. Recent studies have examined the usefulness of LSM compared with liver biopsy to identify fibrosis in NAFLD patients. Table 4 shows details of eight studies that have examined the usefulness of LSMs in the identification of different stages of liver fibrosis in NAFLD patients compared with liver biopsy[83,84,87-92]. In these studies, for F ⩾ 2, the LSM cut-off values range from 6.2 to 11 kPa, with 62%-90% sensitivity and 74%-100% specificity. For F ⩾ 3, the LSM cut-off values range from 8 to 12 kPa, with 84%-100% sensitivity and 83%-97% specificity. For F4, the LSM cut-off values range from 9.5 to 20 kPa, with 90%-100% sensitivity and 75.9%-98.4% specificity.

Table 4 Usefulness of liver stiffness measurement compared with liver biopsy in the detection of fibrosis in nonalcoholic fatty liver disease patients

Study	Probe	Cut-off (kPa)	Sensitivity	Specificity	Number of patients with liver biopsy
Fibrosis stage ⩾ F2					
Imajo *et al*[83] (2016)	M	11.0	61.7	100	142
Pathik *et al*[84] (2015)	M	9.1	Not reported	Not reported	110
Yoneda *et al*[87] (2007)	M	6.65	81.8	91.2	67
Cassinotto *et al*[88] (2015)	M	6.2	⩾ 90	Not available	291
Wong *et al*[89] (2010)	M	7.0	88	74	246
Lupsor *et al*[90] (2010)	M	6.8	67	84	72
Yoneda *et al*[91] (2008)	M	6.65	88	74	97
Kumar *et al*[92] (2013)	M	7.0	78	79	205
Fibrosis stage ⩾ F3					
Imajo *et al*[83] (2016)	M	11.4	85.7	83.8	142
Pathik *et al*[84] (2015)	M	12.0	90	80	110
Yoneda *et al*[87] (2007)	M	8.0	87.5	84.3	67
Cassinotto *et al*[88] (2015)	M	8.2	⩾ 90	Not available	291
Wong *et al*[89] (2010)	M	8.7	84	83	246
Lupsor *et al*[90] (2010)	M	10.4	100	97	72
Yoneda *et al*[91] (2008)	M	9.8	85	81	97
Kumar *et al*[92] (2013)	M	9.0	85	88	205
Fibrosis stage F4					
Imajo *et al*[83] (2016)	M	14.0	100	75.9	142
Pathik *et al*[84] (2015)	M	20.0	90	80	110
Yoneda *et al*[87] (2007)	M	17.0	100	98.4	67
Cassinotto *et al*[88] (2015)	M	9.5	⩾ 90	Not available	291
Wong *et al*[89] (2010)	M	10.3	92	97	246
Yoneda *et al*[91] (2008)	M	17.5	100	97	97
Kumar *et al*[92] (2013)	M	11.8	90	88	205

A meta-analysis[93] in 2014 has indicated that TE is excellent in diagnosing F ⩾ 3 (85% sensitivity, 82% specificity) and F4 (92% sensitivity, 92% specificity), and it has a moderate accuracy for F ⩾ 2 in NAFLD

patients. LSMs were performed with an M probe, and obesity was the major reason for unsuccessful LSM. This problem may be avoided with the use of the novel XL probe. In a study by Wong *et al*[94], the XL probe was used to identify fibrosis in 193 NAFLD patients with a BMI ⩾ 30 kg/m^2 compared with liver biopsy, "the gold standard". Ten valid measurements were obtained in 93% of the patients, with AUROCs of 0.80, 0.85 and 0.91 for F ⩾ 2, F ⩾ 3 and F4, respectively. In a study conducted by Friedrich-Rust *et al*[85], the AUROC for significant fibrosis diagnosis (F ⩾ 2) for the XL probe was 0.82 compared with 0.84 for a severe fibrosis diagnosis (F ⩾ 3) and 0.95 for an F4 diagnosis. This study demonstrates that when measured with the XL probe, the median LSM is significantly lower than that measured with the M probe (6.9 kPa *vs* 8.4 kPa, respectively). According to these two studies, the LSM cut-off values should be approximately 1.5-2 kPa lower when the XL probe is used rather than the M probe for the same stage of fibrosis. This issue justifies the need for more studies on this topic because the existing cut-off values, which are defined for the M probe, cannot be used for the XL probe. Available data indicate that in patients with NAFLD, TE is a highly accurate, non-invasive method for advanced fibrosis exclusion and a moderately accurate method for significant fibrosis exclusion.

The use of paired biopsies for monitoring the progression of the disease in NAFLD patients has been reported. A prospective four-year study has been conducted by Suzuki *et al*[95], in which the disease progression in NAFLD patients was evaluated using TE. Ninety-seven NAFLD patients (demonstrated by liver biopsy) had their LSM obtained at the beginning of the study; of the 97 patients, 36 patients were available for reevaluation after four years, in which their stage of fibrosis was compared with that from their initial assessment. The authors concluded that LSM may be used to monitor hepatic fibrosis severity in patients with NAFLD. Nevertheless, additional prospective studies regarding the monitoring of LSM progression in patients with NAFLD are necessary.

CAP FOR THE PREDICTION OF STEATOSIS GRADES IN NAFLD

Liver steatosis may be defined radiologically as a fat mass comprising ⩾ 5% of the wet weight of the liver or histologically as a fatty deposit presence in ⩾ 5% of hepatocytes. Metabolic dysfunction of the liver may develop over time as a result of liver steatosis, which may consequently progress into irreversible damage to the liver, with the development of fibrosis, cirrhosis and HCC[96]. Steatosis of the liver is a key parameter in liver transplantation. Because any liver with a fat content > 30% is automatically ineligible for donation, determining the liver steatosis level is of substantial importance for the evaluation and clinical prognosis of patients with NAFLD[83]. Efforts regarding the development of reliable non-invasive methods for liver steatosis detection and quantification have been made over the past 10 years[96,97]. A strong correlation of CAP with fat accumulation in the liver (demonstrated by liver biopsy) has been identified in clinical studies investigating TE with CAP; moreover, CAP has been reported to be useful in the diagnosis of steatosis of the liver in numerous chronic liver diseases[83,96-104]. Table 5 shows that (similarly to the LSM cut-off values) the different CAP cut-off values presented by different studies for distinct grades of liver steatosis defined by biopsy (range from S0, which indicates no steatosis, to S3, which indicates the highest level of steatosis); for S ⩾ 1 (⩾ 10% of hepatocytes with fat), the CAP cut-off values range from 214 to 289 dB/m, with a 64%-91% sensitivity range and a 64%-94% specificity range; for S ⩾ 2 (⩾ 33% hepatocytes with fat), the CAP cut-off values range from 255 to 311 dB/m, with a 57%-96% sensitivity range and a 62%-94% specificity range; finally, for S3 (⩾ 66% hepatocytes with fat), the CAP cut-off values range from 281 to 310 dB/m, with a 64%-100% sensitivity range and a 53%-92% specificity range. According to these studies, CAP is useful in the detection of S ⩾ 1, S ⩾ 2, and S3 steatosis as a result of its good sensitivity and specificity; however, the exact cut-off values remain to be defined[83,98-103,105].

De Lédinghen *et al*[104] conducted a study in 2014 regarding the diagnosis of S1, S2 and S3. The cumulative AUROCs of CAP were 0.79 (95%CI: 0.75-0.84), 0.84 (95%CI: 0.80-0.88) and 0.84 (95%CI: 0.80-0.88), respectively. The study included 440 patients who had undergone a liver biopsy. Compellingly, obesity (defined as a BMI >30 kg/m^2) was determined to be the main cause of CAP measurement failure. It must be taken into account that both the 2014 de Lédinghen study and all studies described in Table 5 excluded the benefits of the CAP-enabled XL probe by using only the M probe. Furthermore, these studies demonstrate that the CAP cut-off values are not affected by the cause of the chronic liver disease, in contrast to LSM, in which the cut-off values depend on the type of liver disease[101].

WHAT IS THE POSITION OF TE WITH CAP IN THE ASSESSMENT OF NAFLD?

The significance of metabolic factors in the pathogenesis of NAFLD has been emphasized by numerous studies. As previously discussed, at least one component of MetS is present in approximately 90% of patients with NAFLD, whereas all diagnostic criteria for MetS are met in 35%-75% of patients. Furthermore, the risk for NAFLD is increased 4-11 times by the presence of MetS[3,106-109]. Mena *et al*[110] have identified an association between different MetS components and fibrosis in chronic hepatitis B patients. The pre-

Table 5 Performance of controlled attenuation parameter compared with liver biopsy for the detection of various steatosis grades

Study	Etiology of CLD	Probe	Cut-off (dB/m)	AUC	Sensitivity (%)	Specificity (%)	Number of patients with liver biopsy
Steatosis grade ⩾ 1							
Sasso *et al*[98] (2010)	CLD, ALD, NAFLD	M	238	0.91	91	81	115
de Lédinghen *et al*[100] (2012)	NAFLD, HCV, ALD, other	M	266	0.84	69	85	112
Shen *et al*[102] (2014)	NAFLD, HBV	M	253	0.92	88	83	189
Kumar *et al*[101] (2015)	HBV, HCV, NAFLD	M	214	0.68	64	64	317
Myers *et al*[99] (2012)	Hepatitis, NAFLD, other	M	289	0.79	68	88	153
Chan *et al*[103] (2014)	NAFLD, control	M	263	0.97	91	94	101
Imajo *et al*[83] (2016)	NAFLD, control	M	236	0.88	82.3	91	127
Lupşor-Platon *et al*[105]	HCV, HBV, NAFLD, other CLD	M	260	0.81	64.8	82.3	201
Steatosis grade ⩾ 2							
Sasso *et al*[98] (2010)	CLD, ALD, NAFLD	M	259	0.95	89	86	115
de Lédinghen *et al*[100] (2012)	NAFLD, HCV, ALD, other	M	311	0.86	57	94	112
Shen *et al*[102] (2014)	NAFLD, HBV	M	285	0.92	93	83	189
Kumar *et al*[101] (2015)	HBV, HCV, NAFLD	M	255	0.79	77	80	317
Myers *et al*[99] (2012)	Hepatitis, NAFLD, other	M	288	0.76	85	62	153
Chan *et al*[103] (2014)	NAFLD, control	M	263	0.86	96	67	101
Imajo *et al*[83] (2016)	NAFLD, control	M	270	0.73	64.3	73.6	127
Lupşor-Platon *et al*[105]	HCV, HBV, NAFLD, other CLD	M	285	0.82	69.7	85.1	201
Steatosis grade 3							
Sasso *et al*[98] (2010)	CLD, ALD, NAFLD	M	292	0.89	100	78	115
de Lédinghen *et al*[100] (2012)	NAFLD, HCV, ALD, other	M	318	0.93	87	91	112
Shen *et al*[102] (2014)	NAFLD, HBV	M	310	0.88	92	79	189
Kumar *et al*[101] (2015)	HBV, HCV, NAFLD	M	305	0.91	71	92	317
Myers *et al*[99] (2012)	Hepatitis, NAFLD, other	M	283	0.70	94	47	153
Chan *et al*[103] (2014)	NAFLD, control	M	281	0.75	100	53	101
Imajo *et al*[83] (2016)	NAFLD, control	M	302	0.70	64.3	73.6	127
Lupşor-Platon *et al*[105] (2015)	HCV, HBV, NAFLD, other CLD	M	294	0.83	83.3	82.5	201

CLD: Chronic liver disease; ALD: Alcoholic liver disease; NAFLD: Nonalcoholic fatty liver disease; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

sence of multiple MetS components is associated with fibrosis development, whereas significant fibrosis is uncommon in the absence of MetS.

The occurrence of NAFLD has increased as a result of the rapid increase in the prevalence of metabolic risk factors. Patients with NAFLD are at risk for liver-related morbidity and mortality. Numerous recent studies have indicated a significantly increased incidence of HCC in obese patients and patients with T2DM, and moreover, increasing evidence suggests an increased risk of HCC in NAFLD patients. As a result of the increasing incidence of NAFLD, an increase in NAFLD-related HCC is expected in the future[111].

The risk of developing CVD, CKD and T2DM in long-term follow-up is increased by the presence of NAFLD. The course of these diseases is also affected by NAFLD, because it increases the CVD and CKD risk. Moreover, NAFLD and MetS are more tightly associated because the presence of one condition increases the risk of developing the other condition. From a therapeutic standpoint, the prevention and treatment of hepatic IR, MetS and related complications represent a rational approach in reversing NAFLD, which is why various specialists, such as hepatologists, nephrologists, endocrinologists, cardiologists, general internists, and primary care physicians, should be involved in the care of NAFLD patients[108,112]. The strongest predictor of the progression of liver disease in NAFLD patients is the presence of NASH at the initial liver biopsy. In addition, the main determinant of all-cause and cause-specific mortality in patients with NAFLD is the severity of liver fibrosis[105-107,113,114]. Complicating matters, a recent study conducted by McPherson *et al*[114] has reported that 44% of the patients with simple steatosis at the index liver biopsy progressed to NASH, whereas in 37% of the patients, fibrosis progression was present at the follow-up biopsy. Furthermore, at the follow-up biopsy, 22% of the patients with baseline simple steatosis reached stage three fibrosis. The progression potential from simple steatosis to fibrosis and NASH has also been confirmed by other small studies[115,116]. An association between non-cirrhotic NAFLD and HCC risk has been demonstrated in recent studies[117,118]. According to a study in the United States, conducted between 2002 and 2008, the most common underlying risk factor for HCC is NAFLD, followed by T2DM and HCV infection[119]. Thus, contrary to current opinion, simple steatosis may progress to NASH and significant fibrosis, which, in turn, would indicate that most patients with NAFLD are at long-term risk of progressive liver disease[114-116]. Liver biopsy is the gold standard for liver fibrosis assessment; however, its limitations, complications and cost, given that approximately one-third of the population has NAFLD, make it unreasonable to perform it on all patients. A substantial number of physicians consider liver biopsy to be a diagnostic tool in patients who persistently exhibit increased liver function tests because of the substantial

number of patients at risk (T2DM, obesity, arterial hypertension, dyslipidemia and/or MetS). However, it must be considered that in more than half of NAFLD patients, aminotransferases are within the normal limits; therefore, deciding which patients with NAFLD are candidates for liver biopsy and how to monitor their liver disease progression leaves gastroenterologists with more questions than answers[120].

According to the most recent guidelines by the American Association for the Study of Liver Diseases (AASLD) in 2012[121], liver biopsy should be considered in high-risk patients with NAFLD, patients with an increased risk of NASH and advanced fibrosis. The presence of T2DM, MetS and/or an age > 50 years are risk factors. Systematic screening, at least for higher-risk patients (diabetic and obese patients), has been argued for by many authors. According to the AASLD guidelines, "screening for NAFLD in adults attending primary care clinics or high-risk groups attending diabetes or obesity clinics is not advised at this time because of the uncertainties surrounding diagnostic tests and the cost-effectiveness of screening"[121]. That guidelines were published in 2012 must be considered, and given the new data accumulating, updated guidelines are urgently needed.

MetS may be used to identify patients as candidates for liver biopsy, specifically when it is present with a noninvasive marker of liver steatosis/fibrosis because it predicts the presence of steatohepatitis in patients with NAFLD. TE with CAP may find its place in this approach, according to recent investigations. Interestingly, a study conducted by de Lédinghen *et al*[104] has demonstrated that the CAP value significantly increases with the number of parameters of MetS, BMI, waist circumference, presence of arterial hypertension and T2DM. Our recent analysis, including 648 patients with one or more components of MetS, has provided similar results; specifically, the CAP measurements progressively increase with the number of MetS components. In addition, the presence of MetS (or its individual components), IR, increased uric acid levels and an LSM > 7 kPa are factors independently associated with increased CAP (unpublished data). Kwok *et al*[113] have analyzed 1900 patients with T2DM for NAFLD using TE with CAP and have determined prevalence rates of increased CAP and LSM of 72.8% and 17.7%, respectively. Furthermore, that study included 94 T2DM patients with suspected advanced fibrosis or cirrhosis who had undergone liver biopsy, and of these 94 patients, 56% had NASH, 21% had advanced fibrosis and 29% had cirrhosis. Naveau *et al*[122] have analyzed 100 patients who were candidates for bariatric surgery. TE was performed 15 d prior to liver biopsy. The AUROC generated by TE was 0.81 ± 0.05 for the prediction of fibrosis stage F $\geqslant$ 2 and 0.85 ± 0.04 for the prediction of fibrosis stage F3. The authors have concluded that TE may be used for the early diagnosis of fibrosis in severely obese patients. In a similar study of hospitalized diabetic patients, de Ledinghen *et al*[123] have demonstrated an increased prevalence of severe fibrosis (defined by LSM), which was the highest in the > 50 year-old group of T2DM patients. Cho *et al*[2] have tested the feasibility of TE with CAP in 201 children in a comparison of CAP and LSM values in obese children, non-obese healthy controls and non-obese patients with liver disease. The authors found that the CAP values were increased in the obese group compared with the other two groups. Furthermore, they identified significantly higher LSM values in the obese group compared with the healthy control; however, no statistically significant differences in the LSM values were identified between the group with liver disease and the other two groups. In the obese group, the LSM values were highly correlated with the CAP values, whereas there was no correlation in the healthy control group or the group with liver disease[2].

On the basis of the findings in the previously described studies, CAP and LSM have a good correlation with the presence of MetS and its individual components.

The potential effect of NASH on the course and prognosis of NAFLD is a significant issue. The gold standard for the diagnosis and follow-up of NASH is liver biopsy. According to the study by Friedrich-Rust *et al*[85], the AUROC of TE for steatohepatitis diagnosis (according to the NAFLD activity score) was 0.79 for the M probe and 0.74 for the XL probe. In the study by Cho *et al*[2], LSM was mildly increased in patients with steatohepatitis, which may be attributed to inflammation, whereas similar results have been obtained in patients with alcoholic liver disease[124]. Thus, there is a possibility that LSM values in obese patients may be affected by steatohepatitis[2]. The obtained results indicate the urgency to conduct additional research to further clarify the position of TE with CAP in steatohepatitis management.

A subgroup of NAFLD patients (in the population of patients with one or more MetS components) who are at high risk of developing progressive liver disease may be identified by using TE with CAP because CAP and LSM show good correlations among MetS and its individual components and liver biopsy findings. The presence of MetS, which predicts the presence of steatohepatitis in NAFLD patients, in combination with a non-invasive method for liver fibrosis and steatosis detection may be used to identify candidate patients for liver biopsy. The identification of patients who are at risk for the development of NASH and advanced fibrosis and who require a liver biopsy may be performed through using MetS with high CAP values and particularly with increased LSM values. Available data suggest that to exclude advanced fibrosis in patients with NAFLD, TE is a highly accurate, non-invasive method, whereas it is a moderately accurate method for excluding significant fibrosis in patients with NAFLD, which is why TE with CAP may eventually replace liver ultrasound in the initial evaluation of patients with NAFLD. Taking into account the early observations that MetS and its individual components

(T2DM and obesity) are risk factors for the progression of liver disease in NAFLD patients, the identification of patients in need of a liver biopsy may be accomplished when MetS and its components are present together with increased CAP and particularly increased LSM. As a result of the parallel increasing incidence of both NAFLD and obesity, T2DM and MetS, including the consequences of MetS and NAFLD, *i.e*., the associated morbidity and mortality, the consideration of screening for NAFLD in all patients with one or more MetS components by a non-invasive method, such as TE with CAP, appears reasonable[125,126]. Per our analysis, increased CAP values have been found in patients with only one MetS component[126]. There is a disproportionately small number of studies conducted that have investigated TE in the setting of NAFLD and MetS, given that NAFLD is a common disease, and TE is becoming an increasingly used non-invasive method. Moreover, NAFLD is the most common cause of increased liver enzymes; however, it is critical to consider that AST and ALT may be within their normal ranges even in advanced NAFLD. Thus, the earlier opinion that NAFLD patients with persistently increased liver enzymes should be the only patients who undergo liver biopsy should be revised[125,126].

In parallel to the increasing need for a noninvasive assessment of liver fibrosis and steatosis, several imaging methods have emerged. Two other methods, in addition to TE, have shown promising results. The first method, acoustic radiation force impulse imaging (ARFI), is based on shear wave propagation, similarly to TE. Compared with TE, the inspected liver volume is smaller (1 cm in length); however, ARFI can be used on modified commercial ultrasound machines. Thus, the point of interest can be pinpointed using an ultrasound's B-mode. The downside of this method includes a narrow range of results (0.5-4.4 m/s) with unclear cut-off values for different fibrosis stage levels. Bota *et al*[127] have summarized the studies comparing the two methods in a meta-analysis, indicating comparable sensitivity (0.87 with 95%CI: 0.79-0.92 for ARFI *vs* 0.89 with 95%CI: 0.80-0.94 for TE) and specificity values (0.87 with 95%CI: 0.81-0.91 for ARFI *vs* 0.87 with 95%CI: 0.82-0.91 for TE) of both methods in the detection of liver cirrhosis. The reliability of the measurements is the principal difference between the two methods. ARFI fares better, with 2.1% unreliable results, compared with 6.6% for TE. The main reason for the unreliable results is obesity, and the studies included in the meta-analysis were based on TE measurements performed by the M probe; thus, these percentages should be interpreted with caution. The unreliability highlighted in that study is why the actual reliability difference between TE and ARFI must be re-assessed, including studies using the XL probe. Compared with TE, the inability to determine the level of steatosis is the clear disadvantage of AFRI. The other significant field of noninvasive liver fibrosis and steatosis assessment involves magnetic resonance imaging (MRI)-based methods. These approaches assess the liver in its entirety. The main advantage of MRI methods is that they are not affected by obesity or the presence of ascites. However, distinct methods are required to properly and independently assess liver fibrosis (*e.g.*, MRI elastography) and steatosis (*e.g.*, proton density fat fraction MRI). The high performance of MRI-based methods in assessment of advanced fibrosis (AUROC 0.957 with 95%CI: 0.918-0.996 for 2D-MRI elastography), as well as steatosis levels (correlation coefficient for the quantification of liver steatosis of 0.82 for proton density fat fraction MRI) in NAFLD patients has been demonstrated in recent studies[128,129]. Although MRI-based methods have demonstrated better diagnostic performances in non-invasive liver fibrosis and steatosis detection in patients with NAFLD compared with TE with CAP, there are major factors that limit this method, particularly in monitoring of the progression of the disease; these factors include cost, patient claustrophobia and duration of the examination[83,125].

Several questions should be addressed with additional studies. First, the question arises as to whether TE with CAP may be used to monitor NAFLD progression and whether the progression of LSM values may be used as a parameter of liver fibrosis severity. Because the only treatment option for NAFLD includes lifestyle changes and individual MetS component treatment, the question arises as to whether monitoring the changes in the CAP and LSM values could be used to assess the treatment of individual MetS components and the effect of treatment on NAFLD.

Second, taking into account portal hypertension, TE may potentially be useful in the identification of patients who are at risk of developing varices, as several studies have demonstrated. Furthermore, some studies have highlighted the potential utility of spleen stiffness measurements in the prediction of esophageal varices and portal hypertension level assessment in liver cirrhosis[124]. Thus, additional studies are required regarding this topic in patients with NAFLD.

The use of TE has been demonstrated to have a good prognostic value regarding the development of HCC in patients who suffer from viral hepatitis[78,79]; however, interestingly, there are no studies regarding the prediction of HCC development in patients with NAFLD *via* an assessment of the value of high LSM measurements. Thus, additional prospective studies are urgently required to answer this question. If the predictive value of TE with CAP were verified, clinicians would be able to assess and monitor the risk of HCC development and to establish optimal and personalized monitoring and treatment strategies in patients with NAFLD. Additional studies should also focus on investigating the accuracy of TE with CAP for all clinically significant events (*i.e*., liver cirrhosis and HCC) in patients with one or more MetS components.

Third, what is the place of TE with CAP in steatohepatitis detection? Yoneda *et al*[87] have demonstrated

a substantial increase of LSM in NASH patients, as confirmed by liver biopsy results; however, additional studies must be conducted. According to Yoneda *et al*[87], the LSM values are not affected by the degree of steatosis; however, additional studies must clarify this issue, and the influence of high grade steatosis on LSM values remains controversial.

Fourth, an investigation regarding whether the increased CAP and LSM values could predict the development of MetS in patients with one or two MetS components is needed. In addition, a question arises as to whether it is possible to monitor the changes in MetS and its individual components by monitoring the changes in CAP and LSM.

Given the associations between NAFLD and CVD and CKD risks, additional studies should determine whether patients with NAFLD with both increased CAP and particularly an increased LSM might benefit from early CKD and CVD screening. Finally, large studies are required for the development of new cut-off values for liver fibrosis staging using the XL probe and to investigate the differences between the CAP cut-off values used for the M and XL probes, respectively[84].

In conclusion, an easy, quick and non-invasive mass screening for NAFLD in patients with one or more MetS components may be reasonably achieved with TE with CAP. Once NAFLD is diagnosed, particularly liver fibrosis using LSM values, these patients should be directed to hepatologists, diabetologists and nephrologists. If TE with CAP is used as a screening method, liver biopsy may consequently be avoided in a substantial number of patients. This approach may also be useful in the early diagnosis of associated metabolic abnormalities and may enable the appropriate treatment of MetS, which is highlighted by its being the only available treatment option for patients with NAFLD to date. The accuracy of TE with CAP in the prediction of clinical events (*i.e.*, liver cirrhosis and HCC) in patients with one or more MetS components should be investigated in additional studies.

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REVIEW

Supportive therapies for prevention of hepatocellular carcinoma recurrence and preservation of liver function

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Abstract

Hepatocellular carcinoma (HCC) is one of the deadliest cancers in the world and is associated with a high risk of recurrence. The development of a wide range of new therapies is therefore essential. In this study, from the perspective of supportive therapy for the prevention of HCC recurrence and preservation of liver function in HCC patients, we surveyed a variety of different therapeutic agents. We show that branched chain amino acids (BCAA) supplementation and late evening snack with BCAA, strategies that address issues of protein-energy malnutrition, are important for liver cirrhotic patients with HCC. For chemoprevention of HCC recurrence, we show that viral control after radical treatment is important. We also reviewed the therapeutic potential of antiviral drugs, sorafenib, peretinoin, iron chelators. Sorafenib is a kinase inhibitor and a standard therapy in the treatment of advanced HCC. Peretinoin is a vitamin A-like molecule that targets the retinoid nuclear receptor to induce apoptosis and inhibit tumor growth in HCC cells. Iron chelators, such as deferoxamine and deferasirox, act to prevent cancer cell growth. These chelators may have potential as combination therapies in conjunction with peretinoin. Finally, we review the potential inhibitory effect of bone marrow cells on hepatocarcinogenesis.

Key words: Hepatocellular carcinoma; Liver cirrhosis; Branched-chain amino acids; Late evening snack; Iron chelators; Bone marrow cells

Core tip: Hepatocellular carcinoma (HCC) is one of the deadliest cancers in the world and is associated with a high risk of recurrence. Because liver function worsens upon repeated treatment for HCC recurrence, therapies that preserve liver function are essential. Here, we survey a variety of different therapeutic agents and then review the current status and prospects for prevention of HCC recurrence, particularly from the perspective of supportive therapy to preserve liver function. The agents included branched-chain amino acids (BCAA) supplementation, late evening snacking with BCAA, antiviral drugs, sorafenib, peretinoin, iron chelators, and bone marrow cells.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the second leading cause of deaths due to cancer in the world[1]. The prognosis of HCC has improved recently as a result of progress in a variety of therapies. These therapies include surgical resection, percutaneous-ethanol-injection (PEI), radiofrequency ablation (RFA), trans-arterial chemoembolization (TACE), administration of the drug, sorafenib, and liver transplantation[2-7]. The greatest problem with HCC is a high risk of recurrence, even when radical treatment is conducted. Recurrent HCC results in a fatal outcome for many patients with hepatic dysfunction. Antiviral therapies, such as nucleic acid analogs and interferon (IFN), have the potential to inhibit HCC recurrence after radical treatment of patients who have hepatitis B virus (HBV)- or hepatitis C virus (HCV)-related liver diseases[8,9]. Recently, however, the occurrence of both HBs antigen-negative and HCV antibody-negative HCC has actually increased in Japan as the development of antiviral agent and IFN treatments has progressed[10,11]. It is therefore necessary to develop other therapies to prevent HCC recurrence. Because liver function worsens upon repeated treatment for HCC recurrence, therapies that preserve liver function are essential. In this paper, we review the current status and prospects for prevention of HCC recurrence, particularly from the perspective of supportive therapy to preserve liver function.

First, we review the use of branched chain amino acids and late evening snack (LES), particularly for HCC patients with liver cirrhosis. These treatments are generally intended to address issues of protein-energy malnutrition (PEM) in these patients. We then review a range of chemoprevention options. These include antiviral therapies (nucleic acid analogs, IFN) for the treatment of hepatitis virus-related HCC, as well as treatment options such sorafenib, peretinoin, and iron chelators. Finally, we review the potential inhibitory effect of bone marrow cells (BMCs) on hepatocarcinogenesis.

BRANCHED-CHAIN AMINO ACIDS

Most patients with HCC have liver cirrhosis. Generally, liver cirrhotic patients suffer from PEM. These patients commonly exhibit decreased nutrient intake, hypermetabolism, and increased branched-chain amino acids (BCAA) consumption associated with ammonia metabolism in the skeletal muscle, leading to a decrease in plasma BCAA levels[12-14]. This decrease in plasma BCAA levels reduces protein synthesis in the liver and causes proteolysis in the muscle, which leads to edema and ascites with hypoalbuminemia and decrease in skeletal muscle mass.

BCAA granules consist of leucine, isoleucine, and valine, which are essential amino acids in humans. The Japanese Nutritional Study Group recommends administration of BCAA to liver cirrhotic patients who have serum albumin levels of 3.5 g/dL or less, a Fisher ratio of 1.8 or less, and a BCAA-to-tyrosine ratio of 3.5 or less[15]. In contrast, in the guidelines set by the American Society for Parenteral and Enteral Nutrition (ASPEN) and the European Society for Clinical Nutrition and Metabolism (ESPEN), oral administration of BCAA is recommended only in patients with liver cirrhosis who have hepatic encephalopathy[16,17].

A wide variety of effects of BCAA on chronic liver disease has been confirmed in previous fundamental research and clinical studies. Improvement of insulin resistance[18,19], improvement of hypoalbuminemia and reduction of oxidative stress[20,21], activation of immune function[22,23], promotion of liver regeneration[24,25], and inhibitory effects on hepatocarcinogenesis[26-28] have been reported. With regards to the mechanism of action, it has been reported that BCAA activates the insulin signal cascade through upregulation of phosphatidylinositol 3-kinase[29]. This serves to decrease circulating insulin levels and reduce the expression of insulin-like growth factors (IGF)-1 and IGF-2 as well as IGF-1 receptors to inhibit the IGF/IGF-1 receptor axis[29].

Given the biological properties referred to above, the following effects of BCAA on HCC can be anticipated: (1) inhibition of hepatocarcinogenesis associated with chronic liver disease; (2) prevention of a reduction in residual liver function caused by HCC treatment; and (3) prevention of recurrence after HCC treatment.

Muto *et al*[26] performed a multicenter, randomized controlled trial (RCT) that included 622 decompensated liver cirrhotic patients. They reported that oral administration of BCAA inhibited hepatocarcinogenesis

Table 1 Cohort studies (5 reports) and randomized controlled trial (3 reports) were available

Ref.	Group	Patient number	Therapy for HCC	Male/Female	Age (yr)	Child-Pugh A/B/C	Maximum tumor size (mm)	Study design
Togo *et al*[41]	BCAA	21	Surgery	17/4	66.5 ± 4.5	15/7/0	ND	RCT
2005	control	22		17/5	64.3 ± 9.1	17/5/0	ND	
Ichikawa *et al*[42]	BCAA	26	Surgery	18/8	64.7 ± 9.8	21/5/0	ND	RCT
2012	control	30		20/10	64.5 ± 11.4	25/5/0	ND	
Nishikawa *et al*[43]	BCAA	115	RFA	64/51	69.3 ± 9.4	83/30/2	19.5 ± 6.0	cohort
2013	control	141		85/58	70.9 ± 7.8	88/52/1	19.6 ± 6.6	
Yoshiji *et al*[44]	BCAA	51	RFA	32/19	63.6 ± 15.3	41/10/0	ND	RCT
2013	control	42		25/17	62.2 ± 14.8	33/9/0	ND	
Saito *et al*[45]	BCAA	13	RFA	8/5	73.4 ± 2.2	83/30/2	ND	cohort
2014	control	27		16/11	70.0 ± 1.9	88/52/1	ND	
Nishikawa *et al*[46]	BCAA	40	TACE	27/13	69.9 ± 8.8	6.4 ± 0.4	33.4 ± 16.7	cohort
2012	control	59		32/27	73.2 ± 10.1	5.4 ± 0.1 (score)	35.9 ± 14.7	
Takeda *et al*[47]	BCAA	34	Sorafenib	27/7	72 (55-88)	16/18/0	ND	cohort
2014	control	44		37/7	68 (46-89)	30/14/0	ND	
Imanaka *et al*[48]	BCAA	55	Sorafenib	45/10	72.2 ± 7.8/73.1 ± 6.4	37/18/0	ND	cohort
2015	control	201		167/34	72.4 ± 8.8/67.2 ± 13.0 (Child-Pugh A/B)	179/22/0	ND	

BCAA: Late evening snack using branched-chain amino acid; RFA: Radiofrequency ablation, TACE: Trans-catheter arterial chemoembolization, ND: Not done; RCT: Randomized control trial.

in obese patients [≥ body mass index (BMI) 25 kg/m^2] who had HCV-related liver cirrhosis.

Currently, there are many options for the treatment of HCC. Appropriate treatments such as surgical resection, PEI, RFA, TACE, hepatic arterial infusion chemotherapy (HAIC), molecular target-based therapy by sorafenib, and radiation therapy may be chosen depending on residual liver function and tumor stage. Improvements in prognosis have been observed using these approaches[1,30-40]. However, because recurrence of HCC can occur in liver cirrhosis patients after radical treatment, it is important to maintain residual liver function and to seek ways to inhibit the recurrence.

To our knowledge, there have been eight reports on the efficacy of BCAA granules in patients being treated for HCC; the HCC treatment in these reports was surgical operation (2 reports), RFA (3 reports), TACE (1 report), and molecular target-based therapy (2 reports). In terms of study design, both cohort studies (5 reports) and RCTs (3 reports) were available (Table 1)[41-48].

Early recovery of protein metabolism after hepatectomy can be achieved by administering BCAA granules[41]. Ichikawa *et al*[42] also reported that BCAA granules were effective in inhibiting early relapse after hepatectomy.

Reduction in the cumulative relapse rate and improvement in survival rate were observed after long-term oral administration of BCAA granules to patients who had received RFA[43-45]. Nishikawa *et al*[43] performed a retrospective study of 256 patients who had received RFA and had serum albumin levels of 3.5 g/dL or less. They reported improvements in overall survival (OS) and recurrence-free survival after oral administration of BCAA granules. The study also reported an improvement in OS in patients with HCC who suffered from obesity (≥ BMI 25 kg/m^2) and diabetes. Yoshiji *et al*[44] performed a RCT involving 93 patients who had received RFA and reported improvement of insulin resistance after oral administration of BCAA granules. They also found a decrease in levels of the plasma soluble form of vascular endothelial growth factor receptor 2 (VEGFR2) and a reduction in the cumulative relapse rate after RFA in liver cancer patients who had insulin resistance (≥ homeostasis model assessment for insulin resistance of 2.5) Thus, BCAA can be considered to inhibit recurrence of HCC and to improve survival rates through improved insulin resistance and an anti-angiogenic effect. This may be the same mechanism by which BCAA inhibits hepatocarcinogenesis in liver cirrhotic patients suffering from obesity.

For unresectable HCC, it is common to perform TACE repeatedly, but it is necessary to pay attention to the liver function after TACE. In this respect, it has been reported that administration of BCAA granules prior to TACE inhibited reduction of serum albumin levels measured three and six months after TACE, and helped maintain residual liver function in patients with Child-Pugh A/B[46].

In molecular target-based therapy using sorafenib for treatment of unresectable HCC, it is important to maintain residual liver function, as any reduction could lead to discontinuation of treatment and a poor prognosis. In patients with Child-Pugh A (but not in patients with Child-Pugh B), administration of BCAA granules when sorafenib is used inhibits reduction of serum albumin levels. The dosing period of sorafenib and the survival period are also prolonged[47,48].

These observations suggest that BCAA is effective in inhibiting hepatocarcinogenesis, maintaining residual liver function after HCC treatment, and preventing recurrence of HCC in patients with chronic liver disease.

Table 2 Cohort studies (2 reports) and randomized controlled trial (3 reports)

Ref.	Group	Patient number	Therapy for HCC	Male/Female	Age (yr)	Child-Pugh A/B/C	Maximum tumor size (mm)	Study design
Okabayashi *et al*[56]	LES-BCAA	40	surgery	29/11	65.7 ± 8.6	33/7/0	ND	cohort
2008	control	72		55/17	68.3 ± 8.1	62/10/0	ND	
Kuroda *et al*[57]	LES-BCAA	20	RFA	13/7	65.6 ± 7.0	8/11/1	20.2 (median)	cohort
2010	control	15		9/6	66.0 ± 8.1	6/8/1	19.8 (median)	
Morihara *et al*[58]	LES-BCAA	10	RFA	8/2	73.5 ± 8.5	9/1/0	20.0 ± 10.7	RCT
2012	Morning-BCAA	10		8/2	66.9 ± 9.7	7/3/0	24.3 ± 7.7	
	control	10		7/3	69.3 ± 8.0	7/3/0	24.4 ± 7.7	
Takeshita *et al*[59]	LES-BCAA	28	TACE	19/9	69.1 ± 8.231	6.107 ± 1.315 (score)	ND	RCT
2009	control	28		21/7	70.6 ± 9.745	5.53 ± 0.516 (score)	ND	
Harima *et al*[60]	LES-BCAA	13	HAIC	11/2	64.5 ± 9.5	6/7/0	77.7 ± 50.5	RCT
2010	control	10		8/2	66.4 ± 12.8	6/4/0	88.0 ± 39.7	

LES-BCAA: Late evening snack using branched-chain amino acid; Morning-BCAA: Morning BCAA administration; RFA: Radiofrequency ablation; TACE: Trans-catheter arterial chemoembolization; HAIC: Hepatic arterial infusion chemotherapy; ND: Not done; RCT: Randomized control trial.

Early administration of BCAA granules is expected to be useful for patients, with or without HCC, whose plasma BCAA levels have decreased. However, many of the findings above are based on reports from retrospective studies. Further evaluation of data from RCTs will be required in the future to corroborate these results.

LES

Patients with liver cirrhosis enter a nocturnal starvation state, LES is recommended in the guidelines of both the ASPEN and ESPEN[16,17]. LES with BCAA nutrients improves serum albumin and energy metabolism more than LES with ordinary food; LES with BCAA nutrients is therefore typical[49]. Therefore, BCAA nutrients has been used in a LES. We have reported previously that LES with BCAA nutrients improve energy malnutrition, amino acid imbalance, and glucose intolerance in liver cirrhotic patients[50-52]. However, at present, there are no guidelines for nutrition care in the treatment of HCC[53-55].

To our knowledge, there are as few as five reports on the effects of LES with BCAA nutrients on liver cirrhotic patients with HCC; the HCC treatment in the studies included surgical resection (1 report), RFA (2 reports), TACE (1 report), and HAIC (1 report). The study designs included cohort studies (2 reports) and RCTs (3 reports) (Table 2)[56-60].

LES with BCAA nutrients prior to surgical resection was shown to significantly improve postoperative liver function, significantly reduce postoperative complications, and significantly shorten hospitalization[56]. In patients treated with RFA, LES with BCAA nutrients improved liver function[57,58], nutritional status, and quality of life (QOL)[57]. Takeshita *et al*[59] reported that LES with BCAA nutrients for two weeks caused a reduction in decreased liver function in patients treated with TACE.

We measured energy metabolism using indirect calorimetry (Figure 1) in liver cirrhosis patients without HCC and in liver cirrhosis patients with HCC at different stages as classified by the Liver Cancer Study Group of Japan criteria. In the Child-Pugh A score, the non-protein respiratory quotient (npRQ) significantly decreased in patients with advanced HCC at stage Ⅳ[60]. In the Child-Pugh B score, nutritional status was generally poor, and npRQ decreased in all patient groups; there were no significant differences between the groups (unpublished data). Therefore, in patients with advanced HCC, LES with BCAA nutrients may be necessary depending on the Child-Pugh A score. In fact, LES with BCAA nutrients (LES group) improved the energy metabolism in advanced HCC patients undergoing HAIC compared with ordinary food (control group)[60]. In the 75-g oral glucose tolerance test (75-g OGTT), the area under the concentration curve for glucose (AUC glucose) showed an improvement in the LES group (P = 0.055). No significant difference in survival was identified between the groups (P = 0.667). However, the survival time of the patients whose therapeutic effect of HAIC was stable disease (SD) or progressive disease (PD) tended to be longer in the LES group (P = 0.156) than in the control group. For patients with SD or PD, a significant improvement in npRQ was observed in the LES group, whereas significant reductions in cholinesterase and natural killer cell activity were observed in the control group[61].

Thus, we consider that nutritional therapy tailored to tumor stage and residual liver capacity is required for HCC patients. However, further investigations are necessary because the previous reports examined only a small number of HCC patients.

CHEMOPREVENTION OF HCC RECURRENCE

In HCC patients, the recurrence rate is approximately 50% even after radical treatment[62,63]. Notably, it is approximately 70% in patients with HCV-related HCC[64]. Therefore, various studies on the inhibition of recurrence after radical treatment have been conducted. However, prevention of recurrence should be addressed based on the causative diseases of

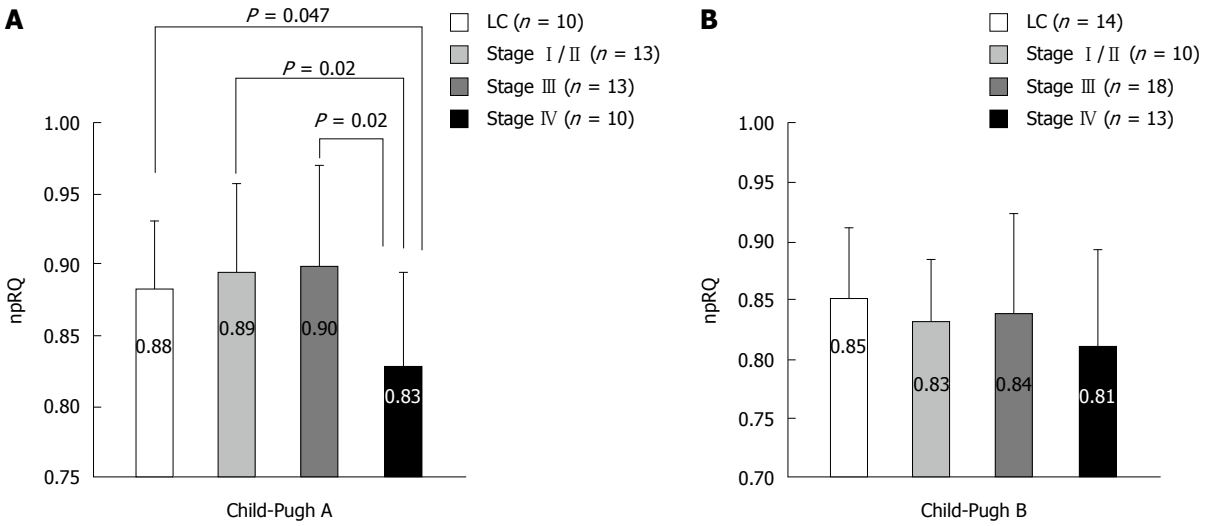

Figure 1 Non-protein respiratory quotient values in cirrhotic patients without hepatocellular carcinoma and with hepatocellular carcinoma at different stages as classified by the Liver Cancer Study Group of Japan criteria. In patients with Child-Pugh A score, no significant difference in npRQ was seen among three groups [liver cancer study (LC) group, hepatocellular carcinoma (HCC) stage Ⅰ/Ⅱ group, and HCC stage Ⅲ group]; however, the npRQ was significantly lower in the HCC stage Ⅳ group than in the LC, HCC stage Ⅰ/Ⅱ, or HCC stage Ⅲ groups (LC group *vs* HCC stage Ⅳ group, $P = 0.047$; HCC stage Ⅰ/Ⅱ *vs* HCC stage Ⅳ group, $P = 0.02$; HCC stage Ⅲ group *vs* HCC stage Ⅳ group, $P = 0.02$). In Child-Pugh B patients, no significant difference in npRQ was seen among the four groups.

HCC. This chapter describes this issue with respect to antiviral treatment for viral (*e.g.*, HBV and HCV) hepatitis and other cancer inhibitors.

Hepatitis virus-related HCC

For HBV- and/or HCV-related HCC, it has been suggested that antiviral treatment for inhibition of HCC recurrence is best administered after radical treatment rather than before. The efficacy of IFNs as antiviral treatments in viral-related HCC has been reported in six meta-analyses[65-70]. However, these meta-analyses had limitations. In the meta-analyses of Zhang *et al*[65] and Breitenstein *et al*[70], HBV and HCV patients were examined together and only IFN-α was evaluated. In the study of Miao *et al*[68], HBV patients and HCV patients were also examined together and various IFNs were assessed. In the study by Singal *et al*[66], only HCV patients were examined, a cohort study was also included, and various IFNs (IFN-α, α-2b, PEGylated IFN, and IFN-β) were evaluated. In the study by Shen *et al*[67], both HBV and HCV patients were examined together, a cohort study was also included, and various IFNs were evaluated. In the study by Miyake *et al*[69], only patients with HCV-related HCC were examined and tumor factors were limited. In any case, as an overall conclusion, it was reported that IFN-α may inhibit postoperative recurrence within the Milan criteria (a generally accepted set of criteria used to assess suitability of patients with cirrhosis and hepatocellular carcinoma for liver transplantation.). Sustained virological response was particularly associated with inhibition of recurrence.

HBV-related HCC

Lee *et al*[71] reported that scores calculated from age, sex, alanine aminotransferase, HBe antigen, content of HBV-DNA, and HBV genotype were informative as predictors of cancer associated with HBV. There are many reports on the cancer inhibition effect of IFN and nucleic acid analogs administered to patients with HBV-related chronic liver disease[69,72]. Hosaka *et al*[73] also conducted a propensity score matching analysis after classifying patients with HBV-related chronic liver disease into an entecavir (a deoxyguanosine analog)-therapy group and a non-therapy group. They reported that the 5-year cancer incidence rate was significantly reduced (3.7% *vs* 13.7%; HR = 0.37, $P = 0.030$) in the therapy group. Sohn *et al*[74] reported that higher amounts of HBV-DNA were associated with higher risk of early recurrence and that a higher amount of HBs antigen was associated with higher risk of late recurrence. These data strongly support the value of antiviral therapy after HCC treatment, and meta-analyses have shown that the use of nucleic acid analogs after HCC treatment can help inhibit HCC recurrence and improve prognosis[75,76]. Although results from an RCT suggested that IFN can inhibit HCC, no firm conclusion was reached, and further investigation will be required[76]. Recently, Lee *et al*[77] reported on the beneficial effect of a nucleic acid analog on inhibition of HCC recurrence after radical treatment with RFA.

Thus, it is suggested that viral control after radical treatment is important for inhibiting HCC recurrence in patients with HBV-related HCC.

HCV-related HCC

The effect of IFN on cancer inhibition in patients with HCV-related chronic liver disease has been reported in many previous studies[78-80]. Miyake *et al*[81] reported in

a meta-analysis that IFN can decrease the carcinogenic risk. In addition, many studies have mentioned the value of IFN even after radical treatment for HCV-related HCC. In these studies, the effect of IFN on prognosis was reported; a trend in inhibiting recurrence was noted but the results did not reach statistical significance[82,83]. However, several studies have reported that IFN-α after radical treatment for HCC inhibited later successive recurrences after a second recurrence[64,84,85]. In addition, the effect of low dosages of IFN in long-term therapy on inhibition of recurrence has been reported. Thus, Kudo *et al*[86] reported that a small amount of IFN-α2b inhibited the first, second, and third recurrence after radical treatment with RFA and contributed to survival (HR = 0.21)[87]. It has also been reported that IFN-β inhibited recurrence after radical treatment for HCV-related HCC (P = 0.0004).

Several direct-acting antivirals (DAAs) have emerged recently as treatments for the safe elimination of viral infections, even in cirrhotic patients. Recently, Reig *et al*[88] administered DAAs to the patients after curative treatment of HCC and investigated subsequent recurrence rate. Although they reported high rate of recurrence after the viral elimination by DAAs, it is a small cohort retrospective study and the reliable opinion is not obtained. In addition, Pol S conducted a multicenter prospective study, and he concluded that there was no evidence that DAAs promote an HCC recurrence[89]. It is still needed future analysis.

OTHER CANCER INHIBITORS

This section describes the current status and prospects of these three agents in the treatment of advanced HCC.

Sorafenib

Sorafenib is a standard therapeutic drug for advanced HCC that was developed as a C-Raf and B-Raf serine/threonine kinase activity inhibitor[7]. It affects both the Raf/MEK/ERK signaling pathway, which influences cell proliferation, and VEGFR, which is associated with neovascularization. Sorafenib is also known to inhibit the tyrosine kinase activity of the platelet-derived growth factor receptor[7].

In 2008, international cooperative group clinical trials involving patients with advanced HCC demonstrated that sorafenib offered a significant prolongation of OS when compared with placebo[7]. To test the hypothesis that sorafenib could prevent HCC recurrence, a RCT targeting patients who had received HCC radical curative treatment (hepatectomy/RFA/PEI) was conducted[88]. This trial (termed the STORM trial) comprised two groups: one that received sorafenib at 800 mg/d and the placebo group. Progression-free survival was set as the primary endpoint. However, sorafenib offered no significant prolongation effect. A major issue in the STORM trial was that long-term oral administration of sorafenib was not possible because of the high incidence of adverse side effects associated with this treatment[90].

Peretinoin

Peretinoin is an orally administered acyclic retinoid with a vitamin A-like structure that targets the retinoid nuclear receptor[91]. It induces apoptosis and inhibits tumor growth in HCC cells[91]. Recently, it has been reported that acyclic retinoids increase the expression of intra-nuclear transglutaminase-2 in JHH-7 cells and induce apoptosis in HCC[92]. Muto *et al*[93] performed a small-scale RCT to determine the effect of peretinoin on inhibition of HCC recurrence after radical treatment (hepatectomy/PEI). They reported that pereretinoin inhibited the second recurrence (adjusted relative risk 0.31; 95%CI: 0.12-0.78)[93]. Based on these results, Okita *et al*[94,95]. performed a randomized double-blind placebo-controlled study in patients after radical treatment for HCV-related HCC (operation/RFA). Recurrence was significantly inhibited in the peretinoin (600 mg/d) group (P = 0.023; multiplicity-adjusted P = 0.048)[94,95]. A double-blind, placebo-controlled, multicenter, randomized, parallel intergroup trial is currently under way (NCT01640808) to verify these findings.

Iron chelators

Iron is necessary for oxygen transport, energy production, and cell metabolism and growth[96,97]. It is especially important in cells with active growth, including cancer cells[98]. A clinical study on hepatocarcinogenesis and iron overload has been conducted; Kato *et al*[99] reported that reduction of iron levels through phlebotomy therapy might significantly inhibit hepatocarcinogenesis. Iron metabolism control may thus become a target for cancer inhibition. An antitumor effect of deferoxamine (DFO) in HCC patients has been reported[100,101]. We have also reported on the antitumor effect of arterial DFO administration in patients with advanced HCC[102]. In a fundamental experiment, we reported that DFO inhibited liver fibrosis and pre-neoplastic lesions in a rat model of hepato-carcinogenesis[103]. Deferasirox (DFX) has also recently emerged as an orally administered iron chelator, and a strong antiproliferative effect associated with DFX has been reported *in vitro*. The effect of DFX on cancer inhibition in combination with losartan has also been reported in an *in vivo* study[104]. By combining DFX and sorafenib, we confirmed not only a therapeutic effect against liver fibrosis and cancer but also a reduction in adverse side effects that were associated with treatment with sorafenib alone. As noted above, long-term administration of sorafenib alone was not possible in the STORM trial because of the high incidence of adverse side effects. In this respect, combined treatment of DFX and sorafenib may prove to be a new therapy to prevent recurrence of HCC.

BMCS

We have reported that infusion of BMCs decreased

livers fibrosis and improved liver function in mice[105,106]. Based on these results, we initiated an autologous BMC infusion therapy for liver cirrhosis in 2003. The safety and efficacy of this therapy have been confirmed in clinical studies[107-110]. Short-term results to date indicate no serious complications associated with reproduction therapy using BMCs. However, longer-term evaluation, particularly evaluation of the potential for hepatocarcinogenesis, is still required.

Ishikawa *et al*[111] generated a rodent model of chemical carcinogenesis by injecting mice with diethylnitrosamine and phenobarbital. They then infused BMCs into these mice. No tumorigenesis associated with the BMC infusion was observed, and the authors reported that the potential for carcinogenesis was low. We examined the influence of BMC infusion on hepatocarcinogenesis using a highly oncogenic cirrhotic murine model. The influence of BMCs on hepatocarcinogenesis was evaluated histologically. The number of liver tumors was smaller and liver fibrosis was inhibited in mice treated with repeated doses of BMCs[112]. This confirmed that BMC infusion contributed to inhibition of hepatocarcinogenesis. Most of the BMCs that engrafted into the damaged liver expressed superoxide dismutase 3, which is an antioxidant protein[112]. It is therefore considered that BMCs inhibit hepatocarcinogenesis by regulating redox homeostasis.

Although it is known that bone marrow-derived mesenchymal stem cells (MSCs) migrate to tumor tissues, their role is mostly unclear. As MSCs secrete a variety of growth factors, there is concern about their effects on tumor progression[113]. In previous studies, it has been reported that growth, invasion, and metastasis of lung cancer and neovascularization are promoted by MSC secretion of factors such as IL-6, VEGF, and IGF-1[114]; that tumor cells cause epithelial-mesenchymal transition[115]; and that tumors are activated as MSCs are differentiated into carcinoma-associated fibroblasts comprising the tumor microenvironment[116].

Conversely, it has also been reported that MSCs inhibit tumor proliferation by controlling WNT signaling and PARP cleavage of tumor cells, thus promoting apoptosis[117,118]. Furthermore, in a clinical study, carcinogenesis was not observed in follow-up at 11 years and five months after cultured MSC was used to reproduce cartilage[119].

Thus, we consider that the potential for carcinogenesis associated with BMCs is low, and that these cells are likely play a minimal role in any newly occurring carcinogenesis. However, the potential for tumor formation through neovascularization or secretion by BMCs of various humoral factors also cannot be neglected. Therefore, it is important to generate further relevant data to determine whether or not tumorigenesis potentially associated with regenerative medicine using BMCs is a realistic concern, or whether BMCs can be developed as a safe and efficacious therapy.

CONCLUSION

We noted that BCAA and LES with BCAA, which address nutritional issues, were important for liver cirrhotic patients with HCC. We also emphasized that antiviral agents, including nucleic acid analogs and IFNs, were effective in the treatment of HCC. In addition, we described the potential of peretinoin, progress in the development of iron chelators, and the promise of BMCs to suppress hepato-carcinogenesis. We showed results on some positive trials supporting the prevention of HCC-recurrence and the preservation of liver function. Therefore, by generating further data and evidence, it is expected that new HCC strategies can be developed by combining the therapies above alongside treatments with anticancer drugs.

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REVIEW

Current hepatitis B virus infection situation in Indonesia and its genetic diversity

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Abstract

Indonesia has a moderate to high endemicity of hepatitis B virus (HBV) infection. The risk for chronic HBV infection is highest among those infected during infancy. Since 1997, hepatitis B (HepB) vaccination of newborns has been fully integrated into the National Immunization Program. Although HBV infection has been reduced by the universal newborn HepB immunization program, it continues to occur in Indonesia. The low birth dose coverage and the presence of vaccine escape mutants might contribute to this endemicity among children. Although limited information is available for an analysis of occult HBV infection (OBI), several variations and substitutions in the pre-S/S region have been detected in Indonesian HBV strains. Additionally, persistent infection and disease progression of chronic hepatitis B are related to not only viral factors but also the host genome. Indonesia is one of the most ethnically heterogeneous nations, with Javanese and Sundanese as the two highest ethnic groups. This multi-ethnicity makes genomic research in Indonesia difficult. In this article, we focused on and reviewed the following aspects: the current hepatitis B immunization program and its efficacy, OBI, HBV infection among high-risk patients, such as hemodialysis patients, and research regarding the host genome in Indonesia.

Key words: Hepatitis B virus; Immunization; Occult infection; Hemodialysis; Indonesia

Core tip: Hepatitis B virus (HBV) infection is still an important health problem in Indonesia. Although HBV infection has been reduced by the universal newborn

hepatitis B immunization program, it continues to occur in Indonesia. The high prevalence of occult hepatitis B infection and HBV infection among hemodialysis patients also contributes to its endemicity. The association between human genetic variations and HBV infection in several Asian countries, including in Indonesia was also identified. We reviewed these important aspects of the current HBV infection situation in Indonesia.

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INTRODUCTION

An estimated 240 million people are chronically infected with hepatitis B worldwide [defined as hepatitis B surface antigen (HBsAg) positivity for at least 6 mo]. A vaccine against hepatitis B has been available since 1982. Although the prevalence of hepatitis B virus (HBV) infection is relatively low in developed countries (*e.g.*, as low as 0.4% in the United States), HBV infection is still quite prevalent in East Asia and Southeast Asia, including Indonesia (2.5%-10%)[1,2]. The endemicity of hepatitis B (marked by HBsAg positivity) in Indonesia is intermediate to high with geographical differences. HBV has been classified into at least 9 genotypes (A through H and J) and has been shown to have a distinct geographical distribution[3,4]. The most common HBV subgenotype in Indonesia is B3, followed by C1. Various novel HBV subgenotypes have been identified throughout Indonesia, and the novel HBV subgenotypes C6-C16 and D6 have been successfully isolated[2] in the general population.

The risk for chronic infection is related to the age at infection; for instance, approximately 90% of infected infants become chronically infected compared with 2%-6% of adults[1]. In addition to HBsAg, HBeAg is an important hepatitis B marker in the field of mother-to-child transmission. HBeAg is a small secretory antigen that can cross the placenta from the mother to the fetus[5]. The vaccine is generally effective in preventing infection[6]. A universal hepatitis B vaccination program for infants was adopted in Indonesia in 1997. What is the current HBV serological status and molecular profile among children in Indonesia fifteen years after the adoption of this universal infant vaccination program?

A specific community with maintenance hemodialysis (HD) is at high risk for blood-borne infections, especially HBV. However, few studies have been conducted on the prevalence of HBV among HD patients in Indonesia, and adequate databases on HBV infection in this population are still limited. Therefore, the HBV subgenotypes among HD patients in Indonesia is also an interesting subject for discussion.

Undetectable HBsAg is generally considered to indicate a lack of HBV infection or the disappearance of viremia and disease remission[7,8]. This belief may result in misinterpretation among patients with occult HBV infection (OBI), which is an HBV infection that lacks detectable HBsAg. Considering the importance of OBIs, the purpose of this review is to provide comprehensive information on OBIs in Indonesia, including infections among HD patients.

In addition to viral factors (*e.g.*, HBV DNA levels, genotypes, and genomic mutations), host factors (*e.g.*, age, gender, race, and immune status) might contribute to the progression of liver diseases[9,10]. Genome-wide association studies have identified associations of genetic variations with diseases related to HBV, including HBV-related hepatocellular carcinoma (HCC).

All of the topics listed above are among the HBV subtopics in Indonesia that will be discussed here.

HEPATITIS B IMMUNIZATION PROGRAM AND ITS IMPACT

Indonesia experiences intermediate to high hepatitis B endemicity that varies between provinces[11] (Figure 1). In countries with a high prevalence of chronic hepatitis B infection, a higher proportion of carriers are infected during infancy or early childhood; historically, 25%-50% of chronic infections in these countries are caused by vertical mother-to-infant perinatal transmission. Surveys of pregnant women in Indonesia have shown prevalence rates between 3% and 8%. This phenomenon generates high potential for perinatal transmission from carrier mothers to their babies[12].

The Lombok Hepatitis B Model Immunization Project (1987-1991) was the first universal infant hepatitis B immunization project[13,14] in Indonesia. This project aimed to integrate the HepB vaccine into the National Immunization Program, including a birth dose targeted as early as possible within the first week after birth. This project achieved > 90% coverage for the administration of the HepB vaccine and was able to demonstrate a decrease in the prevalence of hepatitis B carriage among children under 4 years of age who had received three doses of the vaccine. The carriage rates dropped from 6.2% to 3.0% for infants who received the first dose later than 7 d after birth and to 1.4% in infants who received the first dose within 7 d of birth.

Following the Lombok Hepatitis B project, in 1991, routine HepB immunization was implemented in 4 provinces (West Nusa Tenggara, Bali, Yogyakarta and 5 districts in East Java). In that year, immunization for newborns (birth dose immunization) was recommended. During the period from 1992-1995, routine

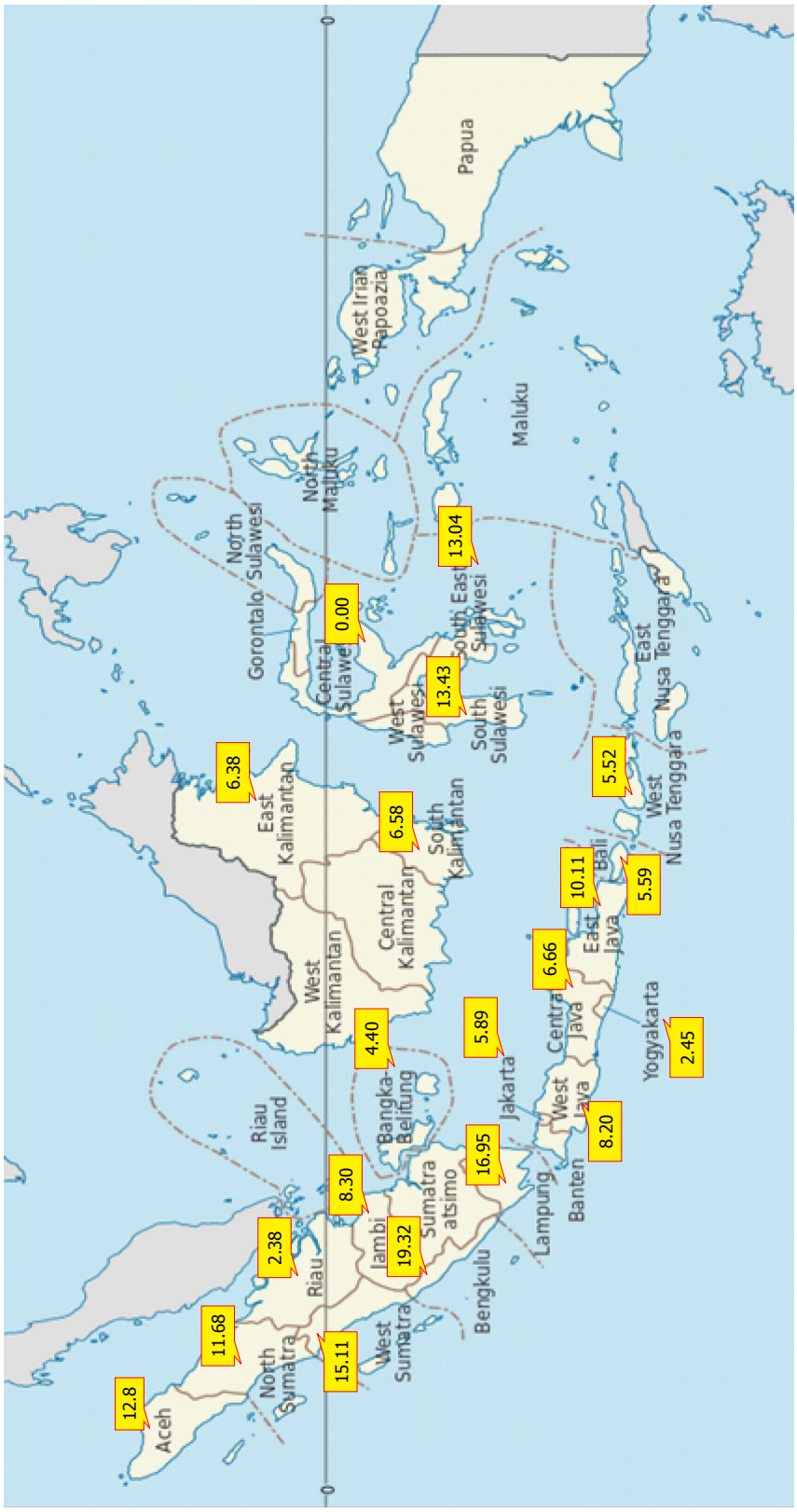

Figure 1 Prevalence of hepatitis B surface antigen in some provinces in Indonesia[11]. The provided number is the average number across all age groups, including the individuals born before and after the introduction of the immunization program. The population were those with more than 1 year of age (general population) in 2007, after 10 years of implementation of national hepatitis B immunization. For the provinces without the prevalence number, there are no available data (https://commons.wikimedia.org/wiki/File:Indonesia,_administrative_divisions_-_en_-_monochrome.sv).

HepB immunization was expanded to 6 other provinces (Lampung, DKI Jakarta, West Java, Central Java, West Sumatera, and West Kalimantan)[13,15]. Finally, in 1997, HepB vaccination of newborns was fully integrated into the National Immunization Program[15]. A plasma-derived vaccine was produced in Indonesia until 1997, when it was replaced by a recombinant hepatitis B vaccine[16]. By 2000, Indonesia was able to use the HB-Uniject, which is a pre-filled single-use injection device for the hepatitis B vaccine that is stable outside of the cold chain, for the birth dose in seven of the provinces in the program. By 2003, the program expanded to target all of Indonesia's five million annual births[17]. According to the National Immunization Program in Indonesia, the birth dose of the hepatitis B vaccine should be given within 7 d after birth and should be followed by three doses of combination vaccines, including Diphtheria, Pertussis, Tetanus and Hepatitis B, within the second, third and fourth months[17,18]. Parents should bring their babies to the Primary Health Center to receive the vaccine; however, many people in remote areas in Indonesia have difficulty reaching these centers due to geographic isolation[12]. Moreover, because screening for hepatitis B in all pregnant women has not been implemented as a national

Table 1 Prevalence of HBsAg, anti-HBc, and anti-HBs in Indonesia

Year	Population group	HBsAg (%)	Anti-HBc (%)	Anti-HBs (%)
2007	In all provinces[11]			
	General population:			
	Male	9.68	36.39	34.37
	Female	9.28	30.14	28.81
	Age groups:			
	1-4 yr	7.32	10.14	50.78
	5-9 yr	6.92	11.56	34.50
	10-14 yr	10.14	14.79	23.30
	In East Java province[16]			
	6-12 yr	3.10	23.80	23.60
2012	In Central Kalimantan, West Timor, South East Sulawesi, provinces[41]			
	1-5 yr	2.1-4.2	3.5-4.8	61.4-65.8
	In Central Kalimantan, West Timor, West Papua provinces[41]			
	6-12 yr	0-5.9	5.2-50.4	20.9-40.4

program in Indonesia, not all infants born to women with hepatitis B receive the hepatitis B immunoglobulin (HBIG)[19]. If the mother is infected and transmits the virus before the child is vaccinated and no HBIG is simultaneously administered within 24 h of birth, vaccination will not protect the child[20].

HBV infection has been reduced by the universal newborn HepB immunization program; nevertheless, it continues to occur in endemic countries[21]. Even with proper immunization, 5%-10% of infants delivered by hepatitis B e-antigen (HBeAg)-positive women become infected[22]. Because HBeAg and hepatitis B core antigen (HBcAg) are highly cross-reactive in terms of helper T cell recognition, transplacental HBeAg from the mother can induce specific helper T cell unresponsiveness to HBeAg and HBcAg in neonates born to HBeAg-positive carrier mothers, who become chronic carriers[5,23]. The maternal HBV DNA load is also strongly associated with HBV intrauterine transmission[24-26]. Up to 90% of infants infected during the first year of life will develop chronic HBV infection[27]. However, more than 90% of perinatal infections can be prevented if HBsAg-positive mothers are identified[28]. Although screening of pregnant women is necessary to prevent further spread of HBV, especially through perinatal transmission, data on the hepatitis B prevalence in pregnant women in Indonesia are limited in number and study coverage[15]. Previous studies reported HBsAg-positive rates of 2.6%, 4.7%, and 5.2% in Bali, West Java and Jakarta, respectively[15,29,30]. In 2003, HBsAg was detected in 1.9% of pregnant women in Bali, which was significantly lower than previously reported data. The prevalence of HBeAg also decreased from 50% to 28% within 10 years[31]. In 2009, the HBsAg prevalence was also significantly lower (2.2%) in Jakarta, with the peak prevalence occurring in women aged < 20 years and between 36-40 years[15].

In Indonesia, a screening test for HepB in pregnant women is not routinely performed, and the price of HBIG, which is unaffordable for many people, makes it difficult to provide for high-risk babies. In 2010, Fitria *et al*[32] reported that the effectiveness of HepB immunization in preventing vertical transmission was 70%-90%. Fourteen of 15 infants born to HBsAg-positive mothers were not infected by HBV, although some of them received their first immunization at more than 24 h of age. The infants were delivered per vaginam and did not receive HBIG. The authors suggested that HepB immunization could prevent vertical transmission in infants born to HBsAg-positive mothers even without HBIG administration. They also found that no infants who had HBsAg-positive umbilical cord blood were infected with HBV (negative HBsAg after HepB immunization). This result is in accordance with other findings[33,34] that transplacental transmission does not play a role in vertical transmission. However, these studies are in contrast with other studies. Beasley *et al*[35,36] found that HepB immunization alone prevented the development of the persistent carrier state in at least 75% of infants born to HBeAg-positive mothers; concurrent use of HBIG and HepB immunization in these infants appears to increase the rate of prevention to as high as 95%. Lavanchy[37] reported that combined HBIG and HepB immunization after birth reduced the risk of transmission from 70%-90% to less than 10% among infants of HBsAg-positive and HBeAg-positive mothers. Zhang *et al*[38] also reported that the immunoprophylaxis failure rate was 3.3% among infants of HBsAg-positive mothers and that the infection rate reached 9.3% in infants of both HBsAg- and HBeAg-positive mothers. Infants born to HBeAg-positive mothers who only received HepB immunization were more vulnerable to HBV infection compared with infants who received HepB immunization plus HBIG, with an immunoprophylaxis failure rate of 16.9% *vs* 7.9%. However, another finding showed that fulminant hepatitis B could occur in infants born to HBeAg-negative and HBsAg-positive mothers if they were not given HBIG, although these mothers were reportedly HBV DNA-negative. Thus, the etiology is most likely unrelated to maternal HBV infection, and the cause is unknown[39].

Primary HepB immunization coverage among Indonesian infants gradually increased from 28% in 1992 to 78% in 2008 and then to 93% in 2009[40]. In 2012, the three doses of HepB vaccine coverage were 73.9%-94.1%, although the birth dose coverage was less than 50% according to the local health office data in 5 provinces in Indonesia (Central Kalimantan, West Timor, West Papua, South East Sulawesi and East Java)[41]. After 15 years of implementation of universal HepB immunization, the HBsAg-positive rates in pre-school- and school-aged children ranged from 2.1%-4.2% and 0%-5.9%, respectively. The anti-HBs seropositivity prevalence among pre-school-aged children was higher (61.4%-65.8%) than among school-aged children (20.9%-40.4%)[16,41] (Table 1). The antibody titer gradually fell to less than 10 mIU/mL by 10 to 15 years of age[42,43]. This decrease

may reflect a decline in the anti-HBs levels; however, the lower immunization coverage in Indonesia during the earlier years of its implementation (before 2003)[17] may have contributed to these results. Utsumi *et al*[16] and Purwono *et al*[41] also found some substitutions (P120S, T126I, M133T/L, T140I, C147S, and S155F) in the S region of HBV isolates from vaccinated children. The T126I substitution involves the largest change in chemical properties and is the most likely substitution to cause structural changes in HBsAg[44-46]. T140I has also been potentially suggested as a vaccine escape mutant. The low birth dose coverage and the presence of a vaccine escape mutant may cause HBV infection among children to remain endemic in Indonesia[41]. The triple-antigen vaccine that includes regions other than the S region may be considered in regions where the anti-HBs prevalence remains insufficient among vaccinated children[16].

VARIATIONS IN THE PRE-S/S REGION

Few studies have investigated pre-S/S variations in Indonesia. In 2011, Utama *et al*[47] reported that the prevalence of pre-S mutations was 2.7% (2/75), 12.9% (8/62), 16.7% (11/66), and 17.7% (11/62) in the asymptomatic carrier, chronic hepatitis, liver cirrhosis, and HCC groups, respectively. The authors concluded that the prevalence of HBV pre-S mutations was relatively low in Indonesian patients compared with patients from Taiwan, Japan, and other Asian countries and that there was a weak association between the pre-S deletion mutation and progressive liver disease. Conversely, Utama *et al*[48] found a mutation in the pre-S2 start codon in 59 samples from 268 subjects (22.0%) with a higher prevalence in patients with cirrhosis (27/66, 40.9%), followed by HCC (18/63, 28.6%), chronic hepatitis (12/66, 18.2%) and asymptomatic carriers (2/73, 2.7%). They reported that the pre-S2 start codon mutation was more common in Indonesian patients than in patients from other Asian countries and that its prevalence was associated with advanced liver disease. In 2015, Yamani *et al*[49] analyzed HBV-infected patients using a deep sequencing method and reported that the accumulation of variations in the major hydrophilic region was associated with a decrease in the HBsAg titer.

OBI

OBI is diagnosed by the detection of HBV DNA and the lack of HBsAg detection with or without anti-HBc or anti-HBs outside of the pre-seroconversion window period[50,51]. OBI usually depends on the difference in the sensitivity of the screening assay; for instance, detection by the HBsAg assay is less sensitive than the detection of HBV DNA by the PCR assay[52,53]. Recent epidemiological studies have detected occult HBV infection worldwide[54]. In 2015, Darmawan *et al*[55] examined 195 healthy young adults who received universal infant hepatitis B vaccination in Banjarmasin and reported that the prevalence of HBsAg, anti-HBc, and anti-HBs was 9 (4.6%), 62 (31.8%), and 96 (49.2%), respectively. Additionally, the authors detected HBV DNA and confirmed occult HBV infection in 9 HBsAg-negative and anti-HBc-positive individuals. Generally, clearance of HBsAg is considered to represent a disappearance of viremia and disease remission[7,8]. However, OBI is reportedly associated with severe liver damage and the development of liver cancer[50,56,57]. Many studies reported that OBI was associated with advanced liver diseases, such as cirrhosis and HCC[58,59]. However, references regarding this information are scarce in Indonesia, and further studies will be necessary.

OBI is sometimes related to the decreased activity of viral replication and mutations in the α determinant region of the S gene, which encodes amino acid residues 124-147 of HBsAg. Utsumi *et al*[16] examined 229 healthy children in East Java and reported that the prevalence of HBsAg positivity was 3.1%; occult HBV infection was detected in 5 out of 222 HBsAg-negative individuals. The authors reported that the T126I amino acid substitution was frequently found. In Indonesia, universal vaccination was introduced in the 1990s, but the efficacy has not been fully investigated. A follow up study is necessary to consider booster immunization. Thedja *et al*[60] examined 309 HBsAg-negative blood donors and reported that the prevalence of anti-HBc and HBV DNA was 134 (43.4%) and 25 (8.1%), respectively. They also examined amino acid substitutions in the α determinant region in HBsAg and reported that several amino acid substitutions, such as T123A, M133L, and T143M, might change the HBs antigenicity.

Occult HBV infection is sometimes related to high-risk patients, such as HD patients, HIV patients and immunosuppressed patients. Utsumi *et al*[61] examined 118 HIV-infected patients in Surabaya and reported that the prevalence of HBsAg and HBV DNA was 15.3% and 27.1%, respectively. HBV reactivation is especially critical in immunosuppressed OBI patients, and many clinicians should take precautions[62]. Although reported in relation to HBV, reactivation in Indonesia is still rare, and the potential risk for reactivation is considered to be high[63,64].

HBV INFECTION AMONG HD PATIENTS

Patients on maintenance HD are among the group at highest risk for HBV infection. Most HBV infection outbreaks in patients in HD units are caused by cross-contamination *via* the following factors: (1) environmental surfaces, supplies (*e.g.*, hemostats and clamps), or equipment that is not routinely disinfected after each use; (2) multiple dose medication vials and intravenous solutions that are not used exclusively for one patient; (3) medications for injection that are prepared in areas adjacent to areas where blood

samples are handled; and (4) staff members who simultaneously care for both HBV-infected and susceptible patients[2,65-68].The risk of HBV transmission from blood-contaminated items in this setting is greater and more serious than would be expected for other common bloodborne viruses[66].

The prevalence of infection is generally lower in developed countries, which experience occasional outbreaks, than in developing countries; this difference might reflect the prevalence of the infection in the general population[65,69,70]. A study in 2003 in Manitoba, Canada, showed that 0.8% of HD patients were positive for the HBsAg[52]. A systematic review of HBV outbreaks in the dialysis units of developed and less-developed countries published between 1992 and 2014 showed fewer European outbreaks compared with other countries (P = 0.0046). Moreover, multiple deficiencies in standard or HD-specific procedures were the most common routes of patient-to-patient HBV transmission (80%). A recent multicenter prospective cohort study among dialysis patients in Korea revealed that 7.1% were HBsAg-positive[71]. In Vietnam, 7% of HD patients tested positive for HBsAg[72]. Several studies have also been performed in Indonesia. In a study conducted in West Java, the rates of HBsAg and anti-hepatitis C virus (anti-HCV) seropositivity among HD patients were 6.8% and 73.5%, respectively[73], whereas those in Yogyakarta were 7% and 81%, respectively[74]. After almost 20 years, more recent data on the rates of infection in Yogyakarta showed 11.2% seropositivity for HBsAg and 80.7% for anti-HCV. Our previous studies in Surabaya showed that the anti-HCV prevalence was between 76.3% and 88%[75-77], whereas our recent study in private hemodialysis units (HDUs) in Surabaya showed that the hepatitis B infection prevalence was 0-8.1%. Interestingly, no HBV- or HCV-infected HD patients were detected in one private HDU that strictly complied with the adherence to standard and dialysis-specific infection control precautions, whereas 24.2% to 60.6% of patients tested positive for anti-HCV in other private HDUs[78].

In general hospitals in Indonesia, dialyzers are commonly reused up to a maximum of eight times for all patients. Following the recommendations for HBV and HCV infection control issued by the Indonesian Society of Nephrology, separate rooms are only available for patients who are HBsAg seropositive but not for anti-HCV-positive patients. Based on the slightly higher prevalence of HBV infection compared with a markedly higher prevalence of HCV infection among HD patients than the general population (2.5%-10% and 2.1%-2.3%, respectively) in Indonesia[2,75,79] and the practice by which patients with hepatitis B but not hepatitis C are isolated in separate rooms, there is a strong possibility that the prevalence of HBV and/or HCV infections among HD patients is caused by nosocomial infections. Our previous study also showed that the HD duration and number of blood transfusions were significantly associated with HCV infection but not with HBV infection[68]. A study in 4 private HDUs in which serological tests were conducted every 3 mo for 9 mo to investigate the new incidence of hepatitis virus infections found no new incidence of HBV in any HDU, whereas the new incidence of HCV was 5.6% during the third sampling in HDU-C and 11.1% and 13.3% during the second and third samplings in HDU-D, respectively[78]. Due to resource limitations, only 5% of the patients in the general hospital and 8.6%-83.3% of patients in the private HDUs with HBsAg seronegativity were vaccinated for HBV[68,78], which made the HD patients more susceptible.

Subgenotype B3 is the most prominent because this genotype is commonly found among the Javanese ethnic group; the Javanese group is the main ethnic group in Indonesia and has mostly settled on Java Island, which is the Indonesian mainland[68,78]. HBV subgenotypes A2, B2-3, B7-9, C1-2, C5-8, C10-16, D6, F, and J are unique to Indonesia, with specific geographic and ethnic distributions[80-86]. Our studies on HBV infection among HD patients showed that all of the HBV/B strains were classified as HBV/B3[68,78]. These studies were performed in Yogyakarta and Surabaya, which are located on Java Island. We presume that a study conducted on HBV infection among HD patients in other parts of Indonesia, especially in East Indonesia where we found several other unique HBV subgenotypes, may result in different prominent subgenotypes.

Patients undergoing dialysis potentially have an increased risk of OBI. OBI harbors a potential risk of HBV transmission through HD[87]. Inadequate data are available concerning OBIs among Indonesian chronic HD patients. In 2013, Rinonce *et al*[68] reported that OBIs were detected in 21 (14.7%) of 143 HBsAg-negative patients, and 7 (33.3%) of these 21 patients tested positive for anti-HBc in Yogyakarta. Most patients who were co-infected with HBV and HCV had lower HBsAg titers than patients with only HBV infection, suggesting that HBV infection was suppressed by HCV co-infection[88]. However, Kanbay *et al*[89] reported in 2006 that HCV positivity was not a contributing factor to OBIs in HD patients. Rinonce *et al*[68] also found that 15 (52%) of 29 HBV DNA-positive patients co-infected with HCV (anti-HCV or HCV RNA-positive) were HBsAg-positive and that 14 (48%) had occult HBV infections.

HOST GENOME RELATEDNESS

Indonesia consists of five major islands and is the largest archipelago in the world. Archaeological evidence revealed that Central and East Java were occupied by the ancestors of modern humans as early as 1.9 million years ago. Currently, Indonesia consists of more than 300 ethnic groups, more than 95% of which are of native Indonesian ancestry. The largest ethnic group in Indonesia is the Javanese, who

Table 2 Human leukocyte antigen region in relation to susceptibility to hepatitis B virus infection

SNP region	rs	Ethnics	Ref.
HLA-DPA1	rs3077	Chinese, Japanese, Thai Korean, Indonesian	[97-101]
HLA-DPB1	rs9277378	Thai	[102]
	rs9277535	Chinese, Japanese, Indonesian, Taiwanese	[97-99,101,103-106]
	rs9277542	Chinese, Japanese, Korean, Thai	[100]
	rs7770370	Korean	[107]
HLA-DQ	rs9275319	Chinese	[108]
HLA-DQA2	rs9276370	Taiwanese	[104]
HLA-DQB1	rs2856718	Chinese, Japanese	[98,99]
HLA-DQB2	rs7453920	Chinese, Japanese, Taiwanese	[98,99,103,104]
HLA-DQ/DR	rs9272105	Chinese	[108]
HLA-DR	rs3135363	Indonesian	[106]
HLA-DRB1		Chinese	[109]

HLA: Human leukocyte antigen.

primarily live on Java Island and make up approximately 40% of the total population.

Although single nucleotide polymorphisms (SNPs) near the interleukin 28B gene (IL28B; IFN-λ-3) are the strongest genetic predictors of the response to interferon-based therapy for chronic hepatitis C patients, whether SNPs are associated with the therapeutic outcome for chronic hepatitis B patients is controversial[90,91]. These SNPs were also reported to be associated with spontaneous clearance of HBV infection, although the data are still limited and have not been confirmed[92,93]. The human leukocyte antigen (HLA) gene is located in region 6p21.3 and plays an important role in antigen presentation. Although technological advancement for sequencing of the human genome has made the analysis of many diseases easier, genomic studies in Indonesia are still limited. Several studies on novel HLA alleles in Indonesian populations have been conducted[94,95]. However, whether these alleles are associated with disease is unclear. Yuliwulandari *et al*[96] examined HLA alleles of 237 Javanese and Sundanese-Javanese ethnic groups and reported that the Western Javanese population was closer to Southwest Asian populations than Northeast Asian populations. These studies would be helpful for future studies in anthropology, organ transplantation, and disease associations in Indonesian populations.

Several studies, especially in Asian countries, revealed that host factors were associated with HBV infection. Host factors, such as age, gender, obesity, diabetes, and genetic variants, are associated with persistent infection and disease progression among HBV-infected individuals. In 2009, Kamatani *et al*[97] first reported that SNPs in the HLA-DP region were associated with chronic HBV in a study of 188 Japanese patients with chronic HBV infection and 934 controls. Thereafter, a large number of studies concerning HLA regions susceptible to HBV infection have been reported from Asian countries (Table 2)[97-109]. A study from the Netherlands reported that several SNPs, including HLA-DR (rs3135363), HLA-DP (rs9277535), and a gene-rich HLA Class Ⅲ interval (rs9267665), were independent risk variants in Indonesian vaccine recipients[106]. For instance, rs3135363 was revealed to be the most significant contributor to the antibody response in Indonesian populations. This result supported the finding that the HLA-DR allele was associated with the host response to HBsAg vaccination[110,111]. More recently, Wasityastuti *et al*[101] reported that the HLA-DPA1 rs3077 variant was associated with a protective effect by increasing spontaneously resolved HBV infection and that combinations of haplotype markers (CA for rs3077-rs9277535 and GA for rs3135021-rs9277535) were associated with HBV susceptibility.

CONCLUSION

The HBV infection rate has been reduced by a universal newborn HepB vaccination program, but the low birth dose coverage and the presence of a vaccine escape mutation might cause HBV infection among children to remain endemic in Indonesia. OBIs have also been reported among the general population, patients with chronic liver disease and patients with immunosuppressive conditions, such as HD. Additionally, some mutations in the pre-S/S region play an important role. Genetic and other data show possible cross-HBV infections among patients in HDUs. Occult hepatitis B cases might also play an important role in HBV transmission in Indonesian HDUs.

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REVIEW

Perspectives on the combination of radiotherapy and targeted therapy with DNA repair inhibitors in the treatment of pancreatic cancer

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Abstract

Pancreatic cancer is highly lethal. Current research that combines radiation with targeted therapy may dramatically improve prognosis. Cancerous cells are characterized by unstable genomes and activation of DNA repair pathways, which are indicated by increased phosphorylation of numerous factors, including H2AX, ATM, ATR, Chk1, Chk2, DNA-PKcs, Rad51, and Ku70/Ku80 heterodimers. Radiotherapy causes DNA damage. Cancer cells can be made more sensitive to the effects of radiation (radiosensitization) through inhibition of DNA repair pathways. The synergistic effects, of two or more combined non-lethal treatments, led to co-administration of chemotherapy and radiosensitization in *BRCA*-defective cells and patients, with promising

results. ATM/Chk2 and ATR/Chk1 pathways are principal regulators of cell cycle arrest, following DNA double-strand or single-strand breaks. DNA double-stranded breaks activate DNA-dependent protein kinase, catalytic subunit (DNA-PKcs). It forms a holoenzyme with Ku70/Ku80 heterodimers, called DNA-PK, which catalyzes the joining of nonhomologous ends. This is the primary repair pathway utilized in human cells after exposure to ionizing radiation. Radiosensitization, induced by inhibitors of ATM, ATR, Chk1, Chk2, Wee1, PP2A, or DNA-PK, has been demonstrated in preclinical pancreatic cancer studies. Clinical trials are underway. Development of agents that inhibit DNA repair pathways to be clinically used in combination with radiotherapy is warranted for the treatment of pancreatic cancer.

Key words: Radiotherapy; Pancreatic cancer; DNA damage; DNA repair; Molecular targets

Core tip: Radiotherapy causes DNA damage, including double-strand breaks, which is more readily repaired in normal cells than in cancerous cells. Radiosensitization, using DNA repair pathway inhibitors, has been well documented in various cancer types, including pancreatic cancer. Further development of optimal protocols, for the combined use of these inhibitors with radiotherapy, with/without chemotherapy, is warranted for the clinical treatment of pancreatic cancer.

Yang SH, Kuo TC, Wu H, Guo JC, Hsu C, Hsu CH, Tien YW, Yeh KH, Cheng AL, Kuo SH. Perspectives on the combination of radiotherapy and targeted therapy with DNA repair inhibitors in the treatment of pancreatic cancer. *World J Gastroenterol* 2016; 22(32): 7275-7288 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i32/7275.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i32.7275

INTRODUCTION

Pancreatic cancer is not a common disease; there are approximately 330000 cases a year worldwide. However, it is highly lethal, with nearly equal numbers of new cases and deaths. The majority (> 80%) of pancreatic cancers are ductal adenocarcinoma, for which the prognosis remains poor, even with recent advances in detection, supportive care, and therapeutics[1]. It remains challenging to make an early diagnosis for sporadic pancreatic cancer, because of the low lifetime risk of developing pancreatic cancer and the lack of adequate screening methods[2]. Endoscopic ultrasonography and MRI screening tools may play a limited detection role in patients with high risks, such as family history or known germline mutations[3,4].

Only 20% of pancreatic cancer patients are good candidates for curative resection at diagnosis. The 5-year survival rate doubled from 10.4% to 20.7%, after 6-mo of treatment with adjuvant gemcitabine, in the CONKO-001 study[5]. This randomized trial revealed that application of adjuvant gemcitabine significantly improved median disease-free survival (13.4 mo *vs* 6.9 mo), compared with that by surgery alone[6]. However, at least 50% of the patients eventually developed distant metastases[6]. Systemic dissemination occurs early in the disease process, in a mouse model of pancreatic cancer[7]. Clinical trials, in the past decade, have attempted to administer modern adjuvant radiotherapy, alone or combined with chemotherapy, to decrease the local recurrence and eventual metastasis that remain significant issues for operable pancreatic cancer[6]. However, the local recurrence rate is still more than 30%[8]. In addition, the survival benefit with adjuvant radiotherapy is controversial and may be outweighed by the toxicity of the treatments[9].

Patients with advanced pancreatic cancer frequently suffer from local symptoms. Local control of the main tumor is paramount to palliate these complaints, in addition to surgical bypass, and biliary/intestinal stenting or drainage[10]. The local control rate with chemotherapy alone varies over a wide range, which is probably due to the use of a single agent versus combined chemotherapy[10,11]. It is questionable if local control can translate into a survival benefit. Bolus 5-FU-based chemoradiotherapy (CCRT) concurrent with maintenance chemotherapy was shown, in the 1980s, to double overall survival from 22.9 wk to more than 40 wk compared to that by radiotherapy alone[12]. However, a comparison of CCRT to chemotherapy alone did not show a consistent survival benefit[13,14]. Moreover, the use of modern radiotherapy techniques, to obtain a survival benefit in locally advanced pancreatic cancer, is of great debate[10,15,16].

The underlying reasons for inconsistent benefits in adding radiotherapy to chemotherapy, in the adjuvant or palliative setting, are multifactorial. Potential explanations are poor quality control of the delivery of radiotherapy, the use of old techniques with high radiation-induced toxicity, breaks with divided radiotherapy courses, and the use of agents with poor radiosensitization and high toxicity. However, pancreatic cancer metastasizes early[7]. The choice of the most appropriate medicine added to the radiotherapy, rather than radiotherapy itself, may be the most important answer. The most common current daily practice combines radiosensitizing agents, 5-FU and gemcitabine, with radiotherapy in the adjuvant or advanced setting. However, the single-agent activity of gemcitabine or 5-FU in advanced disease is poor[17]. In addition, significant toxicities of CCRT are always of concern[9]. The aim of this review is to present an overview of the types of DNA damage in pancreatic cancer, summarize new evidence in non-chemotherapy agents, with the focus on DNA repair-related targeted therapy (Table

1). Additionally, we will provide direction for further development of use of these agents combined with radiotherapy in pancreatic cancer.

TYPES OF DNA DAMAGE IN PANCREATIC CANCER

Radiotherapy has a local therapeutic role for pancreatic cancer, however, it is much less frequently used than systemic therapy[18,19]. The theoretical mechanism of cytotoxicity, induced directly or indirectly by radiotherapy, is DNA damage, regardless of whether it is caused by photons, charged particles with protons or carbon ions, or any emerging technique. The types of DNA damage induced by ionizing radiation (IR) include single-strand break (SSB), double-strand break (DSB), base modifications, and DNA-protein cross-linking. The damage can be repaired, in normal mammalian cells, through several mechanisms. The repair mechanism can be homologous recombination (HR), nonhomologous end joining (NHEJ), nucleotide excision repair (NER), base excision repair (BER), and mismatch repair (MMR), regardless of whether the damage was induced by IR or occurred spontaneously (Figure 1).

The pancreatic cancer genome is unstable. Telomere shortening was obvious in pancreatic intraepithelial neoplasia (PanIN) lesions, even in the earliest PanIN-1A lesions. However, this phenomenon was not found in atrophic or inflammatory pancreatic lesions[20]. The telomeres were much shorter in cancer cells than in PanIN-1 or PanIN-2 lesions[21]. The activation of telomeres expression seen in the majority of pancreatic cancers may be resulted from the protective mechanism of telomeres against catastrophic DNA damage[22]. Previous studies also demonstrated that telomere shortening was closely associated with the DNA repair impairment[23,24]. In addition, widespread DNA damage was found in PanIN lesions. Increased γH2AX^{Ser139}, phospho-ataxia-telangiectasia mutated (ATM)^{Ser1981}, and phospho-cell cycle checkpoint kinase 2 (Chk2)^{Thr68} signals were noted in PanIN lesions, compared to that in the normal pancreatic epithelium[25]. Intraductal papillary mucinous neoplasm, another type of pancreatic cancer precursor, was also shown to have an increased phospho-Chk2^{Thr68} nuclear signal, compared to that in the normal pancreatic epithelium[26]. γH2AX^{Ser139}, phospho-ATM^{Ser1981}, phospho-Chk1^{Ser345}, phospho-Chk2^{Thr68}, phospho-DNA-PKcs^{Ser2056}, Rad51, and Ku70 expression levels are increased in invasive pancreatic cancer tissues compared to that in normal pancreatic tissues[27]. Therefore, DNA damage lesions, induced by exogenous or endogenous reagents, must accumulate early in the carcinogenic process. These lesions induce universal activation of DNA damage responses; however, the repair machinery does not work perfectly and may itself be the victim of mutation. This hypothesis is supported by the observation that some familial pancreatic cancers are associated with genetic defects in DNA damage responses and repair machinery, such as *TP53, BRCA2, ATM, PALB2*, and MMR-related genes (*e.g*., *hMLH1*, *hMSH2,* and *hMSH6*)[28].

Notably, a recent study, which applied whole-genome sequencing and copy number variation analysis, demonstrated that four subtypes of structural variation could be identified in pancreatic cancer[29]. Among them, the "unstable" subtype was characteristic of defects in genomic stability with considerable structural variations. Ten of 14 patients in this subgroup were in the top quintile of the *BRCA* signature, with deleterious mutations in *BRCA1*, *BRCA2*, or *PALB2* genes. Most importantly, five patients in this subtype responded very well to platinum-based therapy[29]. In fact, the DNA repair mechanisms implicated in platinum or IR treatment are overlapping, including DSB repair, SSB repair, NER, BER, and MMR. This study provides a strong rationale for radiosensitization, using agents to inhibit the DNA repair machinery in pancreatic cancer cells treated with IR, so that lethal DNA lesions will go unrepaired. We present a comprehensive review of the mechanism and clinical histories of these agents.

POLY (ADP-RIBOSE) POLYMERASE INHIBITORS

Poly (ADP-ribose) polymerases (PARPs) are nuclear proteins that play important roles in SSB repair. DNA breaks induce PARP to bind to the lesions, through its N-terminal zinc finger motifs, which causes massive ADP-ribose polymerization. PARP hydrolyzes nicotinamide adenine dinucleotide to generate ADP-ribose units. It covalently adds the units to the side chains of aspartate, arginine, lysine, and glutamate amino acids on the surfaces of nearby protein substrates and PARP itself. Then, DNA repair machinery, which has a high affinity for ADP-ribose polymers, is recruited to the DNA nicks and performs DNA repair[30]. Preclinical and clinical studies demonstrated that cancers, with mutated *BRCA1* and/or *BRCA2*, were highly sensitive to PARP inhibitors[31-37]; this confirms the "synthetic lethal" hypothesis[38]. BRCA1 binds to CtBP-interacting protein and the MRE11-RAD50-NBS1 (MRN) complex, when double strand DNA breaks occur. This forms a functional unit that senses and resects the damaged DNA. Then, BRCA1, BRCA2, and PALB2 mediate RAD51 recombinase-dependent HR[39]. However, cancer cells with defective *BRCA1*, *BRCA2,* or *PALB2* have high genomic instability[29]. Therefore, these HR-defective cancer cells are vulnerable to PARP inhibitors that interfere with SSB repair. They suffer from error-prone DNA repair, cell cycle arrest, and ultimately cell death.

Pancreatic cancer, with defective HR, is highly sensitive to PARP inhibitors[34,37,40-42]. Capan-1, a prototypical pancreatic cancer cell line with defective *BRCA2* (6174delT), has *in vitro* sensitivity to molecular

Table 1 Summary of compounds entering clinical trials of pancreatic cancer or radiotherapy

Compound	Target	RT	Clinical trial
Rucaparib	PARP	-	A Study of Rucaparib in Patients With Pancreatic Cancer and a Known Deleterious BRCA Mutation (NCT02042378)
Olaparib (AZD2281)	PARP	-	Ph Ⅱ Olaparib for BRCAness Phenotype in Pancreatic Cancer (NCT02677038) Olaparib in gBRCA Mutated Pancreatic Cancer Whose Disease Has Not Progressed on First Line Platinum-Based Chemotherapy (POLO)(NCT02184195) Trial of ICM With or Without AZD2281 (Olaparib) in Patients With Advanced Pancreatic Cancer (NCT01296763) Efficacy and Safety of PARPi to Treat Pancreatic Cancer (NCT02511223) Study to Assess the Safety and Tolerability of a PARP Inhibitor in Combination With Gemcitabine in Pancreatic Cancer (NCT00515866)
		+	Olaparib Dose Escalating Trial + Concurrent RT With or Without Cisplatin in Locally Advanced NSCLC (olaparib) (NCT01562210) Olaparib and Radiotherapy in Inoperable Breast Cancer (NCT02227082) Olaparib and Radiotherapy in Head and Neck Cancer (NCT02229656) Phase I Study of Olaparib Combined With Cisplatin-based Chemoradiotherapy to Treat Locally Advanced Head and Neck Cancer (ORCA-2) (NCT02308072)
BMN673 (Tazaloparib)	PARP	-	Study of Talazoparib, a PARP Inhibitor, in Patients With Advanced or Recurrent Solid Tumors (NCT01286987)
Veliparib (ABT-888)	PARP	-	Gemcitabine Hydrochloride and Cisplatin With or Without Veliparib or Veliparib Alone in Treating Patients With Locally Advanced or Metastatic Pancreatic Cancer (NCT01585805) Veliparib, Oxaliplatin, and Capecitabine in Treating Patients With Advanced Solid Tumors (NCT01233505) Veliparib, Cisplatin, and Gemcitabine Hydrochloride in Treating Patients With Advanced Biliary, Pancreatic, Urothelial, or Non-Small Cell Lung Cancer (NCT01282333) Veliparib in Treating Patients With Malignant Solid Tumors That Did Not Respond to Previous Therapy (NCT00892736) Veliparib and Irinotecan Hydrochloride in Treating Patients With Cancer That Is Metastatic or Cannot Be Removed by Surgery (NCT00576654) ABT-888 With Modified FOLFOX6 in Patients With Metastatic Pancreatic Cancer (NCT01489865)
		+	A Phase I Study of Veliparib (ABT-888) in Combination With Gemcitabine and Intensity Modulated Radiation Therapy in Patients With Locally Advanced, Unresectable Pancreatic Cancer (VelGemRad) (NCT01908478) A Study Evaluating the Efficacy and Tolerability of Veliparib in Combination With Paclitaxel/Carboplatin-Based Chemoradiotherapy Followed by Veliparib and Paclitaxel/Carboplatin Consolidation in Subjects With Stage Ⅲ Non-Small Cell Lung Cancer (NCT02412371) Veliparib With or Without Radiation Therapy, Carboplatin, and Paclitaxel in Patients With Stage III Non-small Cell Lung Cancer That Cannot Be Removed by Surgery (NCT01386385) A Clinical Study Conducted in Multiple Centers Comparing Veliparib and Whole Brain Radiation Therapy (WBRT) Versus Placebo and WBRT in Subjects With Brain Metastases From Non Small Cell Lung Cancer (NSCLC) (NCT01657799) A Phase I Study of ABT-888 in Combination With Conventional Whole Brain Radiation Therapy (WBRT) in Cancer Patients With Brain Metastases (NCT00649207) ABT-888, Radiation Therapy, and Temozolomide in Treating Patients With Newly Diagnosed Glioblastoma Multiforme (NCT00770471) Pre-Operative Radiation and Veliparib for Breast Cancer (NCT01618357)
Iniparib (BSI-201)	PARP	+	A Trial Evaluating Concurrent Whole Brain Radiotherapy and Iniparib in Multiple Non Operable Brain Metastases (RAPIBE) (NCT01551680)
VX-970 (VE-822)	ATR	+	VX-970, Cisplatin, and Radiation Therapy in Treating Patients With Locally Advanced HPV-Negative Head and Neck Squamous Cell Carcinoma (NCT02567422) VX-970 and Whole Brain Radiation Therapy in Treating Patients With Brain Metastases From Non-Small Cell Lung Cancer (NCT02589522)
AZD6738	ATR	+/-	Phase I Study to Assess Safety of AZD6738 Alone and in Combination With Radiotherapy in Patients With Solid Tumours (Patriot) (NCT02223923)
UCN-01	Chk1	-	UCN-01 and Gemcitabine in Treating Patients With Unresectable or Metastatic Pancreatic Cancer (NCT00039403) UCN-01 and Fluorouracil in Treating Patients With Metastatic Pancreatic Cancer (NCT00045747) 7-Hydroxystaurosporine and Irinotecan Hydrochloride in Treating Patients With Metastatic or Unresectable Solid Tumors or Triple Negative Breast Cancer (NCT00031681)
LY2603618	Chk1	-	A Study for Patients With Pancreatic Cancer (NCT00839332)
MK-1775 (AZD1775)	Wee1	-	Paclitaxel Albumin-Stabilized Nanoparticle Formulation and Gemcitabine Hydrochloride With or Without WEE1 Inhibitor MK-1775 in Treating Patients With Previously Untreated Pancreatic Cancer That Is Metastatic or Cannot Be Removed by Surgery (NCT02194829)
		+	Dose Escalation Trial of MK1775 and Gemcitabine (+Radiation) for Unresectable Adenocarcinoma of the Pancreas (NCT02037230)
LB-100	PP2A	-	Phase I Study of LB-100 With Docetaxel in Solid Tumors (NCT01837667)
MSC2490484A	DNA-PK	+	Phase 1 Trial of MSC2490484A, an Inhibitor of a DNA-dependent Protein Kinase, in Combination With Radiotherapy (NCT02516813)

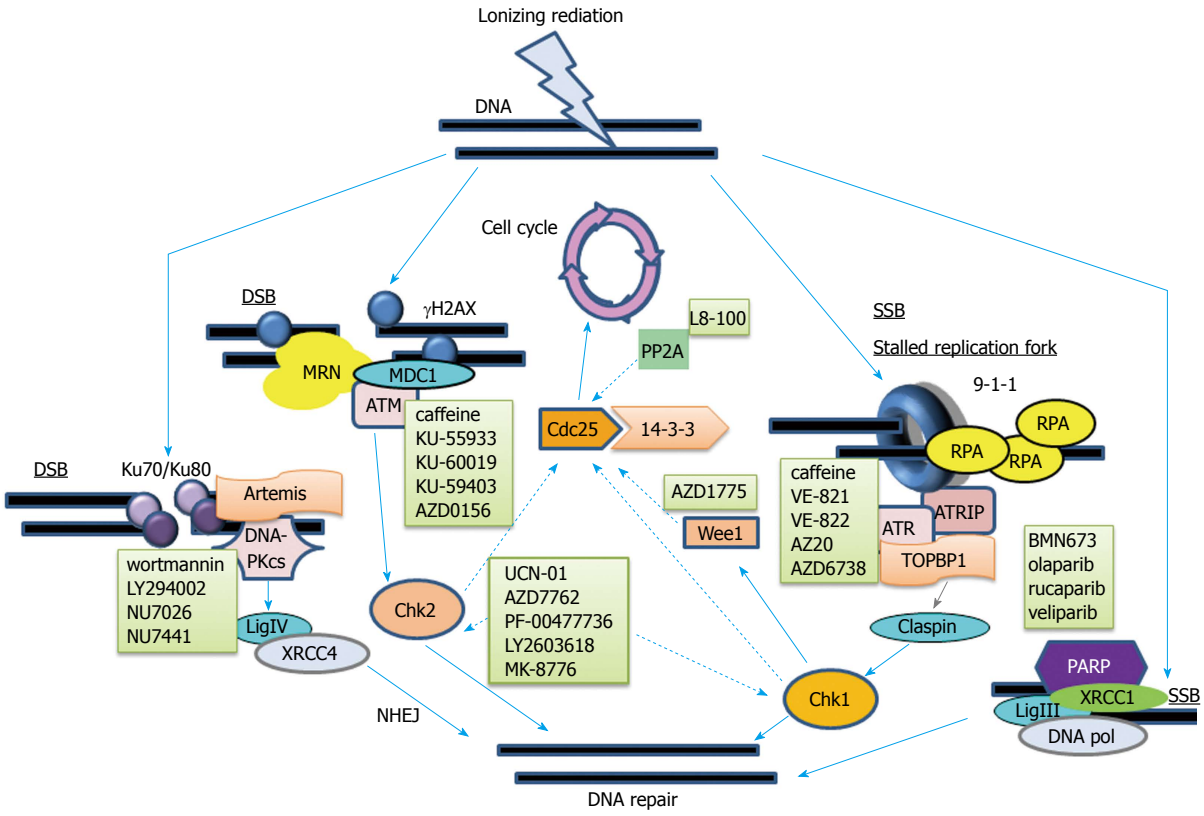

Figure 1 Major DNA repair pathways and molecular agents that inhibit DNA repair. Here we demonstrate the main DNA repair pathways that are activated by IR-induced DNA damage. DNA repair machinery, with high affinity for ADP-ribose polymers, is recruited to DNA nicks after PARP binds SSB lesions; this executes DNA repair. RPA binds to ssDNA and recruits ATR and ATRIP. This is followed by recruitment of the 9-1-1 complex, TOPBP1, and claspin. Finally, Chk1 activates downstream repair machinery and phosphorylates Cdc25 proteins, which are inhibited by 14-3-3 proteins; this arrests the cell cycle. Wee1 and PP2A are also able to modulate the activity of Cdc25. DSB repair requires H2AX phosphorylation and recruitment of the MRN complex, MDC1, and ATM. Then Chk2 is phosphorylated, activated, and mediates repair and cell cycle arrest. NHEJ is the main IR-induced DSB repair method. It starts with Ku70/Ku80 heterodimer recruitment, which is followed by DNA-PKcs to form the active DNA-PK. Then DNA ligase Ⅳ and XRCC4 mediate DNA ligation. The respective molecular inhibitors for PARP, ATR, Chk1/Chk2, ATM, and DNA-PK are indicated. IR: Ionizing radiation; PARP: Poly ADP ribose polymerase; SSB: Single-strand break; DSB: Double-strand break; RPA: replication protein A; ATR: Ataxia-telangiectasia and Rad3 related; ATRIP: ATR-interacting protein; TOPBP1: Topoisomerase-binding protein-1; MRN: MRE11-RAD50-NBS1; NHEJ: Non-homologous end joining; ATM: Ataxia-telangiectasia mutated.

targeted agents, including rucaparib, olaparib, and BMN 673[37,40,41]. Increased formation of nuclear γH2AX foci was found[37,41] after treatment of Capan-1 with rucaparib or BMN 673, which indicated an increased number of DNA breaks. In contrast, nuclear RAD51 foci did not increase[37,41]. A study, in the xenograft model of Capan-1, combined rucaparib with carboplatin, a DNA-damaging agent. The results showed better efficacy than that observed with either agent alone[37]. A patient-derived xenograft model, with mutated *BRCA2*, received BMN 673 treatment, which decreased cancer cell mitosis and increased cell apoptosis[41]. A phase Ⅱ clinical trial of olaparib enrolling 23 pancreatic cancer patients with germline *BRCA1/2* mutations, who were heavily pretreated; the response rate and stable disease were 21.7% and 35%, respectively[34]. Hematological toxicities became a matter of concern, in a phase Ⅱ study that had enrolled patients without enrichment of *BRCA* mutations, despite clinical responses to the combination of gemcitabine and olaparib that seemed to be promising[43].

IR can induce SSB and DSB and may be synergistic with PARP inhibitors, especially in cancer cells with defective DNA repair abilities[44]. In fact, preclinical studies demonstrated that PARP inhibitors had radiosensitizing effects in various cancer types[37,45-52], including pancreatic cancer[53-57], regardless of the DNA repair machinery integrity. Increased apoptosis and DNA breaks, accompanied by decreased proliferation of cancer cells, were observed in a lung cancer model using veliparib[45]. Importantly, veliparib caused comparable radiosensitization in oxic and hypoxic conditions[46]. Diminished angiogenesis was also observed in the lung cancer model that used veliparib[45]; however, increased vascular perfusion was noted in another lung cancer model using olaparib[52]. The discrepancy, in the effects of different PARP inhibitors on blood vessels, may be an epiphenomenon that is not truly associated with radiosensitization.

A synergistic effect was also shown when MiaPaCa-2, a *BRCA*-intact pancreatic cancer cell line, was treated with IR, veliparib, or both. The combination increased apoptosis *in vitro* and inhibited tumor growth in an animal model compared to that by either treatment

alone[56]. S phase arrest and then G2/M arrest were induced in MiaPaCa-2, by combining olaparib with either γ-irradiation or carbon-ion irradiation[54]. Rad51 foci were increased after rucaparib, IR, or combined treatment, which indicated the presence of functional HR[55]. Rucaparib induced more γH2AX foci in Capan-1 than in MiaPaCa-2; however, the magnitude of γH2AX foci induction after IR, or IR combined with rucaparib, was similar in the two cell lines[55]. Differences in DNA repair machinery, other than PARP-related SSB repair and BER, between Capan-1 and MiaPaCa-2 may partially explain the finding.

Gemcitabine, oxaliplatin, irinotecan, and 5-FU are all radiosensitizing agents and are current standards for advanced pancreatic cancer[11,17]. Platinum drugs, such as cisplatin, oxaliplatin, and carboplatin, have the potential to enhance the radiosensitizing effects of PARP inhibitors in patients with defective HR[29]. Synergistic effects between PARP inhibitors and oxaliplatin have been observed already in colon cancer models[58,59]. In addition, IR combined with oxaliplatin and veliparib showed further enhanced synergistic effects *in vitro* and *in vivo*[59]. Chemoradiosensitization with PARP inhibitors was also noted with irinotecan[48,59], 5-FU[59], and gemcitabine[55], in different cancer models. However, the underlying mechanisms for these agents are not clear.

ATM/ATR INHIBITORS

Increased ATM activation was observed in premalignant and invasive lesions of pancreatic cancer[25,27]. In addition, oncogenic Ras can lead to increased oxidative DNA damage[60] and DNA replication stress-induced DNA damage. It eventually activates ataxia-telangiectasia and Rad3-related (ATR)/Chk1-related DDR[61,62]. ATM and ATR belong to the phosphatidylinositol 3-kinase-related kinase (PIKK) family of serine/threonine protein kinases; they share a number of substrates. The MRN complex is recruited upon DNA DSB, induced by IR, chemicals, or endogenous processes. It processes damaged DNA ends and initiates NHEJ through all cell cycle phases or HR in late S/G2 phase only[63]. The MRN complex aids the conversion of inactive ATM homodimers to active monomers, after autophosphorylation at serine^{1981}[64,65]. Then, ATM and DNA-PK phosphorylate H2AX at serine^{139}, which forms γH2AX at sites close to the DNA DSB[66,67]. The association of the MRN complex, MDC1, and γH2AX enhances the accumulation of phosphorylated ATM and further phosphorylation of H2AX at DNA DSB sites[68]. Next, the DSB repair machinery is recruited and p53 and Chk2 are phosphorylated[69-71]. The replication protein A (RPA) coated ssDNA structure recruits ATR and ATR-interacting protein (ATRIP) to bind with RPA, at sites of damaged DNA or stalled replication forks[72,73]. The RAD9-HUS1-RAD1 (9-1-1) clamp complex[74] localizes to the damaged DNA sites, with the aid of RAD17 clamp loader. This event brings the ATR/ATRIP complex activator topoisomerase-binding protein-1 (TOPBP1) to the complex[75]. This step is essential for ATR/ATRIP activation and further signaling. Claspin functions as the adaptor that brings Chk1 to the ATR/ATRIP complex[76]. Phosphorylation of the ATRIP, 9-1-1 complex, TOPBP1, the minichromosome maintenance protein (MCM) complex, and RPA also follow ATR activation.

Inhibitors of ATM or ATR are under active development for the treatment of various cancer types. Caffeine, a methylxanthine alkaloid, inhibited the kinase activities of ATM and ATR with an IC_{50} in millimolar ranges. It subsequently induced Ser^{15} phosphorylation of p53, 2 effects that can contribute to radiosensitization[77]. Notably, caffeine also induced radiosensitization in p53-deficient cells, through the activation of Cdk1[78]. However, the clinical use of caffeine as a radiosensitizer is limited, due to its low serum level and high systemic toxicity. KU-55933, the first potent and selective ATM inhibitor, was shown to induce radiosensitization and inhibit IR-induced ATM-mediated phosphorylation of p53, H2AX, and Chk1. However, the radiosensitizing dose of KU-55933 was much higher than the dose required to inhibit ATM[79]. KU-60019, an analog of KU-55933 with better pharmacokinetics, bioavailability, and selective potency for ATM, had a higher radiation dose to enhancement ratio in glioma cells than KU-55933 did. The radiosensitizing effect of KU-55933 was attributed to the indirect inhibition of AKT phosphorylation[80]; the aforementioned effect was more pronounced in xenograft tumors with mutant *p53*[81]. However, the oral bioavailability of KU-60019 was still poor[81]. KU59403, another analog of KU-55933 with higher potency and oral bioavailability had plasma and intra-tumor concentrations in therapeutic ranges in the xenograft model compared with those of KU-55933. However, its radiosensitizing effects were not addressed[82]. A few ATM inhibitors are entering clinical trials; an example is a phase I trial for AZD0156 (ClinicalTrials.gov: NCT02588105), which was developed by AstraZeneca.

Unlike ATM, the loss of ATR results in early embryonic lethality. ATR is essential for proliferating cells to ensure proper DNA replication and genomic integrity. Schisandrin B, a herbal ingredient isolated from *Fructus schisandrae*, is the first selective ATR inhibitor with an IC_{50} in the micromolar range. Schisandrin B inhibited phosphorylation of p53 and Chk1 following UV irradiation; thereby, providing radiosensitization in A549 lung adenocarcinoma cells[83]. VE-821, developed by Vertex Pharmaceuticals, was the first selective and potent ATR inhibitor[84]. It conferred radiosensitization among all 12 cell lines that were tested. Notably, it could induce radiosensitization and reduce Chk1 phosphorylation in hypoxic conditions[85]. Radiosensitization was observed, in pancreatic cancer cell lines with defective p53, when VE-821 was used concurrently with IR or 24 h after IR; it occurred under normoxic and hypoxic conditions. G2/M phase was delayed and reduced in this study, which indicated that IR-induced checkpoint activation was inhibited by VE-821[86]. Foci of 53BP1 and γH2AX increased

following IR and VE-821 treatment. Interestingly, Rad51 foci were reduced, which suggested inhibition of HR repair[86]. VE-822, also known as VX-970, is an analog of VE-821. It is the first selective ATR inhibitor to enter clinical trials. In a pancreatic cancer cell line model, VE-822 induced radiosensitization, by downregulating Chk1 phosphorylation and Rad51 foci and upregulating 53BP1 and γH2AX foci[87]. VE-822 did not have an antitumor effect *in vivo*; however, it enhanced the efficacy of IR, without significant weight loss in animals[87]. Moreover, tumor growth delay was more significant with gemcitabine-VE-822 plus IR, compared with that by either agent used singly with IR[87]. AZD6738, derived from ATR and mTOR inhibitor AZ20[88], is an orally available, selective, and potent ATR inhibitor; it is under phase Ⅰ clinical trial development in combination with radiotherapy (ClinicalTrials.gov: NCT02223923). AZD6738 was shown to inhibit Chk1 phosphorylation and *in vitro* and *in vivo* radiosensitization[89].

CHECKPOINT KINASE INHIBITORS

Chk1 and Chk2 are functionally overlapping serine/threonine protein kinases. Chk1 or Chk2, activated by phosphorylation (Ser^{317} and Ser^{345} on Chk1, Thr^{68} on Chk2), binds to, and phosphorylates Cdc25 phosphatases. Then, 14-3-3 proteins bind to, sequester, and inhibit Cdc25 phosphatases[90,91]. In addition, Chk1 activates (never in mitosis gene A)-related kinase-11 (Nek11), which phosphorylates Cdc25A and mediates its polyubiquitination and degradation[92]. Human cells have 3 isoforms of Cdc25, all of which can dephosphorylate and activate Cdks. Cdc25s are rapidly degraded when DNA is damaged and the activities of Cdk1 and Cdk2 are inhibited; this results in cell cycle arrest[93].

UCN-01 (7-hydroxystaurosporine) is the first Chk1 inhibitor that has a non-specific inhibitory spectrum, low volumes of distribution, and systemic clearance. Unexpectedly, UCN-01 strongly binds to α1-acid glycoprotein[94]. The long half-life, decreased bioavailability, and pharmacokinetics that are highly variable among patients, may be attributed to α1-acid glycoprotein; this precluded UCN-01 from more advanced clinical development[95]. In addition, the activity of UCN-01 in pancreatic cancer was poor[96]. AZD7762, an ATP competitive and non-selective inhibitor of Chk1 and Chk2, was shown to have *in vitro* and *in vivo* chemosensitization, through stabilization of Cdc25A, following gemcitabine treatment[97]. IR plus AZD7762, in the HT-29 colon cancer cell model, delayed tumor growth more than IR alone, due to impairment of DNA repair by AZD7762[98]. AZD7762 showed better radiosensitization, with or without concurrent treatment with olaparib, in pancreatic cell lines with defective *P53*, including MiaPaCa-2[53]. A combination of IR and AZD7762, in the same model, resulted in Ser^{345} phosphorylation of Chk1, Cdc25A stabilization, decreased Rad51 foci, and delayed *in vivo* tumor growth[99]. However, cardiac toxicities, including increased troponin Ⅰ, myocardial ischemia, abnormal electrocardiogram, and decreased ejection fraction, precluded AZD7762 from further clinical development[100,101].

PF-00477736, a selective and potent ATP competitive Chk1 inhibitor, was shown to inhibit Chk2. It abrogated gemcitabine-induced S-phase arrest, increased γH2AX expression, and induced apoptosis in a HT-29 cell line[102]. In addition, the sequential administration of gemcitabine and PF-00477736, in an *in vivo* model, resulted in more inhibition of tumor growth than gemcitabine alone[103]. Remarkably, three-component chemoradiation, with PF-00477736, gemcitabine, and Lutetium-177 Lu–labeled anti-EGFR antibody, completely eradicated pancreatic cancer xenografts[102]. However, the phase Ⅰ clinical trial was prematurely terminated, because of business-related reasons (ClinicalTrials.gov: NCT00437203).

LY2603618 and MK-8776 (SCH 900776) are other Chk1 and/or Chk2 inhibitors entering clinical trials. Studies showed that both agents cause chemosensitization with gemcitabine[104-106]. Expanded studies showed that a combination of MK-8776, gemcitabine, and radiotherapy was the most promising treatment for inhibition of tumor growth, in animal models of pancreatic cancer[105]. The most common grade 3 or more toxicity, encountered with the use of gemcitabine or LY2603618, was hematological[107]. Grade 3 or higher hematological toxicities and fatigue were the most common negative effects in response to gemcitabine and MK-8776 treatment; however, such occurrences were rare with MK-8776 alone[108]. A variety of Chk1 and/or Chk2 inhibitors are under active preclinical development, including EXEL-9844 (also called XL-844), CEP-3891, PD-321852, Chir-124, CCT241533, LY2606368, CCT245737, SAR-020106, and GNE-900[109-118].

WEE1 AND PP2A INHIBITORS

Chk1 activates Wee1 kinase1 at the G2-M checkpoint, upon DNA damage. Activated Wee1 phosphorylates CDC2 $(Cdk1)^{Tyr15}$, which enables CDC2/Cdk1 inactivation; this process contributes to G2-M arrest[119]. At the same time, activated Chk1 phosphorylates and inactivates Cdc25 to prevent the dephosphorylation and inactivation of CDC2/Cdk1. In contrast, protein phosphatase 2A (PP2A) is able to dephosphorylate and inhibit Cdc25, through 14-3-3 protein[120]. Therefore, inhibitors of Wee1 or PP2A can be used to maintain the activity of Cdc25; thus, they theoretically allow cell cycle progression, without adequate time for DNA repair.

In vitro studies, in p53-defective MCF-7 cancer cells derived from breast cancer cell lines, showed that compared to p53-intact cells, these cells were much more sensitive to Wee1 inhibition by MK-1775

(AZD1775)[121]. In addition, radiosensitization was observed in the p53-defective MCF-7 cells in a clonogenic assay. Cells pretreated with MK-1775 (AZD1775) had a reduction in $Cdk1^{Tyr15}$ phosphorylation and 53BP1 foci; however, they had an increase in γ-H2AX after IR[121]. The defective DNA repair was through inhibition of HR, but not NHEJ[121]. MK-1775 (AZD1775) monotherapy, in patient-derived pancreatic cancer xenografts, was ineffective[122]. However, a combination of gemcitabine and MK-1775 (AZD1775) abrogated G2-M checkpoint arrest, which was accompanied by pancreatic tumor regression, increased mitotic entry and apoptosis in pancreatic cancer cells[122]. MK-1775 (AZD1775) increased gemcitabine-induced radiosensitization, in MiaPaCa-2 pancreatic cancer cell lines, through inhibition of $Cdk1^{Tyr15}$ phosphorylation and upregulation of γ-H2AX expression[123]. However, the radiosensitization phenomenon was not observed in HR and *BRCA2*-defective Capan-1 cells. A combination of AZD1775, gemcitabine, and radiotherapy enhanced a delay in tumor growth and impaired RAD51 focus formation, in xenografts derived from patients with pancreatic cancer[124]. In fact, MK-1775 (AZD1775) is the first-in-class Wee1 inhibitor, with high specificity and potency, to enter clinical trial development. A phase I study, which used single agent MK-1775 (AZD1775) to treat refractory solid tumors, had activities in patients with a *BRCA* mutation; however, myelosuppression and supraventricular tachycardia were dose-limiting toxicities[124]. Clinical trials combining MK-1775 (AZD1775) and radiotherapy in various cancer types are underway.

Knockdown of the PP2A and PPP2R1A subunit, in MiaPaCa-2 and Panc-1 cell lines, resulted in significant radiosensitization and persistent γ-H2AX expression. The main mechanisms, of radiosensitization by PP2A inhibition, are through the activation of CDC25C/$Cdk1^{Tyr15}$ and inhibition of HR[125]. The aforementioned phenomenon was reproduced using the PP2A inhibitor, LB-100, which increased $CDC25C^{Thr130}$, but decreased $Cdk1^{Tyr15}$ phosphorylation[126]. The synergistic effects of LB-100 and radiotherapy on delayed tumor growth were also observed in the MiaPaCa-2 xenograft model[126]. At present, a phase Ⅰ clinical trial, in which LB-100 is administered alone or in combination with docetaxel, is ongoing. Initial outcomes show that one patient, with stage Ⅳ disease, had a long, stable disease[125].

DNA DEPENDENT PROTEIN KINASE INHIBITORS

A member of the PIKK family of serine/threonine protein kinases, along with ATM and ATR, DNA-PK, is essential for NHEJ, the major repair mechanism for IR-induced DSB in human cells. A catalytic subunit, DNA-PKcs, and a regulatory heterodimer (Ku70 and Ku80 subunits) combine to form active DNA-PK, which is an ATM and ATR target. It can phosphorylate Ku70/Ku80, RPA, γH2AX, Chk2, Artemis, DNA ligase Ⅳ, XRCC4, p53, and itself. The Ku heterodimer binds to the ends of dsDNA, which become available because of DNA DSB, and recruits DNA-PKcs[127]. DNA ligase Ⅳ and XRCC4 are recruited to join the DNA ends, after the blunt DNA ends are processed and in place[120].

Wortmannin is the first identified DNA-PK inhibitor; it is equipotent to PI3K, therefore, it is non-selective. Radiosensitization has been observed with Wortmannin[128]; however, its lack of specificity and *in vivo* toxicity precluded its clinical use. Another radiosensitizer[129], LY294002 (structurally unrelated to Wortmannin), is a reversible kinase domain inhibitor with non-selective *in vivo* toxicity. Repair of IR-induced DNA DSB, in pancreatic cancer cell lines, was delayed by Wortmannin. Its effect was comparable between cell lines, with or without defective *BRCA2*, which indicated that NHEJ, but not HR, was successfully inhibited[130]. Wortmannin and LY294002 have demonstrated activities, as single agents, in a pancreatic cancer cell line. In addition, chemosensitization of gemcitabine by LY294002 was shown in xenograft models of pancreatic cancer. However, the involvement of DNA-PK inhibition was not addressed[131]. NU7026, which is structurally related to LY294002, was shown to have better potency and selectivity for the PIKK family kinases. It chemosensitized cells for gemcitabine[132] and impaired NHEJ repair of DNA DSB, following IR in pancreatic cancer cell lines; however, HR repair was inefficiently increased and cells showed prolonged γH2AX expression. These data indicated that radiosensitization occurred through inhibition of DNA-PK[133]. NU7441, developed from the LY294002 backbone, sensitized cells to gemcitabine in PANC-1 cells[132]. In addition, radiosensitization was only demonstrated in V3-YAC cells, with proficient DNA-PKcs, but not in V3 cells without it; this confirmed the mechanism of NU7441[134]. However, the poor pharmacokinetics of NU7026 and NU7441 precluded them from further clinical development[135]. Other DNA-PK inhibitors, including IC86621, IC87102, IC87361, OK-1035, SU11752, and KU-00600648, are currently in preclinical development[135,136].

CONCLUSION

In general, the clinical development of PARP, ATM, ATR, Chk1, CHk2, Wee1, PP2A, and DNA-PK inhibitors is being pursued actively (Table 1). DNA damage is a universal characteristic of pancreatic cancer cells from the premalignant to invasive stages; therefore, the use of DNA repair inhibitors, either singly or in combinations, is of great potential. The concept of synthetic lethality has been supported by the impressive clinical success of PARP inhibitors in *BRCA*-defective cancers. The optimal combination of each of these agents with radiotherapy has yet to be determined for pancreatic cancer, because of limited clinical data. Reliable biomarkers for these agents, with or without radiotherapy, are largely unknown. Chemosensitization, between these agents and genotoxic chemotherapy

drugs, has been well defined in pancreatic cancer preclinical studies. However, the best regimen and administration sequence, of a combination of chemotherapy, radiotherapy, and these agents, remain to be elucidated. Finally, immune checkpoint inhibitors have the potential for creating neoantigens, through the combined action of radiation, inhibitors of DNA repair enzymes, and genotoxic chemotherapeutic agents. This approach may open a new field of therapeutics in cancers without high mutation loads, such as pancreatic cancer[137].

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REVIEW

Management of hepatocellular carcinoma with portal vein tumor thrombosis: Review and update at 2016

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Abstract

Portal vein tumor thrombosis (PVTT) is a common phenomenon in hepatocellular carcinoma (HCC). Compared to HCC without PVTT, HCC with PVTT is characterized by an aggressive disease course, worse hepatic function, a higher chance of complications related to portal hypertension and poorer tolerance to treatment. Conventionally, HCC with PVTT is grouped together with metastatic HCC during the planning of its management, and most patients are offered palliative treatment with sorafenib or other systemic agents. As a result, most data on the management of HCC with PVTT comes from subgroup analyses or retrospective series. In the past few years, there have been several updates on management of HCC with PVTT. First, it is evident that HCC with PVTT consists of heterogeneous subgroups with different prognoses. Different classifications have been proposed to stage the degree of portal vein invasion/thrombosis, suggesting that different treatment modalities may be individualized to patients with different risks. Second, more studies indicate that more aggressive treatment, including surgical resection or locoregional treatment, may benefit select HCC patients with PVTT. In this review, we aim to discuss

the recent conceptual changes and summarize the data on the management of HCC with PVTT.

Key words: Liver cancer; Vascular invasion; Targeted agent; Surgery; Radiotherapy

Core tip: Conventionally, the presence of portal vein tumor thrombosis (PVTT) indicated an extremely poor prognosis for hepatocellular carcinoma (HCC) patients and was considered a contraindication to both surgery and trans-arterial procedures. Recent studies indicate that HCC with PVTT represents a heterogeneous group with variable prognoses. Several classifications have been proposed to gauge the prognoses of PVTT. For selected patients with less severe PVTT, surgery with curative intent is feasible with favorable outcomes. Further, expanding treatment options, such as radiotherapy, radioembolization and systemic treatment, could improve the outcomes of patients with more severe forms of PVTT in patients with HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is characterized by its propensity to invade the vasculature within the liver. The invasion of the hepatic vasculature is of macroscopic or microscopic type. Macrovascular invasion refers to gross invasion into the main portal veins or their branches, hepatic veins or the inferior vena cava in the liver, while microscopic vascular invasion is defined as tumors within a vascular space lined by endothelium that is identifiable only by microscopy[1].

Portal vein tumor thrombosis (PVTT) is the most common form of macrovascular invasion of HCC. Multiple case series have suggested that the PVTT is a common phenomenon with a prevalence rate ranging from 10% to over 60%[2-5]. The presence of PVTT in patients with HCC has been consistently demonstrated by different series to be associated with poor prognoses, with a hazard ratio of death close to 2[5,6]. The poor prognosis of PVTT in HCC patients is the result of combined factors including impaired hepatic reserves, intrinsic aggressiveness of tumor, reduced intolerance to anti-neoplastic treatment and a high rate of developing complications related to portal hypertension. Clinically, PVTT is associated with large tumor size, increased tumor number, higher tumor grade, worse Child-Pugh class and higher serum alpha-fetoprotein (AFP)[7]. At the genetic level, next generation sequencing identified that mutations of the *KDM6A*, *CUL9*, *FDG6*, *AKAP3*, and *RNF139* genes were associated with the development of PVTT in advanced Hepatitis B virus-related HCC[8].

It has to be noted that not all portal vein thrombosis in HCC patients is due to neoplastic thrombus. It is evident that a portion (ranging from 0.6% to 11%) of cirrhotic patients, particularly those with portal hypertension, are complicated by non-neoplastic portal vein thrombosis (NPVT)[9]. Up to 72.7% of portal thrombi in HCC patients are indeed NPVT[10]. Patients with NPVT have better prognoses than those with PVTT; therefore, the differentiation between NPVT and PVTT is of clinical relevance. Theoretically, image-guided percutaneous fine needle aspiration or a biopsy of portal vein thrombosis could provide a definite pathological diagnosis[9,11]. However, biopsy procedures are not frequently conducted in clinical practice to confirm PVTT because of concerns about life-threatening complications such as injuries to the bile ducts or hepatic arteries[11]. Instead, non-invasive imaging studies are the most frequently used diagnostic tool for PVTT. Contrast-enhanced ultrasonography, dynamic contrast enhanced computed tomography and gadoxetic acid-enhanced magnetic resonance could achieve a diagnosis of PVTT with sensitivities of 82.5%-98%, 68%-86% and 92%-95%, respectively[11-13].

Conventionally, the treatment algorithm proposed by the Barcelona Clinic Liver Cancer (BCLC) system considers the presence of PVTT, regardless of the degree of invasion, as a contra-indication to surgery or transarterial chemoembolization (TACE)[14]. Patients with PVTT are classified in the BCLC stage C category and are considered candidates for systemic agents. However, a recent concept has evolved, which considers HCC with PVTT to consist of heterogeneous populations with different disease behaviors and prognoses, and selected patients with PVTT may benefit from more aggressive treatment modalities. In the current review, we aim to discuss these changing concepts in the management of HCC with PVTT with a focus on the latest data on the adoption of a more aggressive treatment approach for this disease entity.

CLASSIFICATIONS OF PVTT

The management of patients with PVTT can be challenging because the clinical course is typically characterized by poor underlying liver reserve and high portal vein pressure. A complicated operation is usually required if an aggressive treatment approach is contemplated. To devise the best treatment strategy for patients with PVTT, a universally accepted classification of PVTT is necessary for the guidance of treatment and a comparison of outcomes between

Table 1 Common classifications of portal vein tumor thrombosis in hepatocellular carcinoma

Group	Types of PVTT	Survival
Ikai *et al*[80]	Vp0: Absent	59% at 5 yr
	Vp1: Distal to but not in second-order branches	39.1% at 5 yr
	Vp2: In second-order branches	23.3% at 5 yr
	Vp3: In first-order branches	18.3% at 5 yr (Vp3 and Vp4)
	Vp4: In the main trunk or contralateral or both	
Shi *et al*[17]	10: Microscopic	
	1: In segmental branches or above	26.7% at 3 yr (Type 10 included)
	2: In the left or right branch	16.9% at 3 yr
	3: In the main trunk	3.7% at 3 yr
	4: In the superior mesenteric vein	0% at 3 yr
Xu *et al*[18]	A: In the main trunk or both the left and right branches	0% at 5 yr
	B: In only the left or right branch	5.2% at 5 yr

HCC: Hepatocellular carcinoma; PVTT: Portal vein tumor thrombosis.

different groups. At present, various classification systems have been developed in different centers. The more conventional and better-known classification is the one proposed by the Liver Cancer Study Group of Japan (LCSGJ). In their General Rules for the Clinical and Pathological Study of Primary Liver Cancer[15], there is a macroscopic classification of HCC with PVTT: Vp0, no PVTT; Vp1, the presence of a PVTT distal to, but not in, the second-order branches of the portal vein; Vp2, the presence of a PVTT in the second-order branches of the portal vein; Vp3, the presence of a PVTT in the first-order branches of the portal vein; and Vp4, the presence of a PVTT in the main trunk of the portal vein or a contralateral portal vein branch or both. According to the guideline, resection is one of the feasible options for treatment in case of minor portal vein involvement (*i.e.*, Vp1 and Vp2)[16]. For selected Vp3 or Vp4 patients, surgical resection would still be considered, and a 5-year survival of 18.3% has been reported[16]. In view of the guarded prognosis, few centers would adopt this aggressive approach. Moreover, the required expertise is not always available (Table 1).

In 2007, Shi *et al*[17] devised a classification of HCC with PVTT that incorporated microscopic PVTT as Type 1. In this classification, Type 1 is a PVTT involving segmental branches or above; Type 2 is a PVTT involving the right or left portal vein; Type 3 is a PVTT involving the main portal vein; and Type 4 is a PVTT involving the superior mesenteric vein. In general, surgical resection can be applied to Type 3 and to selected patients with Type 4. In their report, the numbers of patients with Types 1, 2, 3 and 4 were 144 (32.7%), 189 (42.9%), 86 (19.5%) and 22 (5.0%), respectively. The 1-, 2-, and 3-year overall survival rates were 54.8%, 33.9% and 26.7% for Type 1 patients, respectively, 36.4%, 24.9% and 16.9% for Type 2 patients, respectively, 25.9%, 12.9% and 3.7% for Type 3 patients, respectively, and 11.1%, 0% and 0% for Type 4 patients, respectively ($P < 0.0001$).

A simplified classification by Xu *et al*[18] divided patients with PVTT into two groups: Group A, with involvement of the main portal vein trunk or both the left and right portal veins, and Group B, with involvement of only the left or right portal vein. In their report, the Group A 1-year overall survival rate was 31.5% after resection, and the 1-, 3- and 5-year overall survival rates of Group B patients were 62.3%, 16.1% and 5.2%, respectively. This simple classification could be used as a quick reference when counseling patients (especially Group A patients) about surgical treatment. No matter which of the above classifications is used, the prognosis would be determined by the extent of the PVTT and its proximity to the main, or even the contralateral, portal vein.

SURGERY (*EN BLOC* PORTAL VEIN RESECTION *VS* TUMOR THROMBECTOMY)

The prognosis is notoriously poor for HCC patients with macrovascular PVTT, especially those whose PVTT has extended to the main or contralateral portal vein[19]. The PVTT could propagate further and obstruct the whole vein lumen, resulting in liver failure or life-threatening variceal bleeding. One of the treatment modalities is surgical resection, the two common modes of which are tumor thrombectomy and *en bloc* resection of the thrombus and the portal vein followed by portal vein reconstruction. Tumor thrombectomy is technically less demanding but might result in a residual tumor. As for the latter mode, it is associated with high morbidity and mortality rates despite a "perceived" better oncological outcome[20]. However, there has been no randomized controlled trial to determine the superiority of one over the other, and the choice rests with individual centers and individual surgeons (Figure 1).

A PVTT confined to the hepatic lobe harboring the HCC (ipsilateral PVTT) is usually resected when a hepatectomy is conducted to remove the HCC[21]. For the management of PVTT extending to the portal vein bifurcation or the main or contralateral portal vein,

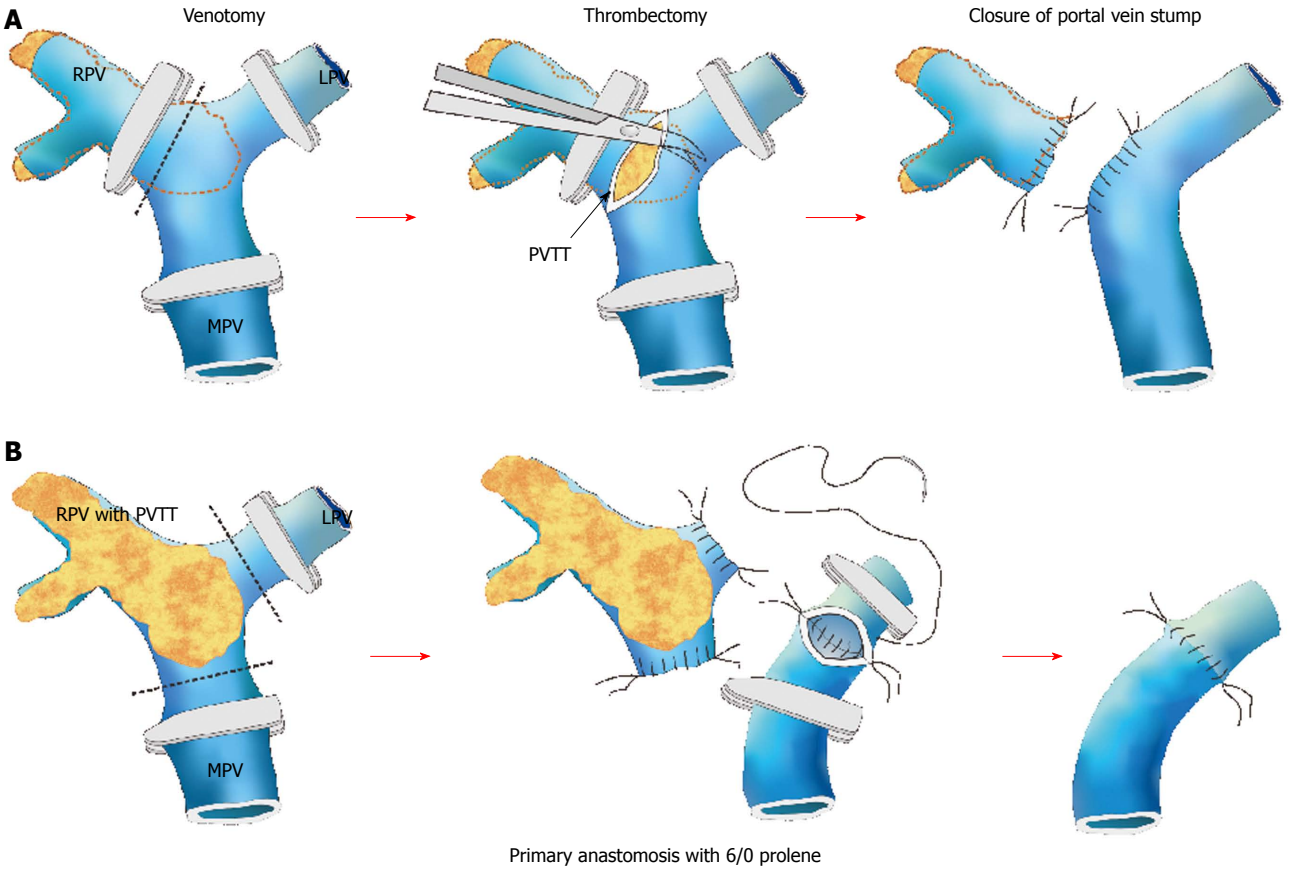

Figure 1 Schematic diagrams demonstrating different types of portal vein tumor thrombosis and the relevant surgical approaches. A: Thrombectomy; B: *En bloc* resection with portal vein reconstruction. RPV: Right portal vein; LPV: Left portal vein; MPV: Main portal vein. Courtesy of Chok *et al*[21].

different approaches have been recommended. *En bloc* resection, including bifurcation with or without the main portal vein and/or the contralateral portal vein, is believed to produce good oncological outcomes[22]. However, it has been documented that thrombectomy can produce similar survival outcomes with lower operative mortality and morbidity[20-22]. In a study from Japan, 979 consecutive patients were evaluated, and 45 of them had Vp3 or Vp4 PVTT[23]. They received hepatectomy with tumor thrombectomy. The 3- and 5-year survival rates in the Vp3 and Vp4 groups were 35.3% and 41.8%, and 21.2% and 20.9%, respectively.

In a study by The University of Hong Kong trying to address the controversy about *en bloc* resection *vs* thrombectomy[21], patients were divided into three groups: Group 1 (n = 71), with ipsilateral PVTT resected in a hepatectomy; Group 2 (n = 10), with PVTT extending to or beyond the bifurcation, treated by *en bloc* resection followed by portal vein reconstruction; and Group 3 (n = 7), with PVTT extending to or beyond the bifurcation, treated by thrombectomy. The median overall survival duration was 10.91 mo in Group 1, 9.4 mo in Group 2, and 8.58 mo in Group 3, and it was shown that *en bloc* resection and thrombectomy were not significantly different in terms of hospital mortality and morbidity. The frequent practice of living donor liver transplantation at this center certainly contributed to the low morbidity rate after portal vein resection[24]. The 1-, 3- and 5-year overall survival rates were 50%, 12.5% and 12.5%, respectively, in Group 2 and 28.6%, 14.3% and 14.3%, respectively, in Group 3. The 1-year disease-free survival rates were 24.3, 0, and 14.3%, respectively. The 3-year DFS rates were 14.3, 0, and 14.3%, respectively. The 5-year DFS rates were 10.7, 0, and 14.3%, respectively. Again, the two approaches had no significant differences in terms of overall survival or disease-free survival. Patients with ipsilateral PVTT also had similar survival outcomes compared with patients with PVTT extending to or beyond the bifurcation. These survival outcomes were satisfactory when compared with those (a median survival duration of only 2.7 mo) of patients with PVTT who were untreated[1]. Peng *et al*[25] compared hepatic resection and transarterial chemoembolization (TACE) for patients with PVTT and found that the resection group had significantly longer overall survival. The 1-, 3-, and 5-year overall survival rates were 42.0%, 14.1% and 11.1%, respectively, in the resection group and 37.8%, 7.3% and 0.5%, respectively, in the TACE group (P < 0.001). In the subgroup analysis, the resection group had better overall survival in regard to Type 1 PVTT, Type 2 PVTT, single tumor, and tumor >5 cm (P < 0.001, P = 0.002, P < 0.001, and P < 0.001,

Table 2 Prospective trials on transarterial chemoembolization for hepatocellular carcinoma with portal vein tumor thrombosis

Group	No. of patients	Treatments	Child-Pugh A	Survival
Luo *et al*[81]	164 (84 *vs* 80)	TACE *vs* Control	Not known	1-yr survival 30.9% *vs* 9.2%
Niu *et al*[82]	150 (115 *vs* 35)	TACE *vs* Control	88 *vs* 21	Median survival 8.67 mo *vs* 14 mo
Kim *et al*[83]	110 (49 *vs* 61)	TACE *vs* TACI	30 *vs* 22	Median survival 14.9 mo *vs* 4.4 mo
Peng *et al*[25]	603 (402 *vs* 201)	TACE *vs* Resection	389 *vs* 197	Median survival 42 mo *vs* 14.1 mo

TACE: Transarterial chemoembolization; PVTT: Portal vein tumor thrombosis; TACI: Transcatheter arterial chemoinfusion.

respectively). No difference in overall survival was shown in regard to Type 3 PVTT, Type 4 PVTT, multiple tumors, and tumor < 5 cm (P = 0.541, P = 0.371, P = 0.264, P = 0.338, and P = 0.125, respectively).

In a number of reports concerning all degrees of PVTT, the median survival durations varied from 8.9 mo to 33 mo, and the operative mortality rates varied from 0% to 5.9%[22,26-30]. For patients with PVTT confined to the ipsilateral first-generation portal vein, resection of the PVTT in a hepatectomy is recommended. With a good resection margin, intrahepatic recurrence could be prevented. For PVTT extending to or beyond the bifurcation, an *en bloc* resection or thrombectomy should be considered. Nonetheless, if the liver is cirrhotic or if the resection would leave an inadequate liver remnant, major resection will not be possible.

TRANS-ARTERIAL CHEMOEMBOLIZATION

Trans-arterial chemoembolization takes advantage of the relatively selective arterial vascularization of hepatic tumors. Chemotherapeutic agents are delivered with simultaneous embolization to increase the local chemotherapeutic dwell time and induce tumor ischemia[31]. The technique of TACE varies; typically, super-selective cannulation of the artery supplying the tumor is performed whenever possible. An emulsion of cisplatin (1 mg/mL) and Lipiodol (Lipiodol Ultrafluide®; Laboratoire Guerbet, Aulnay-Sous-Bois, France) in a volume ratio of 1:1 is injected up to a maximum of 60 mL, depending on the size and number of the tumor[32,33], with or without embolization. TACE is repeated every 8 to 12 wk, and the treatment is to be stopped when there is progressive disease, extrahepatic disease, a severe life-threatening complication, or evidence of liver failure or decompensation (serum total bilirubin > 50 μmol/L, gross ascites with uncontrollable with diuretics, or hepatic encephalopathy)[34]. TACE is considered the primary treatment for patients who have inoperable HCC that is confined to the liver and in the absence of contraindications to TACE. Studies have reported that 35% of patients had a complete or progressive response to TACE, with < 2% of patients having a complete response[35,36]. Lo *et al*[37] compared TACE with symptomatic treatment and found that the patient survival rate at 2 years after treatment was higher with TACE (41%) than that with symptomatic treatment (27%).

The thought behind using TACE for the treatment of HCC with PVTT is evolving. Traditionally, TACE is generally not recommended for patients with PVTT because of the increased risk of liver failure[38,39], but there have been no large trials to validate this recommendation. For the past 5 years, a growing number of studies showed that TACE could be safely conducted in patients with PVTT, provided that there is an adequate hepatic reserve and the establishment of collateral blood circulation around the obstructed PVTT (Table 2)[40,41]. Of note, there are two randomized prospective studies comparing TACE to conservative management in patients with PVTT. Both studies consistently demonstrated improved overall survival in the TACE arm when compared to patients undergoing conservative management. Both studies have also conducted subgroup analyses in a population with different degrees of PVTT. Niu *et al* has shown that the survival benefits in PVTT type 1 (TACE: 19 mo *vs* conservative: 4 mo, P = 0.001); type Ⅱ (TACE: 11 mo *vs* conservative: 1.43 mo, P < 0.001); type Ⅲ (TACE: 7.1 mo *vs* conservative: 1.3 mo, P < 0.001) and type Ⅳ (TACE: 4 mo *vs* conservative: 1 mo, P = 0.005). Luo *et al* showed that the 6-mo survival rates of the TACE arm for branch and main PVTT were 75% and 38.7%, respectively, compared to 45.5% and 20% in the conservative arm. There are also prospective studies comparing TACE *vs* the hepatic arterial infusion of chemotherapy, of which superior survival outcomes were observed in the TACE arm.

Currently, there are no prospective data from a head-to-head comparison between TACE and systemic therapy in patients with PVTT. However, taking the above studies into consideration, it is generally accepted that TACE is feasible and effective in select patients with branch PVTT because the overall survival appeared to be longer than that achieved in most clinical trials of systemic therapy. As shown in Table 2, many more Child-Pugh A patients underwent TACE, and the benefits of TACE were significantly less in Child-Pugh B patients with PVTT. Liver function remains the most important criteria for selecting patients to receive TACE treatment. The role of TACE in the treatment of main PVTT (Vp4 or type Ⅲ/Ⅳ) is more controversial. Although randomized studies indicated the survival benefits of TACE in these groups of patients, overall survival (4-7 mo) was similar, if not inferior, to survival observed in clinical trials on systemic agents such as sorafenib (see below: systemic therapy).

Table 3 Studies on radiation therapy for hepatocellular carcinoma with portal vein tumor thrombosis

n	Treatment	Total RT dose/ fractional dose (in Gy)	Response rate (CR + PR, %)	Median survival (mo)	Toxicity grade ⩾ 3 (%)	Ref.
45	3D-CRT (+TACE/PEI/RFA; 7% RT only)	38-65/1.8-2.5	62.3 (CR 6.7)	11.2	2	Rim *et al*[51]
412	3D-CRT + TACE	21-60/2-5	27.9 (CR 3.6)	10.6	10	Yoon *et al*[44]
40	IGRT + IA 5FU/IFN *vs* IA 5FU/ IFN	30-48/7-16	60 (CR 5)	12 (RT) 9.1 (non-RT)	15	Chuma *et al*[52]
32	IA 5FU/IFN + 3D-CRT *vs* IA 5FU/ IFN	30-45/3	75 (CR 19)	7.5 (RT) 7.9 (non-RT)	G4: 2 G3: 7% (leucopenia)/ 6% (thrombocytopenia)/ 1 (anorexia)	Katamura *et al*[46]
45	PV stenting + TACE + 3D-CRT *vs* PV stenting + TACE	30-60/2	35.6 (CR 0)	16.5 (RT) 4.8 (non-RT)	0	Zhang *et al*[45]
326	3D-CRT (IMRT 14.1%)	60/2-3	18.1 (CR 5.8)	4	0	Huang *et al*[53]
38	3D-CRT	17.5-50.4/1.8-4	44.7 (CR 15.8)	9.6	0	Toya *et al*[47]
59	3D-CRT	30-54/2-3	45.8 (CR 6.8)	7.8	0	Kim *et al*[48]
44	RT + TACE	36-60/2	45.5 (CR 34.1)	8	0	Kim *et al*[49]
19	3D-CRT (+ TACE for liver tumor)	46-60/2	57.9 (CR 0)	7	G3: 5% (thrombocytopenia)/ 2% (leucopenia)/ 2 (GI ulcers)	Yamada *et al*[54]
20	RT + TACE	50/2	50 (CR 0)	5.3	5	Ishikura *et al*[55]
24	RT + TACE	50/2	50 (CR 16.7)	CR/PR (9.7) NR/PD (3/8)	13%	Tazawa *et al*[56]
281	3D-CRT + TACE	30-54/1.8 -4.5	53.8 (CR 3.6)	11.6	20%	Yu *et al*[57]

3D-CRT: 3 dimensional conformal radiotherapy; GI: Gastrointestinal; PR: Partial response; RT: Radiotherapy; TACE: Transarterial chemoembolization; NR: Non-responder; PD: Progressive disease; PVTT: Portal vein tumor thrombosis.

RADIOTHERAPY

External radiotherapy

Historically, external radiotherapy (RT) is not considered a feasible treatment in the management of HCC. This is because the liver is highly radiosensitive, and the delivery of a sufficient high dose RT without excessive hepatotoxicity is challenging. However, as a result of the advances in RT technology, including the conformal RT planning and breathing motion management and image-guided radiation therapy, RT has emerged as a valid treatment option in the treatment of HCC[42,43]. A number of retrospective studies have studied the efficacy of RT as single treatment or in combination with other treatment modalities, particularly TACE, in treating HCC with PVTT (Table 3). These reports have consistently reported a favorable toxicity profile and modest efficacy of RT[44-57]. In one of the largest series, 412 patients with PVTT were treated with 21-60 Gy in 2- to 5-Gy fractions in combination with sequential TACE. The median survival was 10.6 mo with a 2-year survival rate of 23%, while grade 3 or above toxicity was observed in 10% of patients[44]. The radiologic response of PVTT to RT is the most significant prognostic factor, with a median overall survival of 19.4 mo in the responder group *vs* 7.0 mo in the non-responder group.

There is also a growing body of evidence suggesting that the concurrent administration of RT and locoregional treatment could improve the response rate or treatment outcomes of PVTT. In a retrospective study reported by Zhang *et al*[45], patients with PVTT were treated with percutaneous transhepatic PV stenting and TACE, with or without RT. The median overall survival was 16.5 mo in the RT cohort compared with 4.8 mo in the non-RT cohort. In a matched cohort study by Katamura *et al*[46], a significant improvement in the objective response rate in PVTT was observed in the RT group compared with the non-RT group (75% *vs* 25%) in patients treated with intra-arterial 5-fluorouracil and interferon-alpha. Several studies had evaluated the dose-response of RT in the treatment of HCC with PVTT. The response rate was better when the biologically effective dose (BED) exceeded 58 Gy[47,48,50]. Therefore, an attempt to deliver a BED as high as possible is preferred during the planning of RT to HCC with PVTT. However, this can only be achieved in cases of small primary HCC, where both HCC and PVTT could be covered within a high dose target volume without compromising the normal liver. In clinical practice, because bulky HCC is frequently encountered, a combined approach with RT to focus on PVTT and TACE for the treatment of the intra-hepatic tumor is generally preferred to keep the normal liver radiation tolerance within a safe limit. Well-designed prospective studies are required to evaluate this combination for patients with PVTT.

Selective internal radiation therapy

Selective internal radiation therapy (SIRT), or transarterial radioembolization with Yttrium-90, involves the transarterial administration of therapeutic doses of radiation to the hepatic tumor *via* resin or glass particles. Although resin and glass are both considered

permanent embolic agents, their embolic effects are less than those of a TACE procedure due to their small size, as is their effect on hepatic vascular dynamics[58]. During radioembolization, a radioactive microsphere is selectively injected into the hepatic artery or its branch, delivering intense local tumor radiation. SIRT provides adequate radiation to the tumor with little radiation to the rest of the liver and to the patient's body. Because it does not cause ischemia, SIRT can be performed in patients with portal vein thrombosis, making SIRT a feasible choice for HCC with PVTT. Common complications of SIRT include fatigue and deranged liver function[59]. Serious complications such as radiation pneumonitis, radiation cholecystitis, liver abscess and radiation-induced liver disease occur in less than 1% of patients. In general, technetium-labeled macroaggregated albumin scanning is performed prior to SIRT to quantify the fraction of lung shunting and/or the tumor/normal uptake ratio. The accepted safe radiation dose to the lung is less than 30 Gy in a single procedure and less than 50 Gy in total over multiple procedures[59,60].

Studies on SIRT focusing on patients with HCC and PVTT are limited. However, numerous retrospective series have reported the safety and efficacy of SIRT in patients with HCC[61-64]. Subgroup analyses from the three largest series of HCC patients who underwent SIRT involving more than 200 patients with PVTT demonstrated the overall survival to be approximately 10 mo. Ozkan *et al*[65] reported a better median overall survival of 17 mo among 29 HCC patients treated with SIRT, with and without PVTT. There was no significant difference in survival between patients with and without PVT. A report from the largest group of PVT patients showed concordant results[63], in which it was also found that patients with Child-Pugh A cirrhosis, with or without PVT, benefited the most from SIRT. In a recent prospective phase Ⅱ trial of 35 patients with PVT treated with SIRT, the overall survival of Child-Pugh A and B patients were 16 mo and 6 mo, respectively.

The efficacy of SIRT in unresectable HCC was compared with sorafenib in a recent study. Edeline *et al*[66] retrospectively reviewed the records of 151 HCC patients with PVTT. The overall survival of 34 patients treated with SIRT was compared with 117 patients treated with sorafenib only. SIRT was associated with a higher median overall survival compared to sorafenib (18.8 mo *vs* 6.5 mo, $P < 0.001$). A prospective randomized study comparing SIRT and sorafenib has already completed accrual with preliminary results expected to be available in late 2016 (NCT01135056). At present, there have been no randomized controlled trials directly comparing SIRT to TACE in patients with HCC and PVTT. Several small-scale studies have suggested that the efficacy of SIRT is comparable to TACE in unresectable HCC. Of note, She *et al*[67] performed survival analysis of 16 patients who underwent SIRT and compared it with another 16 patients in a matched cohort treated with TACE. Half of the patients in each group had major vascular invasion, and those treated with SIRT had an overall survival of 12.0 mo compared to 8.0 mo in the TACE cohort. These data provide preliminary clues that SIRT might be more effective than TACE in the setting of HCC with PVT, but this hypothesis requires further validation in prospective randomized clinical trials.

SYSTEMIC THERAPY

Compared to other cancer types, the progress in the development of systemic therapy is slower for HCC. Conventional cytotoxic chemotherapy, such as doxorubicin or doxorubicin-based combinations, could lead to significant toxicity, which limits the potential benefits in cirrhotic populations[68]. Other more "novel" regimens of systemic chemotherapy have also been evaluated. In particular, the combination of oxaliplatin and 5-fluorouracil and leucovorin, known as the FOLFOX4 regimen, has been compared to doxorubicin in patients with advanced HCC in an Asian population with HCC[69]. The overall study fails to demonstrate a statistically significant difference between the two regimens, but FOLFOX4 was found to have small survival benefits in the subgroup population of Chinese patients[70,71]. There have been a handful of case reports or series showing that cytotoxic chemotherapy could improve the severity of PVTT, but there are no dedicated prospective studies to validate such efficacy of systemic chemotherapy in the treatment of PVTT. Further, the toxicity of systemic chemotherapy has been shown to be higher in the presence of impaired liver function and portal hypertension, which frequently occur in patients with PVTT.

Sorafenib is a multi-targeted small molecule with specific activity against vascular endothelial growth factor receptor. Its use in the setting of BCLC stage C HCC, including patients with PVTT, extra-hepatic metastases, and ECOG performance status of 1 or higher, is known to modestly prolong median overall survival by approximately 2 mo, compared to a placebo[72,73]. In both Phase Ⅲ clinical trials, namely the SHARP[72] and the Asian pacific study[73], there are no detailed analyses on the efficacy of sorafenib according to the different severities of PVTT. Nevertheless, the efficacy of sorafenib in PVTT may still be indirectly reflected by the subgroup analyses in patients with macrovascular invasion. In both studies, the proportion of macrovascular invasion in the sorafenib arm was 36%, and subgroup analyses unanimously indicated similar survival benefits between patients with and without macrovascular invasion[72,73]. Regarding the specific treatment for patients with Vp3 and Vp4 PVTT, a retrospective review was published by a Korean center, which analyzed the outcome of sorafenib in 6 patients with Vp3 and 24 patients with Vp4 PVTT[74]. It was shown that 10% (3 patients) had a partial response in the PVTT arm with revascularization, and

the median overall survival was 3.1 mo. The above dataset suggested that sorafenib demonstrated a modest efficacy in the treatment of PVTT, but given the low response rate of likely lower than 10%, the treatment is mainly reserved as a palliative treatment for patients with Vp3 or Vp4 who are not amenable to more aggressive treatment.

Recently, the direction of the development of systemic treatment for HCC has shifted from targets along signaling pathways to immunotherapy and, in particular, to the check-point inhibitors. There have been one phase I clinical trial of a cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) inhibitor, tremelimumab, and another ongoing trial on a programmed cell death protein-1 (PD-1) inhibitor, nivolumab, in patients with advanced HCC[75,76]. Both studies have suggested that check-point inhibitors are tolerable and potentially efficacious in HCC. In particular, some remarkable and durable radiological responses have been observed. Given the more mature and robust data of the immune check-point inhibitor in the treatment of HCC, its use in the treatment of PVTT can be better elucidated. Another direction is biomarker-driven drug development, which aims to improve the response *via* patient selection with predictive biomarkers in the tissue or plasma[77].

PORTAL VEIN STENTING

Theoretically, the placement of an endovascular stent into the portal vein could increase the portal blood flow into the liver parenchyma in HCC complicated with PVTT. This may, in turn, relieve the portal hypertension-related complications, especially variceal bleeding, and expand treatment options for the tumor. Most of the studies on portal vein stenting in the treatment of PVTT of HCC are case reports or small-scale series. Two large-scale case series suggest that portal vein stenting is feasible in select patients. One series come from a Japanese group that reported the percutaneous placement of stents in the portal vein following the administration of TACE or trans-arterial infusion of chemotherapy in 21 patients[78]. With a 100% success rate of placement, it was demonstrated that the portal pressure decreased by approximately 3 mmHg. A short course of an anticoagulant was administered in 14 patients, but it was not suitable for the others due to coagulopathy. The patency rate was 53.6% at 1 year with a mean patency period of 12.4 mo. Procedure-related complications appeared to be uncommon, with only a case of pseudoaneurysm reported. Another large case series from a French group reviewed the records of 54 patients with PVTT who underwent portal stenting under general anesthesia by a percutaneous approach or an open approach *via* catheterization of the superior mesenteric vein after exteriorization of the terminal ileum[79]. Twenty percent of patients developed complications after portal stenting, mostly in the form of hepatic failure, and 7% died within 30 d of stenting. Bilirubin levels > 30 μmol/L and an open surgical approach were predictors of short-term mortality. TACE was administered in 48% of patients after stenting, and the 12-mo survival rate was 44%. Both of these series indicate that the placement of portal stents is a feasible procedure with potential efficacy. However, because of the concern of bleeding complications during the anticoagulation period and the potential risk of tumor dissemination during the stenting procedures, in addition to the complicated procedures involved, portal vein stenting has not gained wide acceptance among HCC experts.

FUTURE PERSPECTIVES

The need for high-level evidence

Conventionally, HCC with PVTT is considered to be an advanced disease with clinical management similar to HCC with extra-hepatic metastases. There are no dedicated clinical trials to study the treatment of this population. As a result, the efficacy and safety of different treatment modalities on PVTT are mainly generated from retrospective studies or extrapolated from subgroup analyses of prospective clinical trials. Currently, it is evident that the prognoses and disease behavior of patients with PVTT differ from those of patients without PVTT. In addition, patients with PVTT are more prone to develop complications due to portal hypertension or hepatic failure. Therefore, to define a better treatment strategy for patients with HCC with PVTT, dedicated clinical trials in this population are warranted. Because the prognoses differ between patients with different severities and degrees of PVTT, it is important to accurately gauge the outcome of patients during the design of clinical trials for HCC with PVTT. To achieve this goal, a uniform classification of PVTT is required for the stratification of risk groups during randomization and to facilitate the comparison of results between different studies. At present, at least three classifications have been developed for PVTT in HCC. It is crucial to reach consensus on the classification of PVTT amongst the HCC experts.

Multi-disciplinary approach

Management of HCC with PVTT is a clinical dilemma with challenges. On one hand, despite the recommendation of sorafenib as the standard treatment for HCC with PVTT by the BCLC guidelines, emerging evidence clearly shows that select patients could benefit from more aggressive treatment approaches. On the other hand, not all patients may uniformly benefit from aggressive treatment. PVTT represents an adverse prognostic factor with an underlying more rapid disease course. Aggressive treatment may not lead to better outcomes in some patients; for example, in the setting of poor performance or with impaired hepatic function. Therefore, when facing patients with HCC with PVTT,

clinicians have to balance the potential benefits and toxicity of different treatments in the individual patients. A multi-disciplinary tumor board is required to determine the most appropriate management of patients with PVTT. Furthermore, the cumulative evidence indicates that a single treatment modality is unlikely to achieve a remarkable effect in PVTT, and the combination of different treatment modalities, such as RT to PVTT and TACE or the addition of SIRT to systemic agents, may be more efficacious in select patients. Future research should focus on combinations of different established treatment modalities for PVTT in HCC.

CONCLUSION

The management of HCC with PVTT is evolving. The treatment modality for HCC with PVTT includes surgical resection, TACE, radiation therapy including external RT or SIRT of the liver lesions, and systemic agents, while portal vein stent treatment remains investigational. Dedicated clinical studies on HCC complicated with PVTT are inadequate. The decision of the optimal treatment for individual patients requires multi-disciplinary input. Future research should be geared towards the generation of high-level evidence of novel treatments and combinations of established treatments for this population.

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REVIEW

Pancreatic cancer: Open or minimally invasive surgery?

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Abstract

Pancreatic duct adenocarcinoma is one of the most fatal malignancies, with R0 resection remaining the most important part of treatment of this malignancy. However, pancreatectomy is believed to be one of the most challenging procedures and R0 resection remains the only chance for patients with pancreatic cancer to have a good prognosis. Some surgeons have tried minimally invasive pancreatic surgery, but the short- and long-term outcomes of pancreatic malignancy remain controversial between open and minimally invasive procedures. We collected comparative data about minimally invasive and open pancreatic surgery. The available evidence suggests that minimally invasive pancreaticoduodenectomy (MIPD) is as safe and feasible as open PD (OPD), and shows some benefit, such as less intraoperative blood loss and shorter postoperative hospital stay. Despite the limited evidence for MIPD in pancreatic cancer, most of the available data show that the short-term oncological adequacy is similar between MIPD and OPD. Some surgical techniques, including superior mesenteric artery-first approach and laparoscopic pancreatoduodenectomy with major vein resection, are believed to improve the rate of R0 resection. Laparoscopic distal pancreatectomy is less technically demanding and is accepted in more pancreatic centers. It is technically safe and feasible and has similar short-term oncological prognosis compared with open distal pancreatectomy.

Key words: Laparoscopic; Minimally invasive; Robotic; Pancreaticoduodenectomy; Distal pancreatectomy; Pancreatic cancer

Core tip: Minimally invasive pancreaticoduodenectomy is as safe and feasible as open pancreaticoduodenectomy (OPD) and shows some superiority. The

short-term oncological results are similar between laparoscopic pancreaticoduodenectomy (LPD) and OPD. However, in some experienced hands, better prognosis is detected in the LPD group because the patients can receive adjuvant therapy faster because of the benefits of minimal invasiveness. Minimally invasive distal pancreatectomy is a well-established procedure and widely accepted. It is safe, feasible, and has similar short-term oncological results compared with open distal pancreatectomy.

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INTRODUCTION

Pancreatic cancer ranks as the 4th highest cause of cancer-related death in the United States and the 5-year survival is about 6%[1]. Surgical R0 resection is the best chance for a cure and remains the cornerstone of treatment of pancreatic malignancy[2,3]. However, pancreatic surgery is believed to be one of the most challenging procedures because of the high risks of postoperative morbidity and mortality associated with intraoperative bleeding and postoperative complications including pancreatic fistula[2,4,5]. Another key point for surgical treatment of pancreatic malignancy is oncological adequacy. R0 resection is the best chance for patients to have a good prognosis[3,6].

Minimally invasive techniques, including laparoscopic and robotic approaches, have rapidly evolved and include a variety of abdominal surgical procedures[7-10]. They provide the patients with better short-term outcomes, including smaller incisions, shorter hospital stay and less blood loss. Some surgeons in large-volume pancreatic centers have tried minimally invasive pancreatic surgery[11-16]. However, the short- and long-term outcomes of pancreatic malignancy remain controversial, especially for oncological prognosis.

Many pancreatic surgeons doubt the safety and oncological adequacy of minimally invasive pancreatic surgery. Here, we collected and analyzed the published data about minimally invasive pancreatic surgery.

LAPAROSCOPIC PANCREATICODUODENECTOMY

Background of laparoscopic pancreaticoduodenectomy

Following the first report of laparoscopic pancreaticoduodenectomy (LPD) in 1994[17], Gagner and Pomp[18] subsequently published a series of 10 patients in 1997. In Gagner's series, the conversion rate was 40% and the operating time was 8.5 h. Dependent upon these results, the authors concluded that the minimally invasive approach was not advocated because there was no apparent advantage over traditional open approaches. After that, surgeons spent a decade improving their laparoscopic skills, until a large LPD cohort was reported in France in 2005[12] and then in India in 2009[15]. During 1994-2009, several surgeons tried to apply hybrid, laparoscopic-open approaches to avoid the complexity of a purely laparoscopic procedure[13,19]. Although these approaches may overcome some of the limitations, they may reduce the potential benefits of purely laparoscopic approaches, including less pain, improved postoperative recovery and shorter hospital stay. After Palanivelu *et al*[15] reported 75 cases of LPD in 2009, large cohorts of LPD have been reported in the United States[11,20,21], South Korea[16], China[22], Italy[23] and France[24]. LPD eventually gained momentum, following its 30 years' development, and it has emerged as a well-established procedure with acceptable morbidity and mortality rates in some specialized high-volume pancreatic centers[12,15,16,20,22,23]. Although LPD has been accepted in many specialized minimally invasive pancreatic centers, the short- and long-term results remain controversial. We collected clinical reports with comparative data between minimally invasive PD (MIPD) and open PD (OPD) (Table 1).

Safety and feasibility of LPD

PD is a complex procedure because of the dissection around important vessels and three complex reconstructions. Moreover, it is a procedure with high morbidity[25]. Although LPD has been accepted in some specialized centers, it is still a challenging operation for most pancreatic surgeons; still, there has been a rapid increase in the number of LPDs performed in different centers. Some large-volume centers have published their comparative studies between LPD and OPD[11,15,16,20,21,24,26-31], demonstrating the safety of LPD; although, long-term oncological benefits of this approach remain debatable.

Croome *et al*[20] reviewed their data for patients with pancreatic ductal adenocarcinoma (PDAC) undergoing LPD (n = 108) and OPD (n = 214). A significantly reduced blood loss and blood transfusion requirement and a shorter postoperative stay (6 d *vs* 9 d) were observed in the LPD group compared with the OPD group.

A case match study was performed by Dokmak *et al*[24] comparing 46 LPD and OPD procedures. Patients were matched for demographic data, associated comorbidity and underlying disease. The results suggested that a high rate of severe morbidity due to severe pancreatic fistula was detected in patients with a high risk of pancreatic fistula. In a subgroup of patients with a low risk of pancreatic fistula, the outcome of the two approaches was similar. The result of this study suggested that, in a subgroup of patients with a high risk of pancreatic fistula, LPD was associated with high

Table 1 Safety and feasibility of laparoscopic pancreaticoduodenectomy: Clinical cohorts of minimally invasive pancreaticoduodenectomy and open pancreaticoduodenectomy including comparative results

Ref.	Year	Country	Technique	Cases	Operating time, min	EBL, mL	LHS, D	CD ≥ Ⅲ	PF	DGE	Readmission rate	Mortality
Sharp *et al*[21]	2015	United States	LPD	384	NR	NR	NR	NR	NR	NR	5.0%	5.2% (30 D)
			OPD	4037	NR	NR	NR	NR	NR	NR	9.0%	3.7% (30 D)
Song *et al*[16]	2015	SouthKorea	LPPPD	93	482.5 ± 117.6	609 ± 375	14.3 ± 7.8	7.5%	6 (6.5)	3.2%	5 (5.4)	NR
			OPPPD	93	347.9 ± 87.2	570 ± 448	19.2 ± 8.8	5.4%	6 (6.5)	7.5%	3 (3.2)	NR
Chen *et al*[34]	2015	China	RPD	60	410 ± 103	400 (200-600)	20 ± 7.4	11.7%	13.3%	8.3%	NR	1.7%
			OPD	120	323 ± 80	500 (350-800)	25 ± 11.2	13.3%	24.9%	15.0%	NR	2.5%
Dokmak *et al*[24]	2015	France	LPD	46	342 (240-540)	368 (50-1200)	25 (6-104)	28.0%	48.0%	17.0%	9.0%	2.0%
			OPD	46	264 (120-400)	293 (50-1200)	23 (7-115)	20.0%	41.0%	15.0%	9.0%	0
Baker *et al*[31]	2015	United States	RPD	22	454 (294-529)	425 (50-2200)	7 (4-25)	13.6%	4.6%	13.6%	22.7%	0
			OPD	49	364 (213-948)	650 (150-6100)	9 (5-48)	20.4%	12.2%	30.6%	29.8%	4.1%
Tran *et al*[74]	2015	United States	LPD	681	NR	NR	12 (9-20)	NR	NR	NR	NR	3.8%
			OPD	14893	NR	NR	11 (8-16)	NR	NR	NR	NR	5.0%
Tan *et al*[75]	2015	China	LPD	30	513.17 ± 56.13	NR	9.97 ± 3.74	NR	10/30	2/30	NR	0
			OPD	30	371.67 ± 85.53	NR	11.87 ± 4.72	NR	6/30	3/30	NR	1/30
Adam *et al*[32]	2015	United States	MIPD	983	NR	NR	NR	NR	NR	NR	4.8%	NR
			OPD	6078	NR	NR	NR	NR	NR	NR	3.7%	NR
Chalikonda *et al*[33]	2014	United States	HPD	30	476.00	485	9.79	30.0%	NR	NR	NR	4.0%
			OPD	30	366.48	775	13.26	43.0%	NR	NR	NR	0
Bao *et al*[76]	2014	United States	RPD	28	431 (340-628)	100 (50-300)	7.4 (5.5-17.1)	NR	29.0%	NR	25.0%	7.0% (90 D)
			OPD	28	410 (190-621)	300 (100-800)	8.1 (6.5-15.3)	NR	29.0%	NR	25.0%	7.0% (90 D)
Croome *et al*[20]	2014	United States	LPD	108	379.4 ± 93.5	492.4 ± 519.3	6 (4-118)	5.6% (≥ Ⅲb)	11% (B/C)	9% (B/C)	NR	1.0% (I H)
			OPD	214	387.6 ± 91.8	866.7 ± 733.7	9 (5-73)	13.6% (≥ Ⅲb)	12% (B/C)	18% (B/C)	NR	2.0% (I H)
Speicher *et al*[77]	2014	United States	LPD	25	381 (342-465)	200 (100-425)	8.5 (7-11.2)	NR	16% (B/C)	NR	30.4%	0 (30 D)
			HPD	31	442 (386.5-486.5)	600 (312.5-700)	12 (8.5-18.5)	NR	35.5%	NR	35.5%	3.2%(30 D)
			OPD	84	425.5 (345.8-478.8)	425 (300-700)	10 (8-14)	NR	22.6%	NR	39.3%	1.2% (30 D)
Asbun *et al*[11]	2012	United States	LPD	53	541 ± 88	195 ± 136	8.0 ± 3.2	NR	16.7%	11.3%	NR	5.7% (100 D)
			OPD	215	401 ± 108	1032 ± 1151	12.4 ± 8.5	NR	17.3%	15.3%	NR	8.8% (100 D)
Lai *et al*[28]	2012	China	RPD	20	491.5 ± 94.0	247 (50-889)	13.7 ± 6.1	NR	35.0%	5%	NR	0%
			OPD	67	264.9 ± 63.7	774.8 (50-8000)	25.8 ± 23.1	NR	17.9%	11.9%	NR	3%

CD: Clavien-Dindo; DGE: Delayed gastric emptying; EBL: Estimated blood loss; HPD: Hybrid pancreaticoduodenectomy; LHS: Length of hospital stay; LPD: Laparoscopic pancreaticoduodenectomy; LPPPD: Laparoscopic pylorus-preserving pancreaticoduodenectomy; MIPD: Minimally invasive pancreaticoduodenectomy; NR: Not reported; OPD: Open pancreaticoduodenectomy; OPPPD: Open pylorus-preserving pancreaticoduodenectomy; PF: Pancreatic fistula; RPD: Robotic pancreaticoduodenectomy.

morbidity. Thus, it should be considered only in patients with a dilated pancreatic duct and a hard pancreas texture, who are believed to have a low risk of pancreatic fistula.

Adam *et al*[32] reviewed patients undergoing PD from the National Cancer Database between 2010 and 2011, including 983 MIPDs and 6078 LPDs. Their results suggested that, for patients with PDAC, no difference was detected in number of lymph nodes (LNs) removed, rate of R0 resection, length of hospital stay or readmission. However, the 30-d mortality was lower in the OPD group than in the MIPD group. The authors suggested that the widespread adoption of the technique should be paused. MIPD is a complex procedure that needs comprehensive protocols outlining criteria for implementation.

Asbun and Stauffer[11] presented retrospective clinical data from Mayo Clinic of 215 OPD and 53 LPD patients. They also showed significantly better results in LPD

groups, such as less blood loss ($P < 0.001$) and blood transfusion requirements ($P < 0.001$), and a shorter postoperative hospital stay ($P < 0.001$). While a significantly longer operating time was observed in LPD ($P < 0.001$), the LPD patients had a greater number of LNs removed than the OPD patients ($P = 0.007$). This series also demonstrated that LPD is safe and feasible and showed some benefits for patients.

Results from another cohort with PDAC in the United States treated with LPD were presented at the Western Surgical Association 122nd Scientific Session[21]. The researchers compared 4037 OPDs with 384 LPDs and showed significant differences favoring LPD for length of hospital stay and unplanned readmission. A lower risk of 30-d mortality was found in high-volume centers and in centers with experience of performing more than 10 LPDs; moreover, the 30-d mortality for LPD was similar to that for OPD. Finally, the researchers demonstrated that there is a learning curve for LPD.

Song *et al*[16] compared 137 laparoscopic pylorus-preserving PDs (LPPPDs) with 2055 open PPPDs (OPPPDs) in South Korea. They found that operating time was longer for LPPPD than for OPPPD, and the perioperative complications were similar in both groups. Fewer analgesic injections were administrated in the LPPPD group ($P < 0.001$). The oncological results were similar between the two groups, including number of LNs removed and long-term survival.

In addition to LPD, a few studies have compared robotic PD (RPD) with OPD. Chalikonda *et al*[33] from the Cleveland Clinic reviewed the results of 30 matched laparoscopic RPD (LRPD) and OPD procedures. LRPD and OPD groups were matched with demographics. A similar estimated blood loss and rate of reoperation were found in the two groups. However, there was a significant increase in operating time and shorter hospital stay for the LRPD group.

We found that most of the clinical studies showed that LPD is as safe and feasible as OPD technically, and has some of the superiority associated with minimally invasive surgery, such as less estimated blood loss and shorter hospital stay. However, some authors have suggested that MIPD should be advocated in a subgroup of patients with lower risk of pancreatic fistula. In our opinion, LPD is as safe as OPD. However, due to the complexity of LPD, it is a technically demanding procedure with a learning curve. In small clinical cohorts of LPD at the beginning of the learning curve, there might be higher morbidity and mortality for LPD than for OPD. The problem now is how to reduce the risks of LPD at the beginning of the learning curve. Apart from technical feasibility, the major arguments against LPD are oncological adequacy, especially for patients with PDAC.

Oncological adequacy of LPD for pancreatic malignancy

Pancreatic cancer still has a high fatality rate. Radical resection is required for a good prognosis. Many clinical studies have reported LPD; however, most of those studies have included a variety of diseases requiring LPD. To the best of our knowledge, few studies have compared the oncological prognosis of PDAC treated with LPD or OPD (Table 2).

Song *et al*[16] compared the oncological results of pancreatic cancer treated with OPPPD ($n = 261$) and LPPPD ($n = 11$). TMN stage, R0 resection rate, in-hospital stay and the overall survival were similar between the two groups. In a case-control study from France[24], the results for LPD ($n = 15$) in patients with PDAC were similar to those with OPD ($n = 14$) with regard to tumor size, number of LNs harvested and rate of R0 resection. Croome *et al*[20] reported a large single center study of pancreatic carcinoma treated with LPD. Clinical data of 108 cases of LPD were reviewed retrospectively and compared with 214 cases of OPD performed in the same period at their center. The short-term oncological results, including tumor size, LN positivity, R0 resection and overall survival, were similar between the two groups, and significantly longer progression-free survival was found in the LPD group. The authors thought that this difference might have been because the patients who underwent LPD had the advantage of minimal invasiveness and recovered faster from the operation. This allowed the patients to receive adjuvant therapy in a timely manner and probably led to better prognosis.

A large LPD cohort study[21] from the National Cancer Data Base involved 384 LPDs and 4039 OPDs. The results showed no difference between the LPD and OPD groups with regard to length of stay, margin-positive resection, LN count and readmission rate.

Chen *et al*[34] compared the oncological results of pancreatic cancer treated with RPD ($n = 19$) and OPD ($n = 38$). There was no difference in the R0 resection rate, number of LNs resected, cancer stage, overall survival and disease-free survival between the two groups.

All the results above show that in most of the experienced minimally invasive pancreatic centers, LPD has similar short-term oncological results as OPD. However, Croome *et al*[20] reported the long-term prognostic benefit in the LPD group because of the advantages of minimal invasiveness. Furthermore, Croome *et al*[20] presented the largest cohort with pancreatic cancer treated with LPD, thus, we can probably form the hypothesis that, if surgeons acquire enough experience of LPD, LPD can yield the benefits of minimal invasiveness as well as long-term oncological benefit, compared with OPD. To obtain oncological adequacy, some technical tips are suggested for application during the operation.

Surgical technique to improve rate of R0 resection

Superior mesenteric artery-first approach: To improve the long-term prognosis of patients with

Table 2 Oncological results of pancreatic cancer in minimally invasive pancreaticoduodenectomy and open pancreaticoduodenectomy: Clinical trials including comparative results of pancreatic ductal adenocarcinoma between minimally invasive pancreaticoduodenectomy and open pancreaticoduodenectomy

Ref.	Year	Country	Technique	No. of PDAC cases	Rate of R0 resection	No. of LN	Positive LN	Tumor size, cm
Sharp *et al*[21]	2015	United States	LPD	384	80.0%	18 ± 9.7	NR	3.2 ± 1.3
			OPD	4037	74.0%	16 ± 9.6	NR	3.3 ± 2.4
Song *et al*[16]	2015	South Korea	LPPPD	11	72.7%	15 ± 10	0.8 ± 1.2	2.8 ± 0.6
			OPPPD	261	81.0%	16.2 ± 9.6	1.5 ± 2.2	3.0 ± 1.2
Dokmak *et al*[24]	2015	France	LPD	15	60.0%	20 (8-59)	4.7 (0-32)	2.4 (1.5-4)
			OPD	14	50.0%	25 (8-47)	2.2 (0-12)	2.8 (2.5-4)
Chen *et al*[34]	2015	China	RPD	19	94.7%	18.1 ± 6.6	NR	3.0 ± 0.9
			OPD	38	92.1%	17.8 ± 7.1	NR	3.1 ± 1.0
Croome *et al*[20]	2014	United States	LPD	108	77.8%	21.4 ± 8.1	73.1%	3.3 ± 1.0
			OPD	214	76.6%	20.1 ± 7.5	72.0%	3.3 ± 1.3

LN: Lymph node; LPD: Laparoscopic pancreaticoduodenectomy; LPPPD: Laparoscopic pylorus-preserving pancreaticoduodenectomy; MIPD: Minimally invasive pancreaticoduodenectomy; OPD: Open pancreaticoduodenectomy; OPPPD: Open pylorus-preserving pancreaticoduodenectomy; PDAC: Pancreatic duct adenocarcinoma; NR: Not reported.

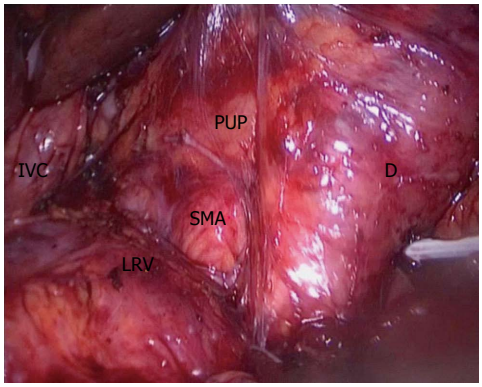

Figure 1 Superior mesentery artery was exposed from the right posterior side after complete kocherization. D: Duodenum; IVC: Inferior vena cava; LRV: Left renal vein; PUP: Pancreatic uncinate process; SMA: Superior mesentery artery.

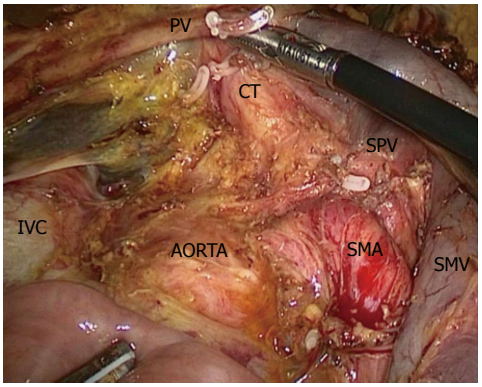

Figure 2 Local vision after removal of the specimen. CT: Celiac trunk; IVC: Inferior vena cava; PV: Portal vein; SMA: Superior mesenteric artery; SMV: Superior mesenteric vein; SPV: Splenic vein.

PDAC, curative (R0) resection is required initially. Many reports have discussed the value of R0 resection in prognosis of PDAC. The consensus among pancreatic surgeons is that positive surgical margins are associated with poor survival[35-38]. The primary site of positive margins is from the right side of the superior mesenteric artery (SMA) (N14) to the right side of the celiac trunk (N9), including the mesopancreas[39]. To improve R0 resection, the SMA-first approach was advocated in OPD. The artery-first approach has been proven as effective in reducing the risk of bleeding and improving the rate of R0 resection in pancreatic cancer.

However, few publications have reported the SMA-first approach in LPD. To the best of our knowledge, only two publications have described laparoscopic SMA-first approaches[40,41]. Pittau *et al*[40] reported the right posterior approach. The authors performed this procedure exactly like the Pessaux procedure in OPD[42], and they dissected the SMA after complete kocherization, including mobilization of the right colon. Cho *et al*[41] described the left posterior SMA-first approach. They dissected the SMA at the ligament of Treitz without mobilization of the duodenum or right colon[43]. In our center, we perform the right posterior SMA-first approach, as described by Pittau *et al*[40]. We expose the SMA from the right side after complete kocherization (Figure 1). After exposure of the SMA, it would be easy to decide the resectability of the tumor. Another benefit is that this approach makes resection of the uncinate process from the SMA easier, and warrants complete removal of the neuro laminar tissue at the right side of the SMA up to the celiac axis (Figure 2).

Major vein resection: Involvement of the portal vein in locally advanced tumor is no longer a contradiction for surgical resection of pancreatic malignancy using traditional open procedures. A lot of data from larger pancreatic centers have provided evidence indicating that *en bloc* resection of tumor with involved vessels is safe and feasible, and can improve the rate of R0 resection[44-51]. Patients who have *en bloc* resection with the involved vein have similar long-term oncological prognosis compared with patients who do not have vascular involvement[44,45,48-50].

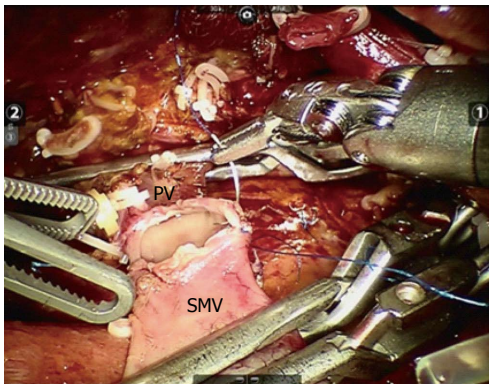

Figure 3 Vein reconstruction *via* robotic system. PV: Portal vein; SMV: Superior mesenteric vein.

Kendrick *et al*[52] reported the first example of LPD with vein resection. Later in that same year, Giulianotti *et al*[53] published data of RPD with major vein resection. Kendrick *et al*[52] reported 11 patients who underwent laparoscopic pancreatectomy with major venous resection. In their series, one segmental resection and 10 tangential venous resections were described. Giulianotti *et al*[53] described three cases of robot-assisted distal pancreatectomy (DP) with vascular resection (two cases of celiac truck resection and one of portal vein resection) and two cases of RPD with portal vein resection. These initial results show that, for surgeons with considerable experience of minimally invasive pancreatic surgery, major vein resection during pancreatectomy is a safe and feasible adjunctive procedure. Kendrick *et al*[52] consequently reported a series of LPD with major vein resection. Thirty-one patients who underwent LPD with vascular resection were compared with 58 patients who underwent OPD with major vessel resection. The LPD group had decreased blood loss and shorter length of hospital stay, but there was no difference between LPD and OPD with regard to severe complications, mortality or overall survival. The authors concluded that LPD with vein resection is safe and feasible, and can achieve similar outcomes compared to patients undergoing OPD with vein resection. Most of the minimally invasive pancreatic centers considered LPD with vein resection a contraindication. However, for further application of LPD in patients with pancreatic malignancy with vein involvement, it is necessary for surgeons to master the minimally invasive technique of vein resection and reconstruction.

In our center, we have performed five MIPD procedures with major vein resection: four patients underwent LPD with tangential venous resection, and one underwent robotic vein segment resection (Figure 3). In our limited experience of LPD with major vein resection, tangential venous resections can be performed safely laparoscopically. However, for segmentectomy of the major vein, a robotic system is advocated. The first RPD was performed by Giulianotti *et al*[54] in 2001. RPD has since been proven to be feasible and safe, with the minimally invasive advantages compared with open procedures[30,31,33,34,55,56]. It is believed that the robotic surgical system provides surgeons with enhanced dexterity, superior magnified high-resolution 3D visualization, and greater precision and ergonomic comfort. This approach enables surgeons to control the surgical instruments with accuracy, flexibility and a wide range of motion, which is suggested for procedures that require complicated resection and reconstruction, such as prostatectomy, coronary surgery and PD. In our opinion, the application of robotic systems in PD with major vein resection can improve the quality of vein reconstruction, and we advocate them if possible.

LAPAROSCOPIC DP

Background

DP is widely accepted as an option for PDAC located in the distal pancreas. However, in past decades, laparoscopic DP (LDP) has been accepted increasingly with evidence of minimally invasive benefits. Compared with LPD, LDP is less technically demanding because there is limited dissection around the vessels and no reconstruction is required[57,58]. So, more surgeons accept LDP than LPD.

Safety and feasibility of LDP

A recently published meta-analysis[59-63] indicated that LDP was a safe and feasible option in terms of operating time and postoperative mortality and morbidities, such as postoperative bleeding and pancreatic fistula. Moreover, minimally invasive superiority was found in LDP, including significantly decreased estimated blood loss, time to first oral intake and length of hospital stay[59-63]. These results clearly show that LDP is as safe and feasible as ODP.

Short-term oncological results

Microscopically, R0 resection is the most important part of treatment of resectable pancreatic cancer. Some non-comparative cohorts have shown that R0 resection of pancreatic cancer can be achieved by laparoscopic resection[64,65]. Most of the comparative studies have shown that there is no difference in the rate of R0 resection in the final pathological results between LDP and ODP[58,66,67]. To the best of our knowledge, only DiNorcia *et al*[68] have reported a decrease in R1 resection in the laparoscopic group; however, their series had mixed pathology, including neuroendocrine tumor and pancreatic adenocarcinoma. Another important short-term oncological marker is LN retrieval. A minimum of 12 LNs is required for resection of pancreatic adenocarcinoma[69,70]. N0 patients with > 12 LNs have better survival than N0 patients with < 12 LNs ($P < 0.001$)[70]. Most studies have found that the number of LNs harvested in laparoscopic and open procedures is similar[58,66-68,71].

The data here demonstrate that most of the minimally invasive pancreatic surgeons have a consensus that LDP has the same short-term oncological results as ODP.

Long-term oncological outcomes of LDP

Only a few studies have described long-term prognosis after LDP, and few comparative data are reported. Mabrut *et al*[64] reported 16 patients with pancreatic malignancy, 4 of whom had pancreatic adenocarcinoma, and 23% of these patients had recurrence during 15 mo. Fernández-Cruz *et al*[72] reported 10 cases of laparoscopic radical antegrade modular pancreatosplenectomy (RAMPS), with 3 having died within a year and a median survival period of 14 mo. Rehman *et al*[67] found a similar 3-year overall survival between 8 LDP and 14 ODP procedures for PDAC. Kooby *et al*[58] reported similar median survival (16 mo) after LDP and ODP in a matched study. Kim *et al*[73] reported 11 LDPs with diagnosis of malignancy in their postoperative pathological results, including 5 cases of PDAC; only 1 patient died of cancer during the follow-up period (3-60 mo). The results to date suggest that the long-term prognosis of LDP for adenocarcinoma is similar to that for open procedures. It was also found that there was no difference in short-term oncological markers, including tumor size, radiological stage, margin-negative resection, power of LN retrieval and LN metastasis between the two groups. The authors concluded that LDP is acceptable for patients with pancreatic malignancy. However, further larger studies are required to give solid evidence of the long-term oncological benefit of LDP.

CONCLUSION

After initial reports of LPD and LDP in the 1990s, laparoscopic pancreatectomy finally became a well-established procedure following 30 years' development of laparoscopic skills and equipment. The data here suggest that minimally invasive pancreatectomy is safe and feasible and has adequate evidence of good short-term outcome. However, randomized controlled trials and long-term oncological results are still lacking. The long-term oncological results should be further addressed by randomized controlled trials. Another problem now is how to generalize this procedure from experienced hands to other centers.

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MINIREVIEWS

Technical advances in external radiotherapy for hepatocellular carcinoma

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Abstract

Radiotherapy techniques have substantially improved in the last two decades. After the introduction of 3-dimensional conformal radiotherapy, radiotherapy has been increasingly used for the treatment of hepatocellular carcinoma (HCC). Currently, more advanced techniques, including intensity-modulated radiotherapy (IMRT), stereotactic ablative body radiotherapy (SABR), and charged particle therapy, are used for the treatment of HCC. IMRT can escalate the tumor dose while sparing the normal tissue even though the tumor is large or located near critical organs. SABR can deliver a very high radiation dose to small HCCs in a few fractions, leading to high local control rates of 84%-100%. Various advanced imaging modalities are used for radiotherapy planning and delivery to improve the precision of radiotherapy. These advanced techniques enable the delivery of high dose radiotherapy for early to advanced HCCs without increasing the radiation-induced toxicities. However, as there have been no effective tools for the prediction of the response to radiotherapy or recurrences within or outside the radiation field, future studies should focus on selecting the patients who will benefit from radiotherapy.

Key words: Hepatocellular carcinoma; Radiotherapy; 3D-conformal radiotherapy; Intensity-modulated radiotherapy; stereotactic ablative body radiotherapy; Charged particle therapy; Image-guided radiotherapy

Core tip: Radiotherapy techniques have greatly improved in the last two decades. After the introduction of 3-dimensional conformal radiotherapy, the use of radiotherapy for hepatocellular carcinoma (HCC) has increased substantially. Currently, more advanced techniques including intensity-modulated radiotherapy, stereotactic ablative body radiotherapy, charged particle therapy, and image-guided radiotherapy are increasingly used for the treatment of HCCs. These techniques

facilitate the delivery of higher dose radiotherapy for early to advanced HCCs, while minimizing radiation-induced toxicities. This review will cover the technical aspects of modern radiotherapy techniques along with their clinical applications.

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INTRODUCTION

In the past, radiotherapy (RT) had a limited role in the treatment of hepatocellular carcinoma (HCC) due to the poor tolerance of the normal liver and the poor RT technique. As a result, the well-known Barcelona Clinic Liver Cancer guidelines for the treatment of HCC did not recommend RT as a treatment option for all stages of HCC[1]. This guideline recommends surgical treatments or local ablative therapies such as percutaneous ethanol injection or radiofrequency ablation (RFA) for the treatment of early small tumor(s) of stage 0 or A. Transarterial chemoembolization (TACE) is recommended for stage B large or multifocal HCCs and new agents like sorafenib are recommended for advanced stage C HCCs, which includes portal vein invasion or lymph node metastases. However, as many patients are not candidates for curative treatment or are not effectively treated with TACE or sorafenib, the use of other effective local modalities are warranted.

With the advancement of RT technologies, including 3-dimensional conformal radiotherapy (3D-CRT), intensity-modulated radiotherapy (IMRT), stereotactic ablative body radiotherapy (SABR), charged particle therapy, and image-guided radiotherapy (IGRT), delivering a higher radiation dose to the tumor in a safer way than before has become possible. To date, many institutions have reported good clinical outcomes for HCC patients receiving high dose radiation[2]. Moreover, increased understanding of the dose-response relationship and radiation-induced liver disease (RILD) facilitates the use of RT for patients with early to advanced HCCs[3-5]. In this topic highlight, we focused on the technical aspects of modern RT techniques for HCC along with their clinical applications.

3D-CRT

In contrast to the conventional 2D-RT technique, which usually uses opposing anterior and posterior radiation fields, 3D-CRT uses multiple coplanar or non-coplanar fields in order to reduce the high-dose exposure of normal tissues including the liver and bowels and to increase the tumor dose coverage (Figure 1). With the use of computed tomography (CT) images for RT planning and a computerized treatment planning system, the tumor and surrounding normal liver can be delineated accurately; the delivered dose and irradiated volume of the tumor and normal liver can be precisely evaluated. As a result, experience in the response of the tumor and normal liver to certain dose levels shapes the current decision making process for the RT regimen.

A high 92% response rate (80% complete response and 12% partial response) was achieved in a French phase 2 trial conducted in 27 patients having Child-Pugh class A or B liver function with a single tumor sized $\leqslant$ 5 cm or 2 tumors sized $\leqslant$ 3 cm after 66 Gy of 3D-CRT delivered in 33 fractions[6]. A Korean multicenter retrospective patterns of care study conducted in 398 HCC patients showed that a biologic effective dose of $\geqslant$ 53.1 Gy_{10} was associated with an improved 2-year overall survival[3]. Seong *et al*[7] treated 158 HCC patients with a dose of 25.2-60 Gy (1.8 Gy per fraction). In their study, the RT dose was identified by multivariate analysis as the only significant factor for survival. The median survival times in patients who received < 40 Gy, 40-50 Gy, and > 50 Gy were 6, 8, and 13 mo, respectively. Other studies also showed that a total RT dose of > 40-50 Gy achieved higher response or survival rates[8-10].

The Korean Practice Guidelines for the Management of Hepatocellular Carcinoma recommend RT for HCC patients as follows: (1) RT can be performed in HCC patients if liver functions indicate Child-Pugh class A or superb B and the irradiated total liver volume receiving $\geqslant$ 30 Gy is $\leqslant$ 60% (evidence level B1); (2) RT can be considered for HCC patients ineligible for surgical resection, liver transplantation, RFA, percutaneous ethanol injection, or TACE (C1); (3) RT can be considered for HCC patients who show incomplete response to TACE when the dose-volume criteria in Recommendation 1 are met (B2); (4) RT can be considered for HCC patients with portal vein invasion when the dose-volume criteria in Recommendation 1 are met (C1); and (5) RT is performed to alleviate symptoms caused by primary HCC or its metastases (B1)[11]. In the meta-analysis of 5 randomized and 12 non-randomized trials, TACE combined with RT achieved a better tumor response and survival than TACE alone[12]. Patients with portal vein thrombosis (PVT) responded to RT in about 45% of the cases[13,14].

However, the tolerance dose of the normal liver often limits the use of higher dose RT for HCC despite the availability of the modern 3D-CRT technique. Many factors including poor liver function with a Child-Pugh B or C score, prior TACE, PVT, and hepatitis B carrier status are known to be associated with a higher risk of RILD[15,16]. Nonetheless, these factors are unavoidable when RT is indicated. Radiation dose modification is recommended according to the liver function, the relative size of the tumor to the whole liver, and the normal liver dose[17,18]. Therefore, more advanced RT techniques are warranted to overcome

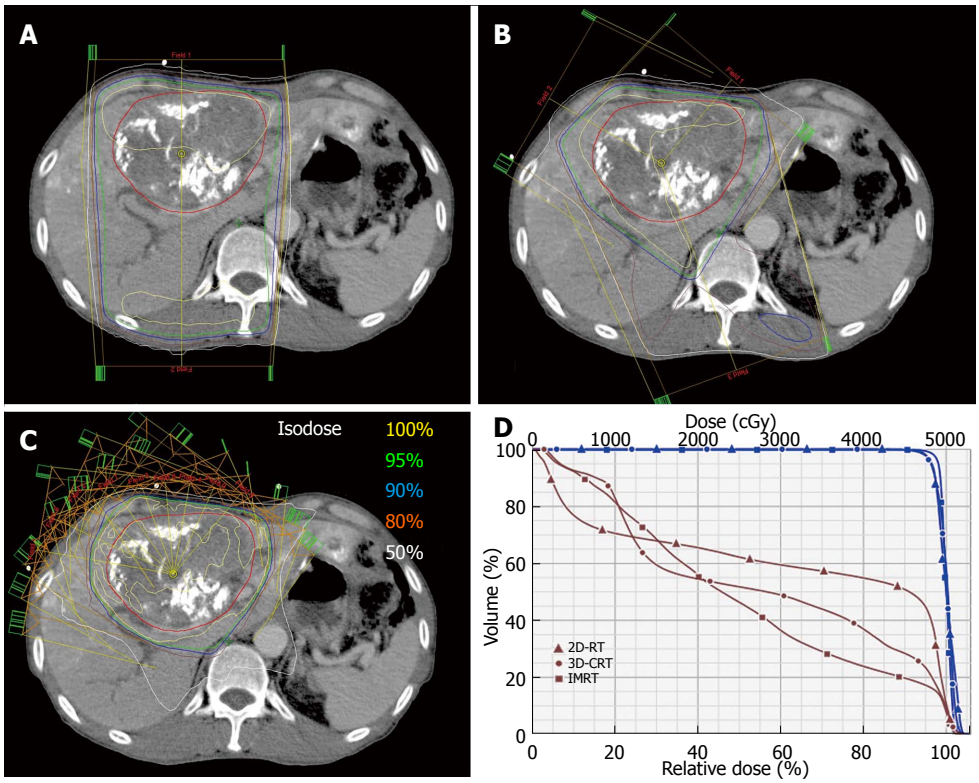

Figure 1 The radiotherapy plans of different radiotherapy techniques for hepatocellular carcinoma. A: 2-dimensional radiotherapy (2D-RT); B: 3-dimensional conformal radiotherapy (3D-CRT); C: Intensity-modulated radiotherapy (IMRT); D: dose volume histogram of the tumor (blue) and normal liver (brown). IMRT plan shows the best liver sparing while 2D-RT shows the worst liver sparing.

these unavoidable obstacles and improve the clinical outcomes in terms of tumor control and normal tissue toxicity.

IMRT

IMRT is an advanced form of conformal RT that facilitates the delivery of a higher radiation dose compared to 3D-CRT. A computer-aided automated optimization process, known as inverse treatment planning, modulates the intensity of each beam to gain the desired target coverage while minimizing the dose to the normal organs (Figure 1). At present, various forms of IMRT, including volumetric-modulated arc therapy (VMAT) and helical tomotherapy (HT), are available. VMAT delivers intensity-modulated beams during gantry rotation, and HT delivers the radiation dose in slices with the help of a rotating gantry similar to a helical CT scanner. Furthermore, IMRT can deliver different doses to different targets at the same time, which is called simultaneous integrated boost-IMRT. Using this technique, a higher dose can be delivered to the gross tumor volume concurrently with a lower dose to areas of subclinical disease. Even though radiation oncologists have been reluctant in using IMRT for moving tumors due to the dosimetric and radiobiological uncertainties related to respiratory movement, recent experimental and clinical studies have rationalized its use for the treatment of HCC.

The distortion of calculated dose distribution of the IMRT plan on the static CT images is inevitable if the target moves during the IMRT beam delivery. The difference between the calculated and measured doses of a single IMRT field or a single fraction with doses of multiple IMRT fields was unacceptably high; however, repeated irradiation negated the effect of motion[19-21]. After the delivery of 30 fractions, the mean dose to a moving tumor differed slightly (< 2%-3%) from that of a static tumor[19]. Volumetric dose measurements by Duan *et al*[21] revealed that the 5-fraction isodose line for the moving phantom was fairly well matched with that of the stationary phantom, and the difference between the tumor control probabilities of the stationary and moving tumors for $\geqslant$ 2 fractions was small (< 2.3%). Kuo *et al*[20] reported that this difference was larger at higher amplitudes of tumor motion and higher dose rates of irradiation (500 MU/min *vs* 300 MU/min); however, it did not differ between the IMRT delivery modes (sliding window *vs* step-and-shoot).

The dosimetric advantages of IMRT over 3D-CRT and the importance of the IMRT techniques were previously reported. Early dosimetric studies comparing IMRT to 3D-CRT suggested that IMRT enabled dose escalation without the risk of increased liver toxicity and potentially reduced the normal tissue complication probability in HCC patients previously diagnosed with RILD after 3D-CRT[16,22]. Chen *et al*[23] compared the techniques of

3D-CRT, fixed-angle IMRT, and VMAT in small to large HCCs and suggested that VMAT might carry the lowest risk of RILD with the lowest V20 and V30 compared to 3D-CRT or IMRT for right lobe tumors. However, the results of comparisons between different RT techniques (3D-CRT *vs* fixed-angle IMRT *vs* VMAT *vs* HT) have been variable. Although some studies reported that the mean liver dose was higher for fixed-angle IMRT or VMAT plan compared to 3D-CRT[16,23], these results could be caused by suboptimal IMRT beam configuration or the routine application of constraints to IMRT planning as a planning study. HT has been reported to provide a better uniformity for the target coverage than fixed-angle IMRT; however, the low dose volume of the normal liver that is related to the risk of RILD was higher for HT compared to that of fixed-angle IMRT[24,25]. Park *et al*[26] reported that the dose-volumetric parameters of VMAT *vs* fixed-angle IMRT differed according to the target location within the liver; central tumors showed higher mean liver dose and lower liver volume receiving 30 Gy for VMAT than for IMRT; however, peripheral tumors showed no difference. When using fixed-angle coplanar IMRT, using fields entering the body near the tumor might be better at reducing the normal liver dose by decreasing the length of beam path through the normal liver compared to the equidistant beam array[27].

The clinical outcomes of IMRT have been reported recently. Yoon *et al*[28] reported that IMRT could deliver higher doses (median, 50 Gy in 20 fractions) and achieved higher 3-year overall survival and progression-free survival than 3D-CRT without the increased risk of RILD in stage Ⅲ or ⅣA HCC patients. Hou *et al*[29] reported similar results in advanced HCC patients with portal vein and/or inferior vena cava tumor thrombi with IMRT of a median total dose of 60 Gy with a fraction size of 2.5-4.0 Gy. Several authors reported that delivering a high dose IMRT was feasible in patients with small to large HCCs without a high incidence of RILD. Wang *et al*[30] delivered 45, 60, or 66 Gy in 1.8 or 2.0 Gy per fraction depending on tumor stage, target location, and the sizes of small to large HCCs ineligible for surgery or ablative treatments. The mean normal liver dose was 19.4 ± 6.3 Gy, and nonconventional RILD was observed in 13% of patients. Kang *et al*[31] delivered a median dose of 50.4 Gy to advanced HCCs with an equivalent sphere size of 11.4 ± 2.6 cm. There was no grade ⩾ 3 RILD in patients treated with IMRT without combination with TACE or intra-arterial chemotherapy. McIntosh *et al*[4] conducted an accelerated IMRT with concurrent capecitabine in 20 patients with unresectable HCC with a mean tumor size of 9 cm (range, 1.3-17.4 cm). The prescribed dose was 50 Gy in 20 fractions and there were no grade > 2 acute or late toxicities. Kim *et al*[32] reported that an accelerated RT with simultaneous integrated boost-IMRT was feasible and safe for patients with inoperable HCC. The tumor and the surrounding area with subclinical disease received 66 Gy and 55 Gy in 22 fractions, respectively. When the tumor was located within < 1 cm of the gastrointestinal structures, 55 Gy and 44 Gy in 22 fractions to the tumor and the surrounding area with subclinical disease, respectively, was delivered.

The results discussed thus far indicated that IMRT has the potential of dose escalation for HCC without an increased risk of RILD, which signals the potential for improved survival and quality of life in patients with HCC. However, because there is no standard technique for IMRT delivery and because the IMRT plan is not always better than the 3D-CRT plan, it is important to individualize the treatment plan for every patient.

SABR

SABR is generally defined as a treatment method for delivering a high dose of radiation to the target in a few fractions (typically 1-5 fractions) with a high degree of precision. SABR with a common linear accelerator usually utilizes multiple coplanar or noncoplanar static beams or multiple arc beams (Figure 2). The CyberKnife system (Accuray, Inc., Sunnyvale, CA, United States) and the VERO system (BrainlLab AG, Feldkirchen, Germany) are specialized machines for SABR, and HT is also used for SABR. To irradiate the tumor more accurately and to increase the sparing of the normal organs, SABR is performed in combination with at least one kind of IGRT technique integrated into the treatment machine; the different IGRT techniques are described in the subsequent section. During the last decade, the use of SABR for HCC has increased substantially and the practice guidelines from the National Cancer Center and Korean Liver Cancer Study Group recommend SABR as an alternative to the ablation/embolization techniques, or when these therapies have failed or are contraindicated[11,33].

Since the reporting of the first clinical experience with SABR by Blomgren *et al*[34] in 1995, many prospective and retrospective studies have been conducted (Table 1). Generally, SABR was used for the treatment of a few, small HCCs (< 5-6 cm) in patients with Child-Pugh class A or B disease. Local control rates at 2-3 years were 84%-100%, excluding two studies in which a relatively low dose was used[35] or large tumors were treated[36]. However, the overall survival and the incidence of severe hepatic toxicities varied due to the heterogeneity of patient and tumor characteristics such as liver function, tumor location, and tumor size. The most recent study by Wahl *et al*[37] showed comparable results between SABR (249 tumors in 161 patients) and RFA (83 tumors in 63 patients) in 224 patients with inoperable, nonmetastatic HCC. The rates of freedom from local progression at 1 and 2 years were 83.6% and 80.2% for RFA *vs* 97.4% and 83.8% for SABR. Notably, increase in tumor size was a predictor of local progression in patients who underwent RFA, but not in patients who underwent SABR.

Although there have been no prospective trials comparing SABR to other ablative modalities, recent

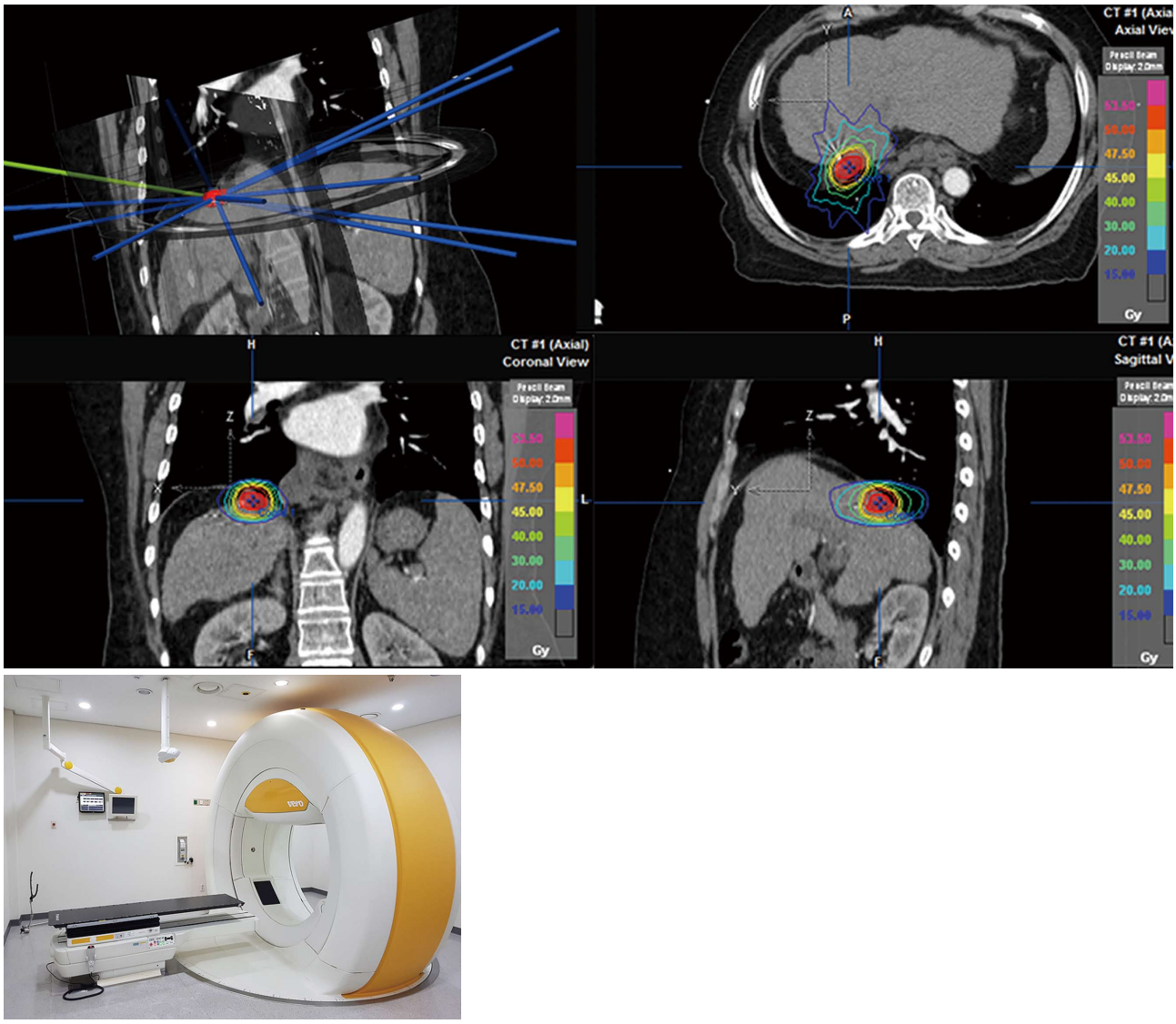

Figure 2 The stereotactic ablative body radiotherapy plan using the VERO system.

Table 1 The summary of the trials conducted using stereotactic ablative body radiotherapy for hepatocellular carcinoma

Study	Year	*n*	Dose/fraction (Gy/Fr)	Local control	Overall survival	Severe hepatic toxicity
Cárdenes *et al*[65]	2010	17	36–48 Gy/3 or 5 Fr	2-yr, 100%	2-yr, 60%	27% in CPC-B
Kwon *et al*[35]	2010	42	30–39 Gy/3 Fr	3-yr, 68%	3-yr, 59%	Gr ⩾ 3, 2%
Andolino *et al*[5]	2011	60	40–48 Gy/3 or 5 Fr	2-yr, 90%	2-yr, 67%	Gr ⩾ 3, 0%
Bujold *et al*[36]	2013	102	24–54 Gy/6 Fr	2-yr, 74%	2-yr, 34%	Gr ⩾ 3, 17%
Kang *et al*[66]	2012	47	42–60 Gy/3 Fr	2-yr, 95%	2-yr, 69%	Gr ⩾ 3, 19%
Yoon *et al*[67]	2013	93	30–60 Gy/3-4 Fr	3-yr, 92%	3-yr, 54%	Gr ⩾ 3, 7%
Sanuki *et al*[68]	2014	185	35–40 Gy/5 Fr	3-yr, 91%	3-yr, 70%	Gr 5, 7% in CPC-B
Kimura *et al*[69]	2015	65	48 Gy/4 Fr	2-yr, 100%	2-yr, 76%	Gr ⩾ 3, 23%
Wahl *et al*[37]	2016	63	27–60 Gy/3 or 5 Fr	2-yr, 84%	2-yr, 46%	Gr ⩾ 3, 2%

CPC-B: Child-Pugh class B; Gr: Grade.

data supports the use of SABR as an alternative ablative treatment option for the treatment of inoperable HCC. However, because SABR cannot be repeated unlike the other treatment modalities and because RILD occurs more frequently in patients with poor liver function, the decision on the best ablative modality should be made using a multidisciplinary approach.

CHARGED PARTICLE THERAPY

Charged particle therapy such as proton and carbon ion therapy offers distinct physical properties. The

Table 2 The summary of trials conducted using charged particle therapy for hepatocellular carcinoma

Study	Year	Particle	*n*	Dose/fraction (Gy/fr)	Local control	Overall survival	Grade ⩾ 3 Liver toxicity
Nakayama *et al*[38], retrospective	2009	Proton	318	55–79.2 CGE/10–22 Fr	NA	5-yr, 45%	None
Komatsu *et al*[40], retrospective	2011	Proton	242	52.8–84 CGE/4–38 Fr	5-yr, 90%	5-yr, 38%	1%
		Carbon	101	52.8–76 CGE/4–20 Fr	5-yr, 93%	5-yr, 36%	3%
Hata *et al*[39], retrospective	2006	Proton	19	50–84 Gy/10–24 Fr	1 failure	2-yr, 42%	None
Bush *et al*[70], phase 2	2011	Proton	76	63 Gy/15 Fr	NA	3-yr, 60%[1]	None
Hong *et al*[42], phase 2	2016	Proton	44	58.05–67.5 CGE/15 Fr	2-yr, 95%	2-yr, 63%	2%
Bush *et al*[43], randomized	2016	Proton	33	70.2 Gy/15 Fr	2-yr, 88%	2-yr, 48%[1]	None

[1]Progression-free survival. CGE: Cobalt gray equivalent; NA: Not available.

absorbed dose rapidly increases and suddenly rises to a peak before the proton is ultimately stopped, called the "Bragg peak effect". This facilitates increased sparing of normal tissues surrounding the tumor compared to conventional photon beam therapy, and thus, dose escalation for HCC can be achieved.

Some retrospective[38-40] and prospective[41-43] studies have reported encouraging outcomes with proton or carbon beam therapy in patients with HCC (Table 2). Local control rates were 88%-98% at 2-5 years with a very low incidence of severe toxicity. Hata *et al*[39] reported that patients with Child-Pugh C cirrhosis also showed no therapy-related toxicity of grade ⩾ 3. Bush *et al*[41] reported that 6 patients showed pathologic complete response and 7 patients showed microscopic residual disease in 18 patients who underwent liver transplantation after proton beam therapy. Recently, the interim analysis of a randomized trial comparing proton beam therapy to TACE for HCC was reported[43]. At the time of analysis, 36 patients in the TACE group and 33 patients in the proton group were available for analysis. Pathologic complete response was achieved in 10% of the 10 patients from the TACE group and 25% of the 12 patients from the proton group, who underwent liver transplantation after treatment. There was a trend toward improved 2-year local control (88% *vs* 45%, $P = 0.06$) and progression-free survival (48% *vs* 31%, $P = 0.06$) favoring proton beam therapy.

Charged particle therapy generally showed better local control and survival rates than the photon-based RT series, although a direct comparison is impossible due to the differences in patient characteristics. Moreover, a recent interim analysis of a randomized trial comparing TACE and proton beam therapy favored the proton beam therapy. Although the facilities for charged particle therapy have been limited thus far, it is anticipated that the use of charged particle therapy will increase in the near future.

IGRT

IGRT is defined as RT that employs imaging to maximize accuracy and precision throughout the whole process, which includes target and normal tissue delineation, radiation delivery, and adaptation of therapy to anatomic and biological changes over time in individual patients[44]. Of these, accurate target delineation, target relocalization to allow proper patient repositioning, and respiratory motion management have been the most challenging in patients with HCC.

Target delineation

The initial step of IGRT is precise tumor delineation. The specific enhancement pattern of HCC (enhancement in arterial phase and washout in portal venous or late delayed phase) can help radiation oncologists delineate gross tumor volume. A radiologic-pathologic correlation study showed that microscopic invasion from HCC was observed up to 4 mm from the gross tumor, and the distance was correlated with the alpha-feto protein level, tumor size, PVT, and TNM stage[45]. This study suggested that a margin of < 5 mm from the gross tumor volume is required for the clinical target volume. The planning target volume (PTV) is defined as the volume that is used for the RT planning to ensure the tumor dose in the presence of breathing motion and set-up uncertainties. The PTV margin from the clinical target volume ranges 5-10 mm or more, depending on the methods of simulation and in-room IGRT.

For the tumor delineation of HCC, 4D-CT images, which are synchronized with the patient's respiratory cycle, are usually acquired to capture the whole trajectory of the moving tumor. Brock[46] recommended the acquisition of contrast breath-hold CT scans followed by 4D-CT in the HCC patients to capture both the early enhancement and washout phases. However, 4D-CT cannot acquire the same quality achieved with the diagnostic scans[47] and have many artifacts preventing accurate tumor delineation[48]. Therefore, diagnostic CT or magnetic resonance imaging (MRI), which shows the extent of HCC better, should be used for tumor delineation. Rigid or deformable registration between the diagnostic and RT planning images can be used. Based on our experience of rigid registration, it is important to match the fiducial markers (*e.g.*, lipiodol) or anatomical landmarks (*e.g.*, liver contour and vessels) near the tumor, instead of the whole liver. Although a difference in target size by a few millimeters was observed after the deformable registration between MRI and CT images[49], deformable registration

between diagnostic MRI and RT planning images could be helpful for target delineation; however, it is still at the investigational phase.

Every effort should be taken to delineate the target precisely using the currently available imaging modalities, and further research is required for the combination of these modalities in order to make the whole trajectory of the tumor more clearly visible on the RT planning system.

Target relocalization and tumor surrogates

Before the radiation beam is turned on, bony landmarks are usually used to position the tumor to its original location at the time of simulation. However, as HCC moves during the respiratory cycle and is often invisible on in-room images, surrogates for the tumor are required for the application of IGRT. High-density materials (*e.g.*, inserted fiducial markers, packed lipiodol, surgical clips), the diaphragm, large vessels, and the entire liver can be used as surrogates. With the help of these surrogates, PTV margins can be reduced and normal tissue doses can be further spared.

Various techniques involving 2D or 3D volumetric image guidance are now available to verify and reposition the location of surrogates[46]. Kilovoltage (kV) or megavoltage (MV) radiography can help visualize the location of diaphragm or fiducial markers, which is subsequently compared to their location on the planning CT image at the specific phase of respiration (*e.g.*, breath-hold or gated). The kV fluoroscopic imaging can show the tumor motion during respiration or breath-hold. Using volumetric imaging by a CT scanner in the treatment room, soft tissues, including the liver, adjacent structures, or fiducial markers, can be used for image guidance. Because the long acquisition time for CT images can lead to image blurring, breath-hold or respiration sorting techniques can be used as well. The specific technique for the target relocalization can be chosen according to the RT delivery technique (free-breathing, breath-hold, gated, or tracking). Recently, non-invasive MRI has been used for IGRT[50,51].

Gold fiducial markers are preferred over other surrogates because they provide better visibility on a standard MV imaging device as well as on kV X-ray images. They can be used for real-time tumor tracking (for gated or tracking treatment) as well as for confirming PTV margins in 2D or 3D images. Interestingly, Wahl *et al*[37] reported that the local failure rate was higher in SABR-treated patients without fiducial markers compared to those with fiducial markers (10% *vs* 0%, respectively, $P = 0.15$), which highlights the importance of using an accurate IGRT technique.

The management of respiratory motion

Another issue in RT for HCC is the control of the respiratory motion, because the liver moves in a significant range during respiration[52]. The ways to treat a moving tumor can be classified into motion-encompassing, forced shallow breathing with abdominal compression, respiratory gating, and real-time tumor tracking[53]. The motion-encompassing method refers to the covering of all possible positions of the moving tumor through the whole breathing cycle and subsequently a large volume of normal tissue may be irradiated. Although breath-hold and forced shallow breathing can reduce the respiratory motion for liver tumors, this might result in significant patient discomfort or inconvenience during treatment. Presently, respiratory gating and real-time tumor tracking are the most advanced techniques. Figure 3 shows the dose distribution for the three techniques of motion management.

The respiratory gating method involves turning on the radiation beam when the tumor is at a given location, which leads to a smaller PTV volume. The current commercially available Real-time Position Management system (Varian Medical system, Palo Alto, CA) detects the respiratory signal *via* the movement of the surrogate on the abdominal surface, which can be correlated with the respiratory movement of the tumor inside the body. The position and width of the gate within a patient's respiratory cycle are determined by monitoring the tumor's respiratory motion that was captured on 4D-CT images. This gating method using an external breathing signal is easy, noninvasive, and radiation-free; however, a potential error might be that the signal does not accurately correlate with the internal target position[54-56]. For this reason, the Hokkaido group developed the real-time tumor tracking radiation therapy system that combines both the external breathing signals and the internal tumor motion signals *via* implanted fiducial markers[57]. Kubo *et al*[58] reported the feasibility of gated IMRT as well. A disadvantage of the gating techniques is the reduced efficiency of radiation delivery, resulting in a prolonged treatment time (or reduced duty cycle). For SABR, where a larger dose is delivered at each treatment, this prolonged treatment time could decrease the patient compliance.

An alternative strategy is to reposition the radiation beam while tracking the tumor's changing position dynamically. Ideally, this method can eliminate the need to compensate for the movement of the tumor and achieve a 100% duty cycle for dose delivery. Iizuka *et al*[59] showed that the tracking technique could reduce the PTV volume by 35% in 11 liver cases, compared to the motion-encompassing method. Currently, there have been two treatment machines capable of tumor tracking: the CyberKnife system and the VERO system. The clinical feasibility of the CyberKnife system has been shown in several studies[60-63]. The CyberKnife system consists of a pair of fluoroscopes in the ceiling coupled to a small X-band linear accelerator mounted on a robotic arm, which can move according to the movement of the inserted fiducial markers. The VERO

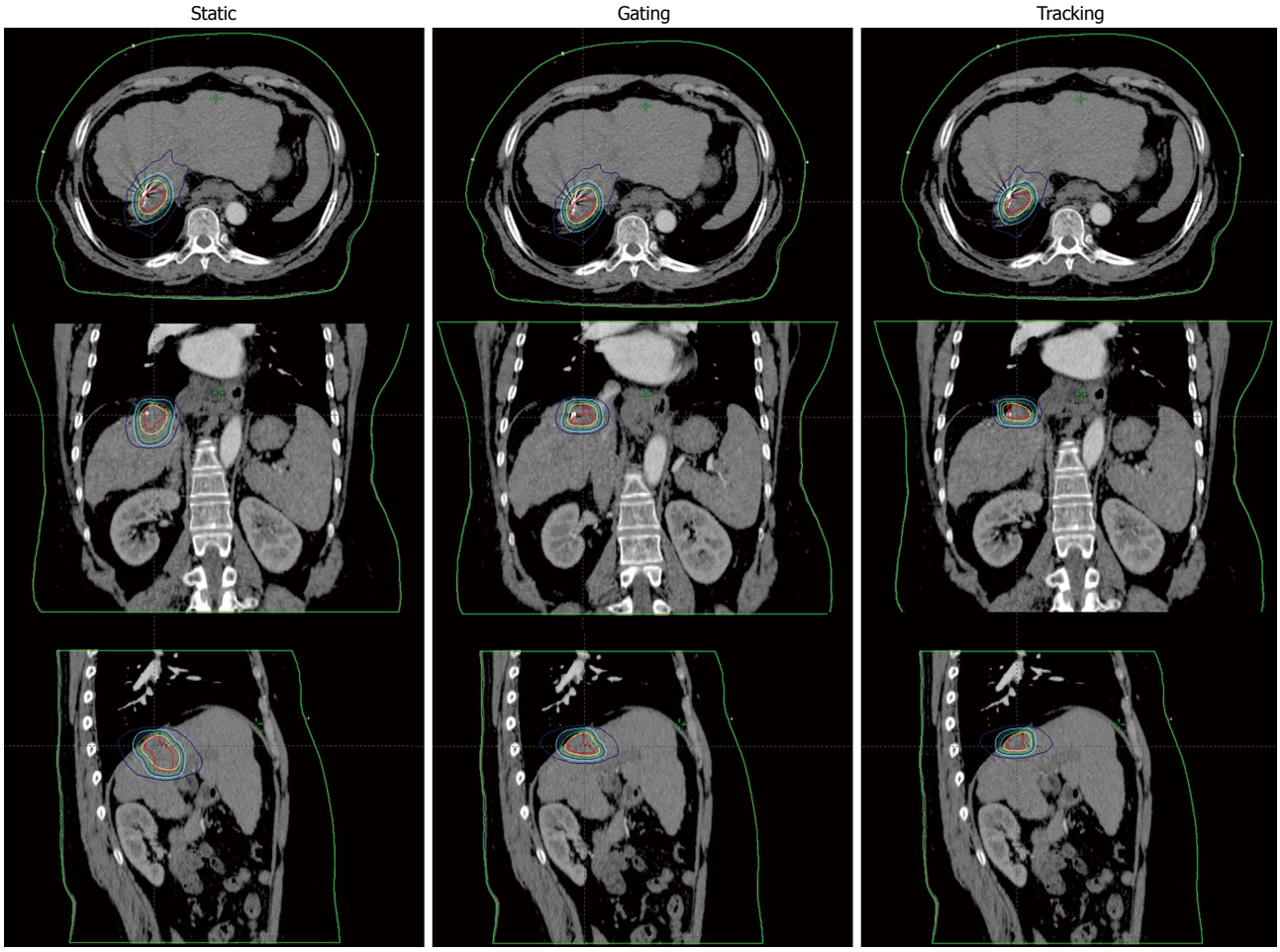

Figure 3 The comparison of dose distributions in 3 stereotactic ablative body radiotherapy plans for a representative case. The static plan using the motion encompassing technique is shown in the left column. The gating and tracking plans are shown in the middle and right column, respectively.

system uses a pair of fluoroscopes mounted in the machine to monitor the movement of inserted fiducial markers and allows the treatment head, gimbal, to pivot in two dimensions according to the movement of the fiducial markers[64].

CONCLUSION

Recent advances in the RT techniques facilitate dose escalation for small to large tumors with the hope of improved local tumor control without increasing normal tissue toxicity. However, local failure is still problematic, especially in advanced HCCs, and intrahepatic or distant metastases often develop, which could offset the impact of increased local control and render the given treatments meaningless. Unfortunately, reliable methods that can predict the tumor response to RT or recurrences within or outside the RT field have not been developed. Therefore, future research should focus on the prediction of the outcomes after treatment to determine the patients who will benefit from RT as well as the novel biologic agents that can prevent recurrences outside the RT field.

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ORIGINAL ARTICLE

Basic Study

Protein and gene expression characteristics of heterogeneous nuclear ribonucleoprotein H1 in esophageal squamous cell carcinoma

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Author contributions: Sun YL and Zhao XH designed the study; Sun YL, Liu F and Liu F performed the research; Sun YL analyzed the data and wrote the manuscript; Zhao XH supervised this work.

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Abstract

AIM

To investigate the expression characteristics of heterogeneous nuclear ribonucleoprotein H1 (HNRNPH1) mRNA and protein in cell lines and tissues of esophageal squamous cell carcinoma (ESCC).

METHODS

Western blotting was used to assess the expression of HNRNPH1 protein in seven ESCC cell lines and 30 paired fresh tissue specimens. The subcellular localization of HNRNPH1 was determined by immunofluorescence in ESCC cells. The RNA sequencing data from 87 patients with ESCC were obtained from the cancer genome atlas (TCGA), and the expression and clinical characteristics analysis of different transcript variants of *HNRNPH1* were evaluated in this dataset. In addition, immunohistochemistry was carried out to detect the expression of HNRNPH1 protein in 125 patients.

RESULTS

The expression of HNRNPH1 protein varied across different ESCC cell lines. It was exclusively restricted to the nucleus of the ESCC cells. There are two tran-

script variants of the *HNRNPH1* gene. Variant 1 was constitutively expressed, and its expression did not change during tumorigenesis. In contrast, levels of variant 2 were low in non-tumorous tissues and were dramatically increased in ESCC (P = 0.0026). The high levels of variant 2 were associated with poorer differentiated tumors (P = 0.0287). Furthermore, in paired fresh tissue specimens, HNRNPH1 protein was overexpressed in 73.3% (22/30) of neoplastic tissues. HNRNPH1 was significantly upregulated in ESCC, with strong staining in 43.2% (54/125) of tumor tissues and 22.4% (28/125) of matched non-cancerous tissues (P = 0.0005). Positive HNRNPH1 expression was significantly associated with poor tumor differentiation degree (P = 0.0337).

CONCLUSION

The different alternative transcript variants of HNRNPH1 exhibited different expression changes during tumorigenesis. Its mRNA and protein were overexpressed in ESCC and associated with poorer differentiation of tumor cells. These findings highlight the potential of HNRNPH1 in the therapy and diagnosis of ESCC.

Key words: Heterogeneous nuclear ribonucleoprotein H1; Esophageal squamous cell carcinoma; Alternative transcript variants; Biomarker

Core tip: Heterogeneous nuclear ribonucleoprotein H1 (HNRNPH1) is an evolutionarily conserved splicing factor. It is involved in alternative splicing, polyadenylation, mRNA export, and translation. This study investigated the expression, localization, and clinical significance of HNRNPH1 in esophageal squamous cell carcinoma (ESCC). We found that this gene possesses two alternative transcript variants; one was constitutively expressed, while the other was regulated and dramatically increased in ESCC. HNRNPH1 protein was overexpressed in ESCC tissues. Strong HNRNPH1 levels were associated with poorer tumor differentiation and alternative splicing of apoptosis-related genes. These findings suggest that HNRNPH1 is a potential diagnostic biomarker and therapeutic target for ESCC.

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INTRODUCTION

Esophageal cancer is the third most commonly diagnosed cancer and the fourth leading cause of cancer death in China[1]. It was estimated that 477900 new cases and 375000 deaths occurred in 2015, accounting for about 13.3% of all cancer deaths. The predominant histological subtype in China is esophageal squamous cell carcinoma (ESCC).

Under various environmental exposures and genetic factors, ESCC develops through progression from normal esophageal epithelium to dysplasia, early stage and then advanced stage esophageal carcinoma with an accumulation of numerous genetic and epigenetic abnormalities[2]. Currently, alternations in thousands of genes have been found in ESCC. Nascent transcripts that are produced by RNA polymerase II undergo precursor mRNA splicing to generate mature mRNAs. Heterogeneous nuclear ribonucleoproteins (hnRNPs) play critical roles in this process. In addition, hnRNPs act as trans-factors to regulate alternative splicing, gene expression, mRNA export, localization, translation, and stability[3]. In humans, the hnRNPs family consists of at least 20 abundant, major hnRNP proteins and other less abundant, minor hnRNP proteins[3]. Most of the major hnRNP proteins have been shown to be overexpressed in ESCC tissues by proteomic analysis[4-7]. Immunohistochemical staining showed that HNRNPB1 was a potential diagnostic marker for squamous cell carcinoma of various organs, including ESCC[8].

Our previous proteomic study found that a major hnRNP protein, HNRNPH1, was upregulated approximately 8.4-fold in ESCC. Its overexpression was also observed in another proteomic study in ESCC[5]. HNRNPH1 was first purified in 1994 as an abundant component of hnRNP complexes[9]. It is ubiquitously expressed in various human tissues and binds only to poly (rG) sequence[9,10]. HNRNPH1 was demonstrated to stimulate pre-mRNA cleavage and polyadenylation, and it is an important determinant of alternative splicing[11-17]. At present, the relevant reports about HNRNPH1 and cancer are still very limited. Upregulation of HNRNPH1 was found in pancreatic adenocarcinoma, hepatocellular carcinoma, gastric carcinoma, head and neck carcinomas, and colon cancer[18,19]. However, the aberrant expression of HNRNPH1 has not been verified and evaluated in large-scale clinical samples. In this study, we investigated the expression and clinical significance of HNRNPH1 mRNA and protein in ESCC using an RNA sequencing dataset from the cancer genome atlas (TCGA), western blotting, and immunohistochemical staining assays.

MATERIALS AND METHODS

Cell lines and cell cultures

The human ESCC cell lines KYSE30, KYSE140, KYSE170, KYSE180, KYSE410, and KYSE510 were the gifts from Dr. Y. Shimada at Hyogo College of Medicine. EC0156 was established by our laboratory[20]. EC0156 was cultured in Dulbecco's modified Eagle's medium

supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, NY, United States). The other cell lines were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium. All the cells were incubated at 37 ℃ in a humidified atmosphere of 5% CO_2.

Clinical specimen collection and preparation

Surgical tissues from ESCC patients were collected after obtaining informed consent and approval from the Institutional Review Board of the Cancer Hospital of Chinese Academy of Medical Sciences (CAMS, Beijing, China). A total of 30 fresh tumor and paired adjacent non-tumor esophagus tissue samples were collected from patients (25 male, five female; median age, 58 ± 10 SD; range 32-72 years) undergoing resection from January 2005 to January 2009. All patients were diagnosed by two senior pathologists without chemo/radiotherapy before surgical operation. The tissue samples were collected and washed right after surgical resection. They were then snap-frozen in liquid nitrogen immediately and stored at -80 ℃.

For immunohistochemical staining, 50 formalin-fixed, paraffin-embedded tissues specimens were collected from surgically resected ESCC in Cancer Hospital of CAMS from January 1999 to 2009. In addition, a tissue microarray that contained 75 ESCC cases were purchased from Shanghai Outdo Biotech Co., Ltd (Shanghai, China).

Protein extraction

Cells in the exponential phase of growth were harvested using a protein lysis buffer (pH 7.4) containing 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% nonidet P-40 (NP-40), 0.1% sodium dodecyl sulfate (SDS), and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). In addition, subcellular protein extraction was performed using ProteoExtract™ Subcellular Proteome Extraction Kit (Calbiochem, Billerica, MA, United States) according to the manufacturer's guidelines. Fresh tissue samples were homogenized, and the proteins were extracted using the protein lysis buffer described above. The protein content was determined by Coomassie Plus Protein Assay (Pierce, Rockford, IL, Untied States).

Western blot analysis

Approximately 15 μg of total proteins or subcellular proteins were diluted in Laemmli buffer containing 10% β-mercaptoethanol and boiled at 95 ℃ for 10 min. Samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride membranes. After blocking, the membranes were incubated with anti-HNRNPH1 (ab10374, Abcam, Cambridge, United Kingdom), anti-Lamin B, anti-AIF (Santa Cruz Biotech., Dallas, TX, Untied States), and anti-β-actin (Sigma-Aldrich, St. Louis, MO, United States) antibodies. Following intensive washing, the membranes were developed with horseradish peroxidase conjugated second antibodies (Jackson Immunoresearch Lab., West Grove, PA, United States) and visualized using an enhanced chemiluminescence system (Santa Cruz Biotech.). The upregulation or downregulation of HNRNPH1 was defined as a change in relative band intensity in tumors compared with their paired adjacent normal tissues.

Immunofluorescence staining

EC0156 cells were grown in 0.01% poly-l-Lysine coated slices for 24 h. After being fixed with 4% paraformaldehyde for 30 min at room temperature and washed three times with phosphate buffered saline (PBS) (pH 7.4), the cells were blocked with 1% bovine serum albumin (BSA) and 0.1% Triton X-100 for 30 min at room temperature. Washed cells were incubated for 30 min with rabbit anti-HNRNPH1 antibody. The cells were then incubated in the dark for 60 min with Alexa Fluor 488-conjugated goat anti-rabbit secondary IgG (Life Technologies, Carlsbad, CA, United States). The fluorescence signals were captured under a Nikon E400 fluorescence microscope (Nikon Instech Co., Tokyo, Japan).

Immunohistochemistry

The tissue array of paraffin-embedded ESCC and their matched adjacent normal tissues were incubated with HNRNPH1 or control antibodies. After washing with 1 × PBS, slices were reacted with the biotin-labeled second antibody and then visualized using an ultrasensitive streptavidin-peroxidases system (Maxim Biotech, Fuzhou, China). Semi-quantitative analysis of the HNRNPH1 immunoreaction was quantified as described previously[21]. A staining index was used in which 0 was considered negative, 1-4 was weak, and > 4 was considered strong expression.

TCGA RNA sequencing data mining and statistical analysis

The ESCC transcriptome dataset was obtained from TCGA. The normalized transcripts (isoforms) sequencing data from 11 non-tumor tissues and 87 tumor tissues were available. The expression of two HNRNPH1 transcripts and their clinical significance were analyzed. The Mann-Whitney *U* test was used to compare the RPKM (Reads per kilobase of transcript per million reads mapped) between the two groups. Spearman rank correlation analysis was used to calculate the correlation coefficient of the two transcripts. *P* values < 0.05 were considered significant. All analyses were performed using GraphPad prism 6.0 (GraphPad Software Inc., La Jolla, CA, United States).

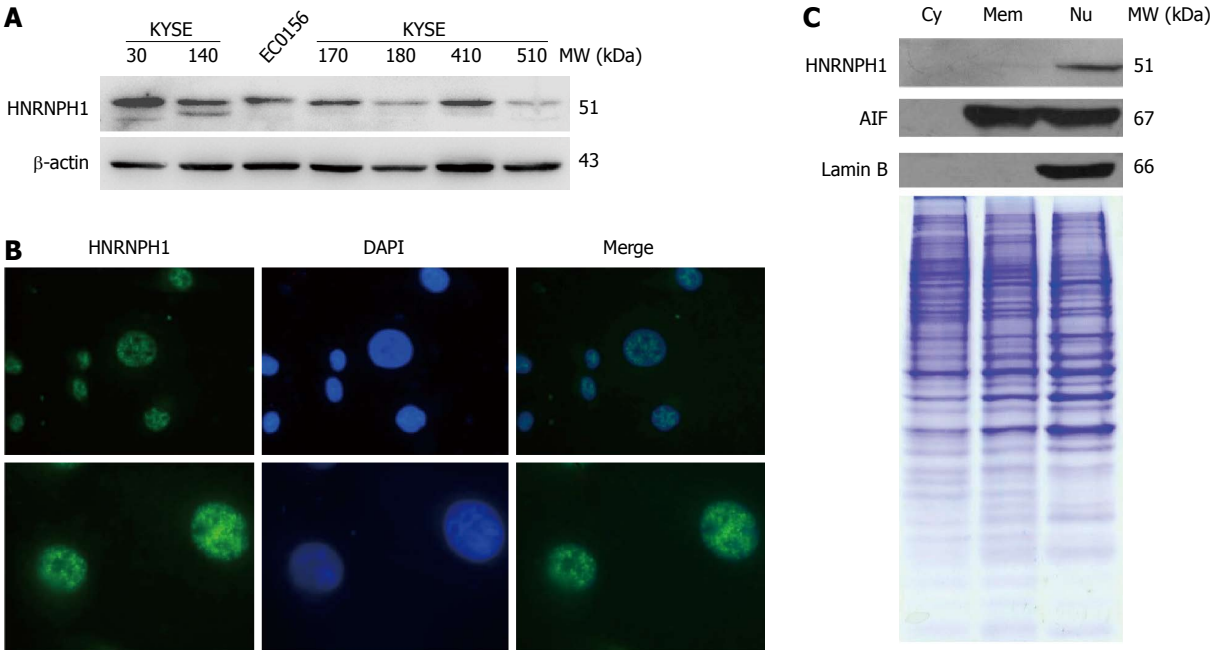

Figure 1 Expression and localization of heterogeneous nuclear ribonucleoprotein H1 in esophageal squamous cell carcinoma cells. A: Protein levels of HNRNPH1 were assessed by Western blots in seven ESCC cell lines. The β-actin protein was used as a loading control; B: Immunofluorescent visualization of HNRNPH1 in EC0156 cells; C: Subcellular protein levels of HNRNPH1 in EC0156 were assessed by western blots. Lamin B is a specific marker for nuclear proteins. AIF is a marker for intracellular membrane and nuclear proteins. Coomassie blue-stained SDS-PAGE gel was used as the loading control. AIF: Apoptosis inducing factor; HNRNPH1: Heterogeneous nuclear ribonucleoprotein H1; ESCC: Esophageal squamous cell carcinoma; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

RESULTS

Expression and localization of HNRNPH1 protein in ESCC cell lines

First, we observed the levels of HNRNPH1 protein in several ESCC cell lines. As shown in Figure 1A, HNRNPH1 expression varied across the different ESCC cells, with KYSE30, KYSE140, KYSE410, KYSE170, and EC0156 showing relatively high expression, whereas KYSKE180 and KYSE510 showing relatively low expression levels. Many members in the HNRNP family shuttle rapidly between the nucleus and cytoplasm. The shuttling capacity of HNRNPH1, however, remains unknown. Therefore, we next investigated the subcellular localization of HNRNPH1 *via* two methods. Immunofluorescence staining showed that it was localized in the nucleus but not the nucleolus (Figure 1B). Furthermore, western blotting analysis of subcellular protein showed that HNRNPH1 was strictly nuclear (Figure 1C). Thus, HNRNPH1 protein is ubiquitously expressed and exclusively sequestered to the nucleus in the ESCC cells.

HNRNPH1 mRNAs are up-regulated in ESCC tissues

Based on the NCBI RNA reference sequences collection (RefSeq) database (hg19), the *HNRNPH1* gene has two transcript variants, NM_001257293 (variant 1) and NM_005520 (variant 2). They are different in the 5' untranslated region (UTR) region but encode the same protein (Figure 2A). Using the TCGA RNA sequencing gene isoforms data from ESCC patients (n = 87), we compared the abundance of these two variants between tumor and non-tumor tissues. In the non-tumorous tissues (n = 11), variant 1 was constitutively expressed, whereas most of the samples barely expressed variant 2. However, in the tumor tissues, the expression of variant 1 was not altered compared to control (P = 0.3211), whereas variant 2 was significantly up-regulated (P = 0.0026, Figure 2B). Because the samples in TCGA were comprised of different races, we compared the differences of variant 1 and 2 in Asians and Caucasians. Caucasians had slightly higher levels of HNRNPH1 than Asians, but there was no significant difference between the two races (Figure 2B). In addition, the expression of variant 1 was not correlated with that of variant 2 in tumor tissues (P = 0.1201, R = -0.1679; Figure 2C), suggesting that the two variants of *HNRNPH1* are regulated by different mechanisms and display different expression characteristics.

Furthermore, we investigated the clinicopathological significance of variant 1 and 2 mRNA levels in Asians. No correlation between variant 1 and clinical features was observed (Figure 2D), whereas the levels of variant 2 were higher in poorly differentiated tumors (P = 0.0287; Figure 2E). Moreover, all of the cases were dichotomized into two groups, a high level group and a low level group, based on the median RPKM values in the tumor tissues. There was no significant relationship between variant 2 expression and overall survival of ESCC patients (Figure 3). Overall, it seems that the variant 1 of *HNRNPH1* is constitutively

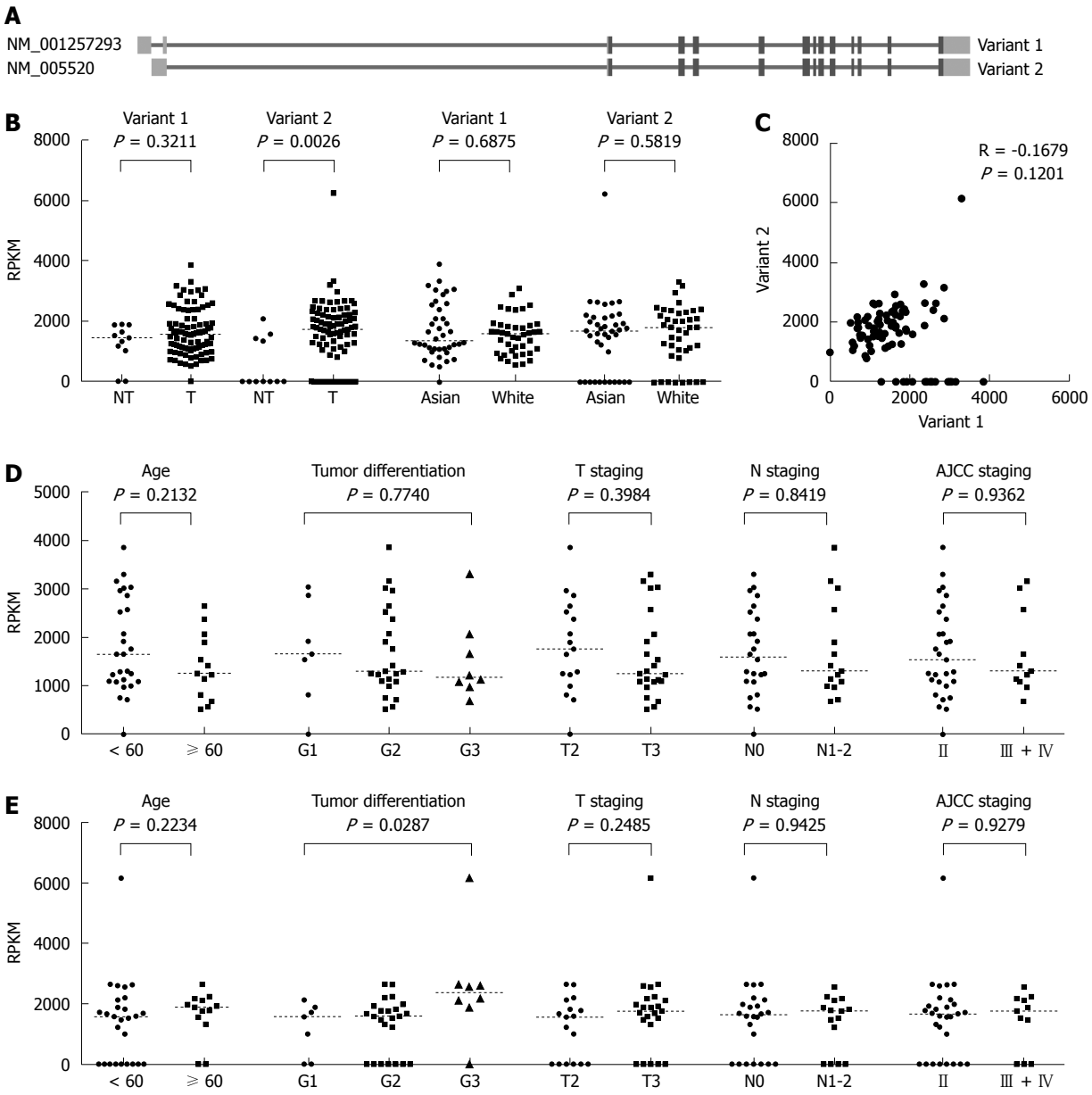

Figure 2 Expression and clinicopathological characteristics of heterogeneous nuclear ribonucleoprotein H1 mRNA presented in the cancer genome atlas RNA sequencing dataset. A: Transcript models for HNRNPH1 in hg19 visualized in the NCBI RefSeq. Human HNRNPH1 was encoded on the reverse strand. Two transcript variants were transcribed with different 5'UTR region. The light grey represents the untranslated region, while dark grey represents the coding region; B: These two transcripts had different expression patterns in ESCC. NT, non-tumor tissues (n = 11); T, tumor tissues (n = 87); C: The correlation analysis between variant 1 and 2 in the tumor tissues of ESCC (n = 87). Pearson correlation coefficients were calculated between their mRNA levels; D and E: The clinicopathological characteristics analysis of variant 1 (D) and variant 2 (E) expression in the Asian ESCC cases (n = 40). T staging, tumor invasive depth; N staging, lymph node metastasis; AJCC staging, the 7th edition cancer staging of American Joint Committee on Cancer. HNRNPH1: Heterogeneous nuclear ribonucleoprotein H1; ESCC: Esophageal squamous cell carcinoma; UTR: Untranslated region.

expressed, whereas the expression of variant 2 is modulated. Variant 2 expression was more associated with tumorigenesis in ESCC than variant 1 expression.

HNRNPH1 protein is overexpressed in ESCC tissues

To confirm the observation at the mRNA level, the protein expression of HNRNPH1 in ESCC patients was analyzed using western blotting and immunohistochemical staining assays. First, western blotting results revealed that HNRNPH1 was overexpressed in 73.3% (22/30) of neoplastic tissues compared to non-tumorous esophageal mucosal tissues (Figure 4A and B).

Immunohistochemistry in 125 paired ESCC and non-neoplastic esophageal mucosa was used to evaluate the expression of HNRNPH1 in more detail. The staining of HNRNPH1 was confined to the nuclei in both tumor and non-tumor cells (Figure 5). HNRNPH1 was strongly stained in 43.2% (54/125) of tumor tissues and in 22.4% (28/125) of matched non-cancerous tissues. In addition, HNRNPH1 expression was weak in 17.6% (22/125) of tumors, while its expression was weak in 35.2% (44/125) of normal

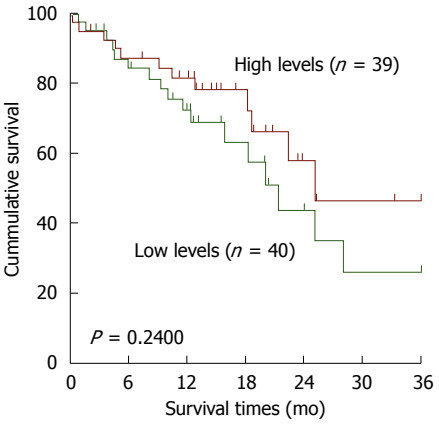

Figure 3 Kaplan-Meier curves of esophageal squamous cell carcinoma patients with low and high levels of variant 2 transcript of heterogeneous nuclear ribonucleoprotein H1 (*n* = 79). All of the cases were dichotomized into two groups, high level group and low level group, by the median RPKM values in tumor tissues. Log-rank test was used to compare the survival curves (P = 0.2400). HNRNPH1: Heterogeneous nuclear ribonucleoprotein H1; ESCC: Esophageal squamous cell carcinoma.

esophageal epithelia. The upregulation of HNRNPH1 in tumor tissues was statistically significant (P = 0.0005). The correlations between the clinicopathologic characteristics of ESCC patients and the expression of HNRNPH1 in their tumors are summarized in Table 1. Its high expression in ESCC correlated significantly with the poorer tumor differentiation degree (P = 0.0337). However, no correlation was found between its expression and age, gender, and lymph node metastasis (P > 0.05). These trends were consistent with the results demonstrated at the mRNA level. Taken together, these findings suggest that HNRNPH1 is overexpressed in ESCC, and its high expression is associated with worse biological behaviors of tumors.

HNRNPH1 regulates cell proliferation and apoptosis-related genes in ESCC

Previously, it was shown that HNRNPH1 modulates the alternative splicing of apoptotic mediators Bcl-x and A-raf[18]. To clarify further the biological significance of HNRNPH1 in ESCC, we performed a correlation analysis between variant 2 of HNRNPH1 and the alternative transcripts of these genes. In addition, the cell proliferation marker MKI67, which encodes Ki-67 protein, was included. As shown in Figure 6A and B, variant 2 was positively correlated with the expression of MKI67 (Rho = 0.3101, P = 0.0035) and the prominent transcript of A-raf (Rho = 0.2787, P = 0.0090). Moreover, variant 2 was inversely correlated with the pro-apoptotic transcript Bcl-X [Bcl-X (S), Rho = -0.2349, P = 0.0285; Figure 6C]. Therefore, overexpression of variant 2 of HNRNPH1 contributes to cell growth and anti-apoptosis in ESCC.

DISCUSSION

The human *HNRNPH1* gene is mapped to the reverse strand at 5q35.3. It encodes two transcript variants based on the annotation of NCBI RefSeq hg19. Variant 1 possesses 14 exons, whereas variant 2 has 13 exons (Figure 2A). They are differed at the 5′ UTR region but encode the same protein. Until now, there were no studies describing the expression characteristics of these two variants. Our results showed that variant 1 is constitutively expressed, and it is not regulated by the disordered signal transduction networks during carcinogenesis. Meanwhile, variant 2 is barely expressed in normal tissues. These results are consistent with previous findings that have shown that the expression of HNRNPH1 was unaffected by treatment with two second messengers and seven cytokines in normal human keratinocytes[10].In addition, we found that variant 2 is significantly overexpressed in ESCC and correlated with poorer tumor differentiation. These results suggest that the transcript variant 1 of HNRNPH1 is responsible for maintaining its invariable intracellular levels, whereas variant 2 is regulated and responds to tumorigenesis. We have identified a new mechanism for the gene expression regulation of HNRNPH1.

HNRNPH1 protein has three RNA binding domains, a glycine-tyrosine-arginine-rich (GYR) domain and a C-terminus glycine-rich domain. The central GYR domain is responsible for its nuclear localization[22]. HNRNPH1 can shuttle between the nucleus and

Table 1 Expression of heterogeneous nuclear ribonucleoprotein H1protein and its clinical significance in 125 esophageal squamous cell carcinomaspecimens *n* (%)

Characteristics	All cases (*n*)	HNRNPH1			*P* value[1]
		Negative	Weak	Strong	
Tissues					
Normal	125	53 (42.4)	44 (35.2)	28 (22.4)	0.0005
Cancer	125	49 (39.2)	22 (17.6)	54 (43.2)	
Age (yr)					0.5422
⩾ 60	71	26 (36.6)	16 (22.5)	29 (40.8)	
< 60	54	23 (42.6)	6 (11.1)	25 (46.3)	
Gender					0.9615
Male	90	34 (37.8)	17 (18.9)	39 (43.3)	
Female	35	15 (42.9)	5 (14.2)	15 (42.9)	
Tumor differentiation					0.0652
Well	29	15 (51.7)	5 (17.2)	9 (31.1)	
Moderately	72	26 (36.1)	16 (22.2)	30 (41.7)	
Poorly	24	8 (33.3)	1 (4.2)	15 (62.5)	
Tumor differentiation					0.0337
Well + moderately	101	41 (40.6)	21 (20.8)	39 (38.6)	
Poorly	24	8 (33.3)	1 (4.2)	15 (62.5)	
Lymph Node Metastasis[2]					0.1839
Present	42	14 (33.3)	9 (21.4)	19 (45.2)	
Not present	36	18 (50.0)	7 (19.4)	11 (30.6)	

[1]The strong expression of HNRNPH1 was compared with negative and weak expression; [2]Only 78 cases had information on lymph node metastasis. HNRNPH1: Heterogeneous nuclear ribonucleoprotein H1; ESCC: Esophageal squamous cell carcinoma.

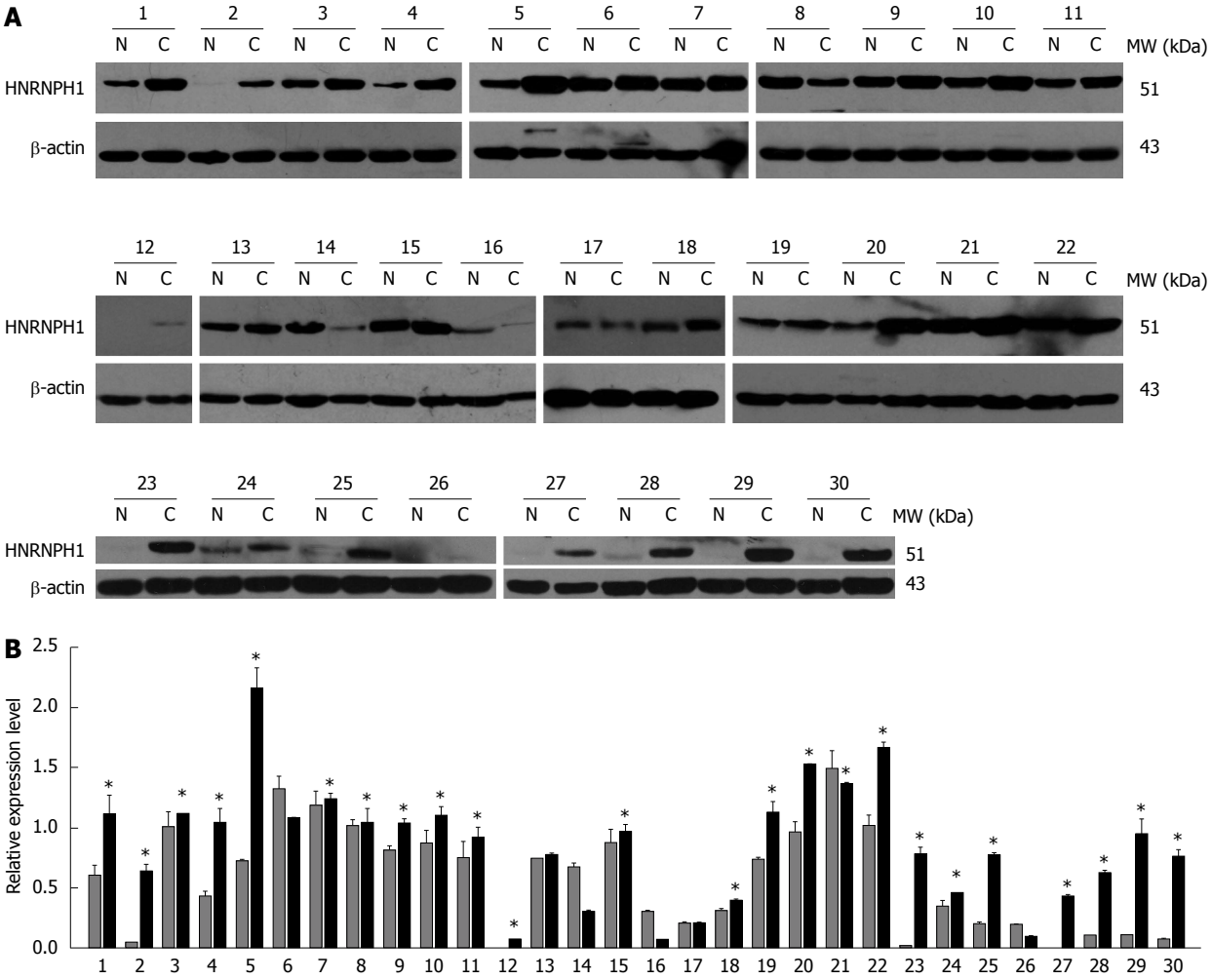

Figure 4 Western blotting analysis of heterogeneous nuclear ribonucleoprotein H1 in paired esophageal squamous cell carcinoma specimens. A: Representative western blotting images of tumor (C) and matched adjacent non-tumor esophageal mucosal tissues (N) from 30 patients with ESCC. β-actin protein levels are shown as a loading control. The patients were coded from 1 to 30. B: Densitometric analysis of 30 ESCC cases. The gray and black bars represent the relative band intensity of HNRNPH1 in non-tumor (N) or tumor (C) tissues. Each data point represents the mean ± SD derived from three independent experiments. The asterisks mark the cases that overexpressed HNRNPH1 in tumor tissues. HNRNPH1: Heterogeneous nuclear ribonucleoprotein H1.

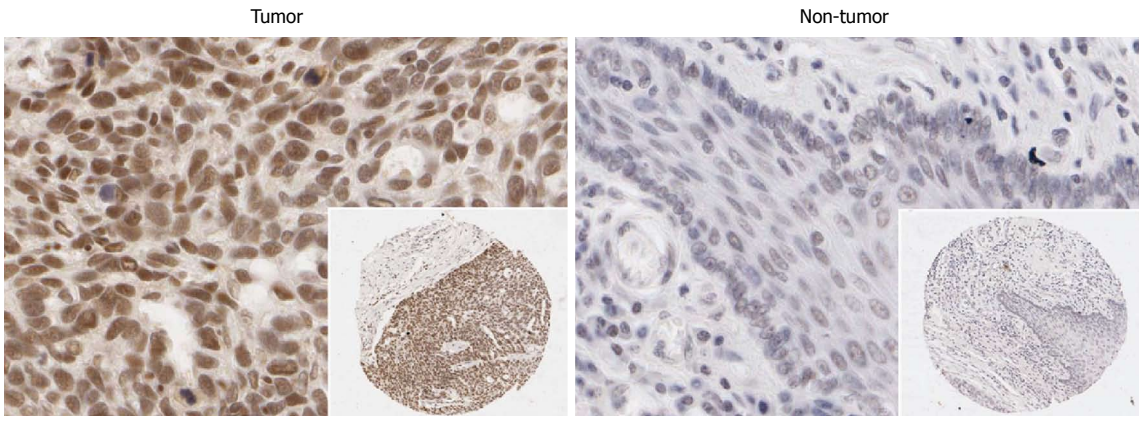

Figure 5 Representative immunohistochemistry staining of heterogeneous nuclear ribonucleoprotein H1 in the tumor and non-tumor tissues from a esophageal squamous cell carcinoma patient. HNRNPH1 was mainly localized to the nuclei. The images are shown at high magnification (× 400), and the lower right panels are magnification × 40. HNRNPH1: Heterogeneous nuclear ribonucleoprotein H1; ESCC: Esophageal squamous cell carcinoma.

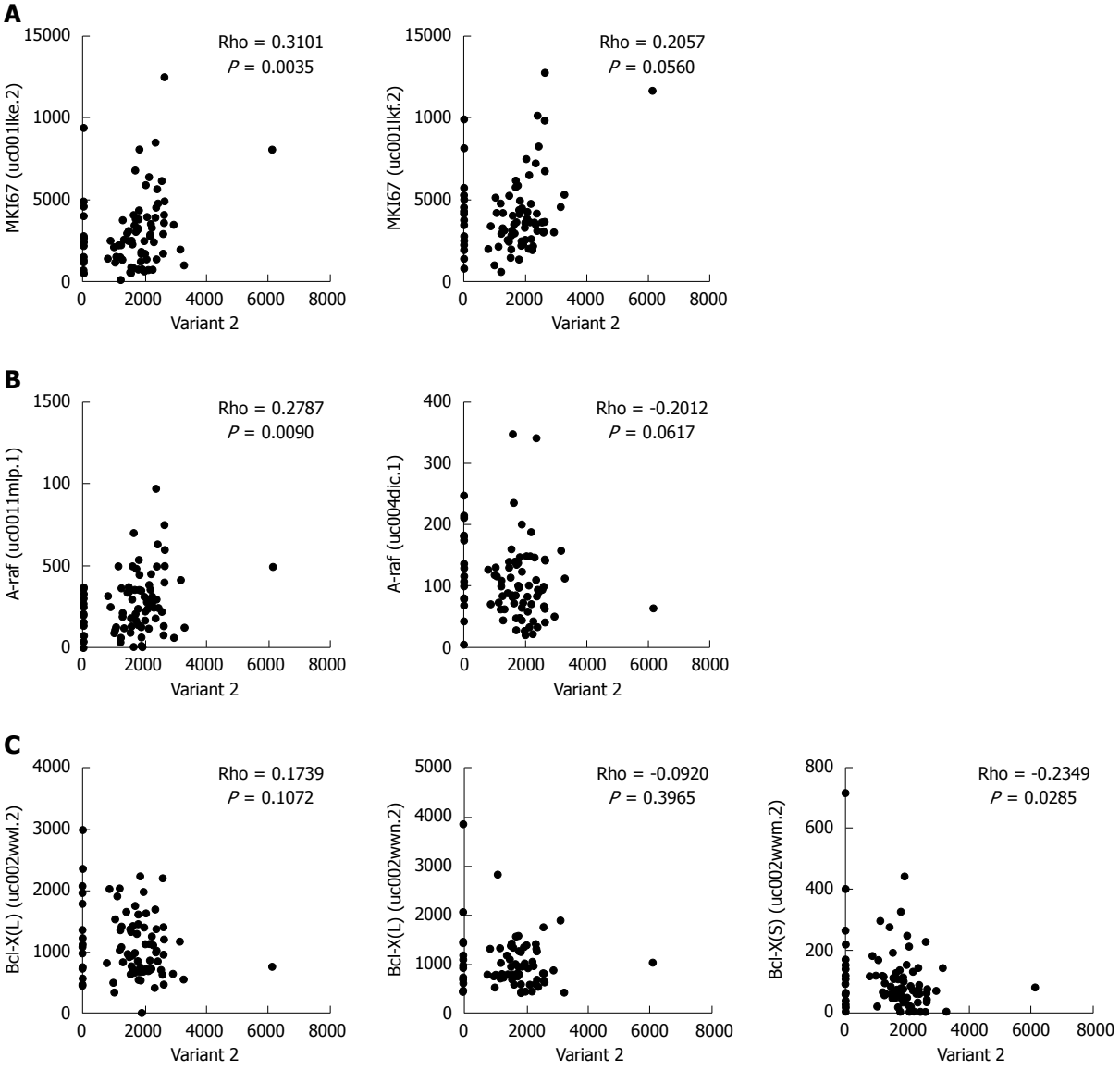

Figure 6 Expression levels of transcript variant 2 of heterogeneous nuclear ribonucleoprotein H1 were associated with the alternative splicing of proliferation- and apoptosis-related genes in esophageal squamous cell carcinoma. A: The correlation analysis between variant 2 and the two transcripts of MKI67 gene; B: The correlation analysis between variant 2 and the A-raf gene; C: The correlation analysis between variant 2 and the Bcl-X gene. HNRNPH1: Heterogeneous nuclear ribonucleoprotein H1; ESCC: Esophageal squamous cell carcinoma.

cytoplasm[22], and strong cytoplasmic staining was observed in some cases of pancreatic, rectal, liver, gastric, and lung cancer[19]. In head and neck cancer, however, HNRNPH1 was only overexpressed in the nuclei[18]. Our results showed that HNRNPH1 was restricted to the nucleus in ESCC. Therefore, the intracellular localization of HNRNPH1 may be tissue or cell type-dependent.

HNRNPH1 is an evolutionarily conserved splicing factor, and it plays a dual role in the activation and inhibition of pre-mRNA processing, polyadenylation, mRNA export, and translation. It can act as a component in the intronic splicing enhancer complex to stimulate gene splicing, including c-src, MAP kinase activating death domain (MADD), and macrophage stimulating 1 receptor (MST1R)[13,17]. In addition, it can be recruited to the exonic splicing silencer to regulate alternative splicing of tropomyosin, collagen-like tail subunit (COLQ), muscle nicotinic acetylcholine receptor alpha subunit, and fibroblast growth factor receptor 2[11,12,16,23,24]. HNRNPH1 can cooperate with other hnRNP proteins to stimulate polyadenylation through a direct interaction with poly (A) polymerase[25]. Moreover, it has been shown to bind some mRNAs to inhibit their nuclear export[26]. In amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD), the RNA foci that result from GGGGCC (G4C2) intronic repeat expansion within C9ORF72 sequester HNRNPH1 and other RNA binding proteins, leading to neurotoxicity[27]. In view of these functions, aberrant expression of HNRNPH1 will alter cell phenotypes. Apoptosis was significantly activated when HNRNPH1 was depleted. Experiments showed that inhibition of HNRNPH1 induced apoptosis by activation of caspase-3,

poly(ADP-ribose)polymerase (PARP) cleavage, and 10-fold increases in DNA fragmentation, whereas its transient overexpression was protective against etoposide-induced apoptosis[18]. The anti-apoptotic role of HNRNPH1 may result from its substrates, A-raf and Bcl-X[18,28]. Bcl-X encodes two isoforms with different functions. The longer isoform Bcl-X (L), which is translated from transcripts uc002wwl.2 and uc002wwn.2, acts as an apoptotic inhibitor, whereas the shorter form Bcl-X (S), which is translated from transcript uc002wwm.2, acts as an apoptotic activator. It also maintains p53 pre-mRNA 3'-end processing to contribute to p53-mediated apoptosis[15]. In this study, we observed that the mRNAs and proteins of HNRNPH1 are all increased in ESCC. These increases in HNRNPH1 levels promote cell proliferation and inhibit apoptosis partially *via* upregulating the pro-proliferative and anti-apoptotic transcripts of MKI67 and A-raf and restraining the pro-apoptotic transcripts of Bcl-X. These findings indicate that it may enhance the chemoresistance of ESCC cells.

In conclusion, our results demonstrated that there are two transcript variants of HNRNPH1. One is constitutively expressed, and the other is regulated. The regulated mRNA variant led to the overexpression of HNRNPH1 protein in ESCC. Its expression was restricted to the nucleus and was associated with poorer differentiation of tumor cells. Our study is the first to investigate the aberrant expression of HNRNPH1 in ESCC and highlights the potential of HNRNPH1 in the therapy and diagnosis of ESCC.

COMMENTS

Background

Esophageal squamous cell carcinoma (ESCC) is the third most commonly diagnosed cancer and the fourth leading cause of cancer death in China. Currently, thousands of gene alternations have been found in ESCC. One of these genes encodes heterogeneous nuclear ribonucleoprotein H1 (HNRNPH1), an evolutionarily conserved splicing factor that is involved in alternative splicing, polyadenylation, mRNA export, and translation.

Research frontiers

Previous proteomic studies have found that HNRNPH1 was upregulated approximately 8.4-fold in ESCC. However, data regarding the association between HNRNPH1 and cancer are still very limited.

Innovations and breakthroughs

This study is the first to investigate the expression, localization, and clinical significance of HNRNPH1 in cell lines and clinical specimens of ESCC using RNA sequencing dataset, western blotting, immunofluorescence, and immunohistochemistry staining.

Applications

There are two alternative transcript variants of HNRNPH1; one was constitutively expressed, while the other was regulated and dramatically increased in ESCC. HNRNPH1 protein was also overexpressed in ESCC tissues. Strong HNRNPH1 levels were significantly associated with poorer tumor differentiation, suggesting that it may be a potential diagnostic biomarker and therapeutic target in ESCC.

Terminology

Heterogeneous nuclear ribonucleoproteins (hnRNPs) play critical roles in precursor mRNA splicing, alternative splicing, gene expression, mRNA export, localization, translation, and stability regulation. In humans, the hnRNP family consists of at least 20 abundant, major hnRNP proteins and other less abundant, minor hnRNP proteins. HNRNPH1 is an ubiquitously expressed major hnRNP protein thatbinds only to the poly (rG) sequence.

Peer-review

This manuscript clearly demonstrated that HNRNPH1 is overexpressed in human ESCC and is associated with poor differentiation. Overexpression of HNRNPH1 was associated with alternative splicing of some proliferation and apoptosis related genes. Overall, the manuscript is well organized, and the data are convincing. However, the functional consequences of HNRNPH1 overexpression in human ESCC still need further exploration.

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ORIGINAL ARTICLE

Basic Study

Effects of sleeve gastrectomy with jejuno-jejunal or jejuno-ileal loop on glycolipid metabolism in diabetic rats

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Abstract

AIM

To explore the effect of sleeve gastrectomy (SG) with jejuno-jejunal or jejuno-ileal loop on glycolipid metabolism in diabetic rats.

METHODS

Diabetic rats, which were induced by high-fat diet (HFD), nicotinamide and low-dose streptozotocin, underwent sham operations, SG, SG with jejuno-ileal loop (SG-JI) and SG with jejuno-jejunal loop (SG-JJ) followed by postoperative HFD. Then, at the time points of baseline and 2, 12 and 24 wk postoperatively, we determined and compared several variables, including the area under the curve for the results of oral glucose tolerance test (AUC_{OGTT}), serum levels of triglyceride, cholesterol and ghrelin in fasting state, homeostasis model assessment of insulin resistance (HOMA-IR), body weight, calorie intake, glucagon-like peptide (GLP)-1 and insulin secretions after glucose gavage at dose of 1 g/kg.

RESULTS

At 2 wk postoperatively, rats that underwent SG, SG-JJ and SG-JI, compared with sham-operated (SHAM)

rats, demonstrated lower body weight, calorie intake and ghrelin ($P < 0.05$ *vs* SHAM), enhanced secretion of insulin and GLP-1 after glucose gavage ($P < 0.05$ *vs* SHAM), improved AUC_{OGTT}, HOMA-IR, fasting serum triglyceride and cholesterol (AUC_{OGTT}: 1616.9 ± 83.2, 837.4 ± 83.7, 874.9 ± 97.2 and 812.6 ± 81.9, $P < 0.05$ *vs* SHAM; HOMA-IR: 4.31 ± 0.54, 2.94 ± 0.22, 3.17 ± 0.37 and 3.41 ± 0.22, $P < 0.05$ *vs* SHAM; Triglyceride: 2.35 ± 0.17, 1.87 ± 0.23, 1.98 ± 0.30 and 2.04 ± 0.21 mmol/L, $P < 0.05$ *vs* SHAM; Cholesterol: 1.84 ± 0.21, 1.53 ± 0.20, 1.52 ± 0.20 and 1.46 ± 0.23 mmol/L). At 12 wk postoperatively, rats receiving SG-JJ and SG-JI had lower body weight, reduced levels of triglyceride and cholesterol and elevated level of GLP-1 compared to those receiving SG ($P < 0.05$ *vs* SG). At 24 wk after surgery, compared with SG, the advantage of SG-JJ and SG-JI for glucolipid metabolism was still evident ($P < 0.05$ *vs* SG). SG-JI had a better performance in lipid metabolism and GLP-1 secretion of rats than did SG-JJ.

CONCLUSION

SG combined with intestinal loop induces better glycolipid metabolism than simple SG, with the lipid metabolism being more improved with SG-JI compared to SG-JJ.

Key words: Sleeve gastrectomy; Jejuno-jejunal loop; Jejuno-ileal loop; Diabetes; Glucolipid metabolism

Core tip: To improve the effect of sleeve gastrectomy (SG), surgeons sporadically use different intestinal loops; however, these innovative surgical procedures lack a theoretical foundation. We explored the effect of SG with jejuno-jejunal loop (SG-JJ) or jejuno-ileal loop (SG-JI) on glycolipid metabolism in diabetic rats. We discovered that SG-JJ and SG-JI were superior to SG in improving glycolipid metabolism. Compared with SG-JJ, the improvement in lipid metabolism after SG-JI was more apparent. These findings might help surgeons select procedures for individual patients.

Zhong MW, Liu SZ, Zhang GY, Zhang X, Hu SY. Effects of sleeve gastrectomy with jejuno-jejunal or jejuno-ileal loop on glycolipid metabolism in diabetic rats. *World J Gastroenterol* 2016; 22(32): 7332-7341 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i32/7332.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i32.7332

INTRODUCTION

Recently, international diabetes organizations made a joint statement in *Diabetes Care*[1]. This statement indicated that patients with type 2 diabetes mellitus (T2DM) with class Ⅲ [body mass index (BMI) ≥ 40 kg/m^2] and class Ⅱ (BMI 35.0-39.9 kg/m^2) obesity as well as those with class Ⅰ obesity (BMI 30.0-34.9 kg/m^2) should consider bariatric/metabolic surgery, if T2DM is inadequately controlled despite optimal treatment with oral or injectable medication. During the past decade, bariatric surgery, especially sleeve gastrectomy (SG), has been increasingly performed in patients. Currently, the most commonly performed procedure in patients of the United States, Canada and the Asia/Pacific region is SG, which outnumbers Roux-en-Y gastric bypass (RYGB)[2]. As a novel metabolic surgery, SG is characterized by a lower complication rate, faster operation, fewer technical requirements, and fewer postoperative nutritional problems[3], and some researchers have reported that SG and RYGB have equal efficacy for diabetes[4,5]. However, long-term randomized controlled comparison of the effects of SG and RYGB in patients with T2DM is surprisingly limited. Other researchers doubt the long-term effect of SG on diabetes patients[3]. In addition, SG has been reported as an independent predictor for the recurrence of diabetes[6].

Surgeons have designed many additional surgical procedures for SG to improve excessive body weight loss and diabetes control, such as SG with duodeno-jejunal bypass[7], loop gastroileostomy[8] jejuno-ileal bypass[9] and duodeno-ileal bypass[10]. Although these surgical procedures showed some improvement with regard to excessive body weight loss and diabetes control, they were performed with resection of different segments of small intestine or different intestinal loops. The procedures were not compared in randomized controlled trials (RCTs) and were limited to clinical retrospective studies. In the present study, we conducted a RCT in a rat model to compare the effect of SG with different intestinal loops on diabetes control.

Several factors of the diabetes postoperative recurrence have been confirmed, including preoperative BMI, patient age, T2DM course and severity, excessive weight loss, body weight regain, and postoperative diet and lifestyle[6,11,12]. Dietary control seems more important for patients with SG[13], and in our previous study, we demonstrated that the improvement in glucose metabolism after metabolic surgery can be reversed by postoperative high-fat diet (HFD) in rats[14]. In this present study, we used rats with postoperative HFD to simulate patients with an undesirable diet. Diabetic rats were treated with sham operation (SHAM), SG, SG with jejuno-jejunal loop (SG-JJ) or SG with jejuno-ileal loop (SG-JI). At the stated time points, we determined and compared the glucose and lipid metabolic profiles, serum parameters including insulin, glucagon-like peptide (GLP)-1 and ghrelin.

MATERIALS AND METHODS

Animals

Under the conditions of constant temperature at 24 to 26 ℃, humidity at 50% to 60% and light/dark

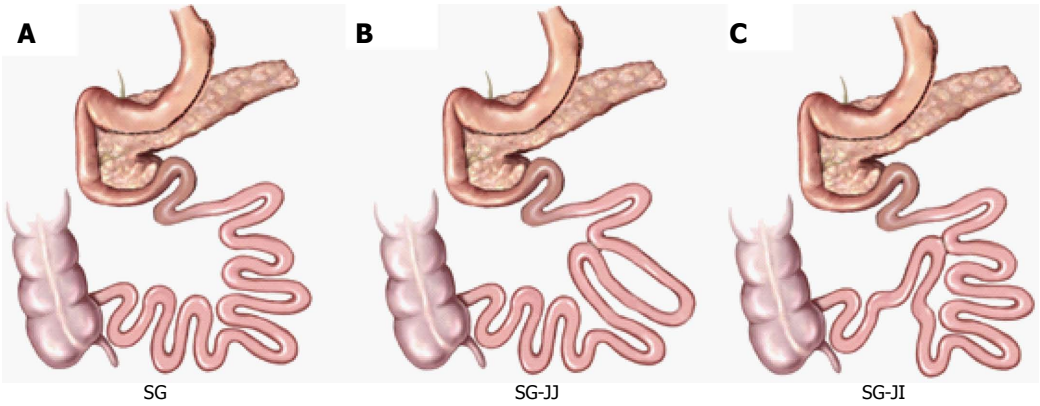

Figure 1 Sleeve gastrectomy (A), sleeve gastrectomy with jejuno-jejunal loop (B) and sleeve gastrectomy with jejuno-ileal loop (C). Sleeve gastrectomy (SG): The lesser curvature of the stomach is preserved and 70%-80% of the stomach is removed from the greater curvature; SG with jejuno-jejunal loop (SG-JJ): Based on SG surgery, a jejuno-jejunal side-by-side anastomosis was made between the jejunum 15 cm distal to the ligament of Treitz and 35 cm distal to the ligament of Treitz; SG with jejuno-ileal loop (SG-JI): Based on SG surgery, a jejuno-ileal side-by-side anastomosis was made between the jejunum 15 cm distal to the ligament of Treitz and the distal ileum 20 cm proximal to the ileocecal valve.

alternation at 12 h interval, 70 8-wk-old Wistar rats, provided by Laboratory Animal Center of Shandong University, were separately housed in independently ventilated cages. All the rats received 1-wk adaptive feeding, followed by 4-wk HFD (Huafukang Biotech Company, China), which contains 40% fat as calories, to induce insulin resistance. A 12-h fasting period was succeeded by a single intraperitoneal injection with nicotinamide at the dose of 170 mg/kg. After 15 min, streptozotocin (Sigma-Aldrich, St Louis, MO, United States), at the dose of 65 mg/kg, was administrated to the rats by injection, so that they could reach a diabetic state[15]. Two weeks later, 39 rats met the criteria for the diabetic state, which included fasting blood glucose level of more than or equal to 7.1 mmol/L or the 2-h blood glucose level of more than or equal to 11.1 mmol/L during oral glucose tolerance test (OGTT). We excluded the rats with extreme hyperglycemia (blood glucose level of more than 16.7 mmol/L)[16]. All animal experimental procedures involved in our study had been approved by the Animal Care and Utilization Committee of Qilu Hospital of Shandong University, Jinan, China.

Experimental schedule

We fed the diabetic rats with low-residue diet for 48 h, then performed SHAM (SHAM group, n = 10), SG (SG group, n = 10), SG-JJ (SG-JJ group, n = 10) and SG-JI (SG-JI group, n = 9) on them under anesthesia with 10% chloral hydrate solution. All rats were continuously provided with HFD, followed by 72 h low-residue diet feeding post-operation.

At the time points of baseline, 2, 12 and 24 wk postoperatively, we measured several variables including the results of OGTT, homeostasis model assessment of insulin resistance (HOMA-IR), levels of triglyceride, cholesterol and ghrelin in fasting serum, body weight, calorie intake and secretion of GLP-1 and insulin after gavage (1 g/kg).

Surgical procedures

The surgical procedures were performed as described in our and other previous studies[17,18] (Figure 1).

SG surgery: We made a 4-cm vertical midline abdominal incision along with abdominal midline from the xiphoid process. After locating the greater curvature, we freed it from the gastric cardium to the pylorus with ligating and transecting relative gastric vessels. The glandular stomach and most of the gastric body were removed (70% of total stomach). Residual stomach was closed using 7-0 silk sutures, and replaced in the abdominal cavity. Then, a 5-0 silk suture (Ningbo Medical Needle) was applied for the closure of the incision.

SG-JJ surgery: On the basis of SG surgery, we located jejunum 15 cm and 35 cm distal to the ligament of Treitz respectively and connected them to form a jejuno-jejunal side-by-side anastomosis.

SG-JI surgery: On the basis of SG surgery, we divided jejunum 15 cm distal to the ligament of Treitz, and connected it to the distal ileum 20 cm proximal to the ileocecal valve to form a jejuno-ileal side-by-side anastomosis.

SHAM surgery: The incision and procedure were similar to the performance of SG except that we kept the glandular stomach and most of the gastric body. Same durations of operation were maintained to ensure similar stress from the surgery and anesthesia.

OGTT

Upon completion of 8-h fasting, all rats received 1 g/kg glucose by oral gavage. Then, we estimated the levels of blood glucose at five time points (baseline, 10, 30, 60 and 120 min after gavage) respectively. The glucometer used was the Roche One Touch Ultra

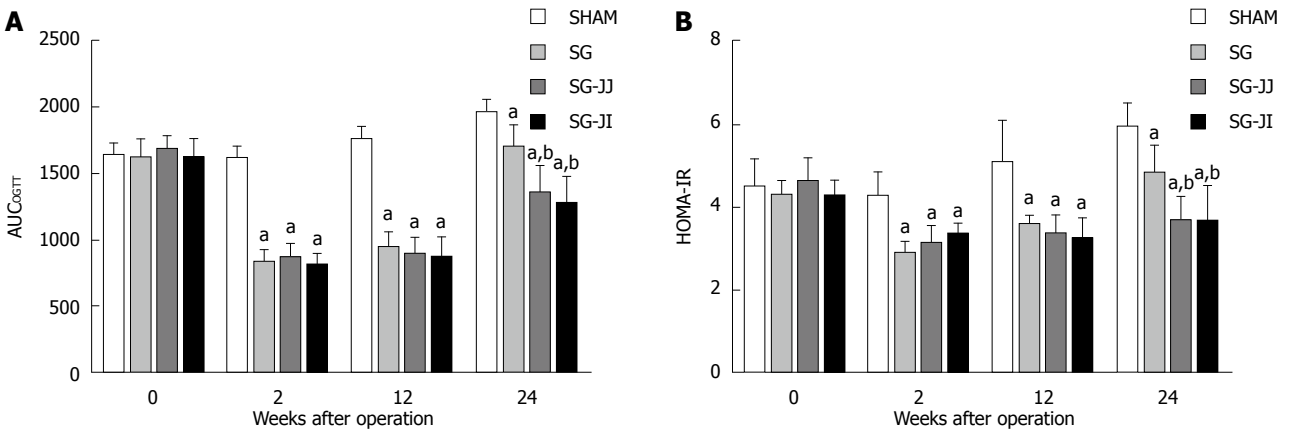

Figure 2 Area under the curve for the results of oral glucose tolerance test (A) and homeostasis model assessment of insulin resistance (B). Area under the curve for the results of oral glucose tolerance test (AUC_{OGTT}) and homeostasis model assessment of insulin resistance (HOMA-IR) were determined at baseline, and 2, 12 and 24 wk after surgery, and were analyzed by one-way ANOVA followed by Bonferroni *post hoc* comparison ($^{a}P < 0.05$ *vs* SHAM; $^{b}P < 0.05$ *vs* SG). SHAM: Sham-operated; SG: Sleeve gastrectomy; SG-JJ: SG with jejuno-jejunal loop; SG-JI: SG with jejuno-ileal loop.

(Lifescan, Milpitas, CA, United States).

Serum parameters

With rats under anesthesia by diethyl ether, we respectively gathered blood samples from retrobulbar venous plexus at time points of baseline, 15, 30, 60 and 120 min after gavage with glucose, similar to OGTT. Serum was collected by centrifugation (1006 × *g*, 4 ℃, 15 min) and stored at -80 ℃ for further measurement. Levels of triglyceride and cholesterol in fasting serum were detected by the Hitachi automatic biochemical analyzer (Japan). Concentrations of insulin, GLP-1 and ghrelin in serum were tested by enzyme-linked immunosorbent assay (ELISA) kits (insulin: Millipore, Billerica, MA, United States; GLP-1 and ghrelin: Uscn Life Science, Wuhan, China).

HOMA-IR

We conducted the calculations of HOMA-IR, according to the levels of insulin and blood glucose in fasting serum, by the following formula: HOMA-IR = fasting insulin (mIU/L) × fasting blood glucose (mmol/L)/22.5[19].

Statistical analysis

All quantitative data are presented as mean ± SD. By the use of trapezoidal integration, we calculated the area under the curves for OGTT (AUC_{OGTT}). We analyzed data of different groups, including the AUC_{OGTT}, HOMA-IR values, triglyceride, cholesterol and ghrelin serum levels, by means of one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* comparison. A mixed model ANOVA followed by Bonferroni *post hoc* comparison analysis was used in insulin and GLP-1 secretions in glucose-gavage rats. All statistical calculations were processed with the use of SPSS version 19.0 (IBM, Armonk, NY, United States), at an alpha level of 0.05.

RESULTS

Ten, nine, eight and nine rats survived in the SHAM, SG, SG-JJ and SG-JI groups, respectively. Three rats died of residual stomach leakage.

OGTT

As shown in Figure 2A, in the early postoperative period (2 wk after surgery), rats in the SG (837.4 ± 83.7), SG-JJ (874.9 ± 97.2) and SG-JI (812.6 ± 81.9) groups showed lower AUC_{OGTT} than the SHAM group (1616.9 ± 83.2, $P < 0.05$), and the lower AUC_{OGTT} was sustained until the end of the study (SG: 1696.6 ± 155.5; SG-JJ: 1343.9 ± 217.3; SG-JI: 1275.9 ± 194.3; SHAM: 1965.9 ± 81.6; $P < 0.05$). At 24 wk after surgery, the AUC_{OGTT} in the SG-JJ and SG-JI groups was lower than that in the SG group ($P < 0.05$), and no difference in AUC_{OGTT} was observed between the SG-JJ and SG-JI groups at any time point during this study.

HOMA-IR

Rats that underwent SHAM had higher HOMA-IR than rats of other metabolic surgery types at every postoperative time point ($P < 0.05$). At 24 wk postoperatively, the HOMA-IR in the SG-JJ (3.72 ± 0.54) and SG-JI (3.73 ± 0.79) groups was comparable, and it was lower than that in the SG group (4.86 ± 0.62, $P < 0.05$) (Figure 2B).

Lipid profiles

Lipid profiles showed a similar trend to AUC_{OGTT}. At 12 wk postoperatively, rats of the SG group had significantly higher fasting triglyceride levels than those of the SG-JJ (2.27 ± 0.21 mmol/L *vs* 1.59 ± 0.17 mmol/L) and SG-JI (2.27 ± 0.21 mmol/L *vs* 1.49 ± 0.25 mmol/L, $P < 0.05$) groups (Figure 3A). The same

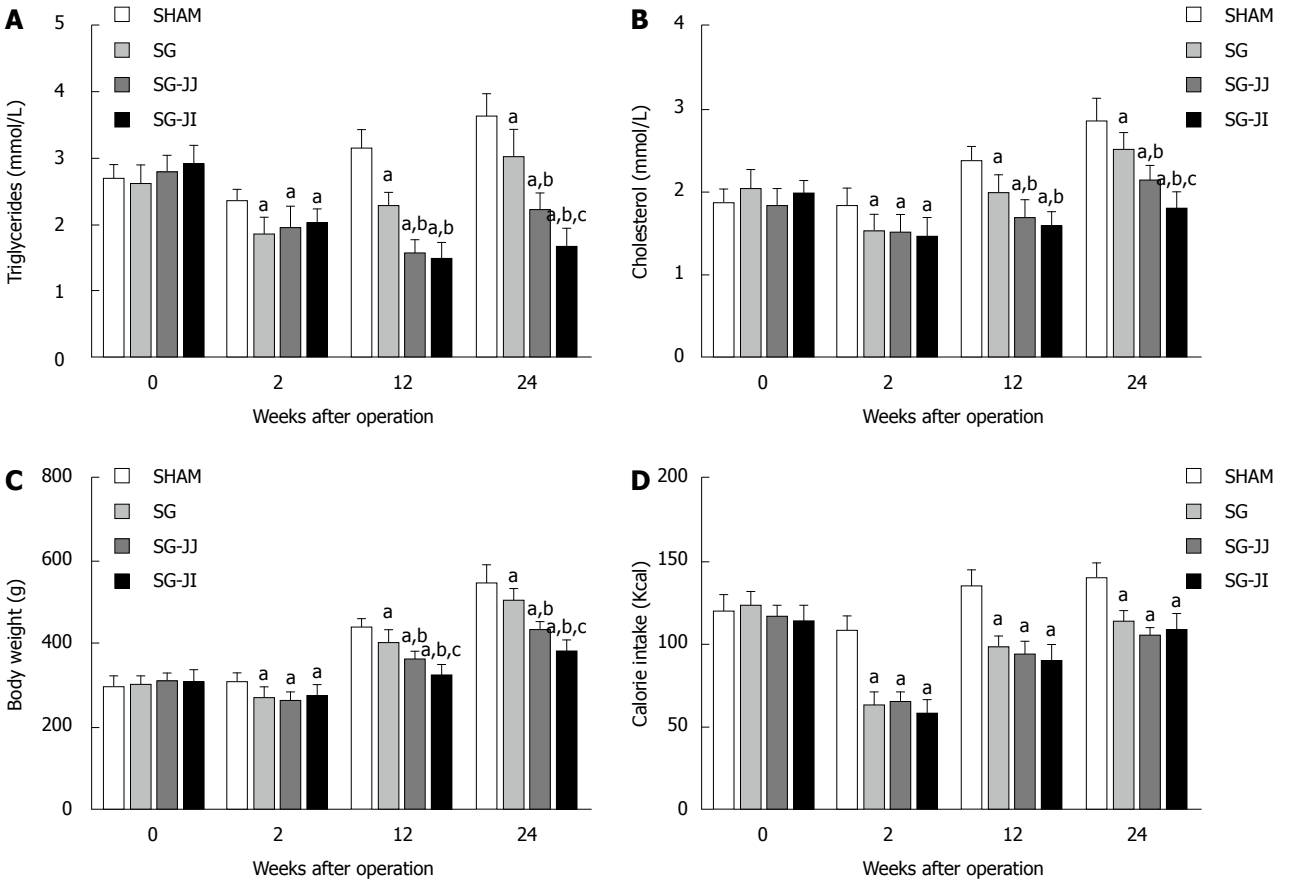

Figure 3 Fasting serum triglyceride (A), cholesterol (B), body weight (C) and calorie intake (D). Analysis was carried out by one-way ANOVA followed by Bonferroni *post hoc* comparison at baseline, and 2, 12 and 24 wk after surgery ($^{a}P < 0.05$ *vs* SHAM; $^{b}P < 0.05$ *vs* SG; $^{c}P < 0.05$ *vs* SG-JJ). SHAM: Sham-operated; SG: Sleeve gastrectomy; SG-JJ: SG with jejuno-jejunal loop; SG-JI: SG with jejuno-ileal loop.

trend was also observed in fasting cholesterol levels among the SG (1.98 ± 0.22 mmol/L), SG-JJ (1.70 ± 0.20 mmol/L) and SG-JI (1.58 ± 0.18 mmol/L, $P <$ 0.05) groups (Figure 3B). Fasting triglyceride levels of the SG-JI group, at 24 wk postoperatively, were lower than in the SG-JJ group (1.68 ± 0.24 mmol/L *vs* 2.22 ± 0.25 mmol/L) (Figure 3A), which showed the same trend in fasting cholesterol levels of the SG-JI group compared with the SG-JJ group (1.81 ± 0.19 mmol/L *vs* 2.14 ± 0.17 mmol/L, $P < 0.05$) (Figure 3B).

Body weight and calorie intake

At every postoperative time point, compared with the SHAM group, the rats that underwent SG, SG-JJ and SG-JI had lower body weight (Figure 3C) and calorie intake (Figure 3D) ($P < 0.05$). The differences of 12-wk postoperative body weight were statistically significant in the SG group, compared with the SG-JJ group (403.8 ± 31.0 g *vs* 366.1 ± 14.2 g, $P <$ 0.05) and with the SG-JI group (403.8 ± 31.0 g *vs* 326.2 ± 24.8 g, $P < 0.05$), and the differences were also observed at 24 wk after surgery. No differences, however, were seen for calorie intake among the SG, SG-JJ and SG-JI groups.

Serum insulin

Insulin secretion after gavage at baseline, and 2, 12 and 24 wk after surgery is described in Figure 4A-D, respectively. Secretion of insulin after gavage in the SG, SG-JJ and SG-JI groups was higher than that in the SHAM group at any time postoperatively ($P < 0.05$). At 24 wk after surgery, compared with the SG group, rats had higher insulin secretion in the SG-JJ ($P < 0.05$) and SG-JI groups ($P < 0.05$). No statistical difference was seen for insulin secretion between the SG-JJ and SG-JI groups.

GLP-1

Serum GLP-1 at baseline, and 2, 12 and 24 wk after surgery is shown in Figure 5A-D, respectively. There was no difference in GLP-1 secretion at baseline among the four groups. At baseline, rats that underwent different surgeries had similar GLP-1 secretions, which were higher in the SG ($P < 0.05$), SG-JJ ($P < 0.05$) and SG-JI ($P < 0.05$) groups compared with the SHAM group at 2 wk postoperatively. Then, at 12 wk postoperatively, rats in the SG group secreted less GLP-1 than those in the SG-JJ ($P < 0.05$) and SG-JI groups ($P < 0.05$). Rats in the SG-JJ group had lower GLP-1 secretion than those

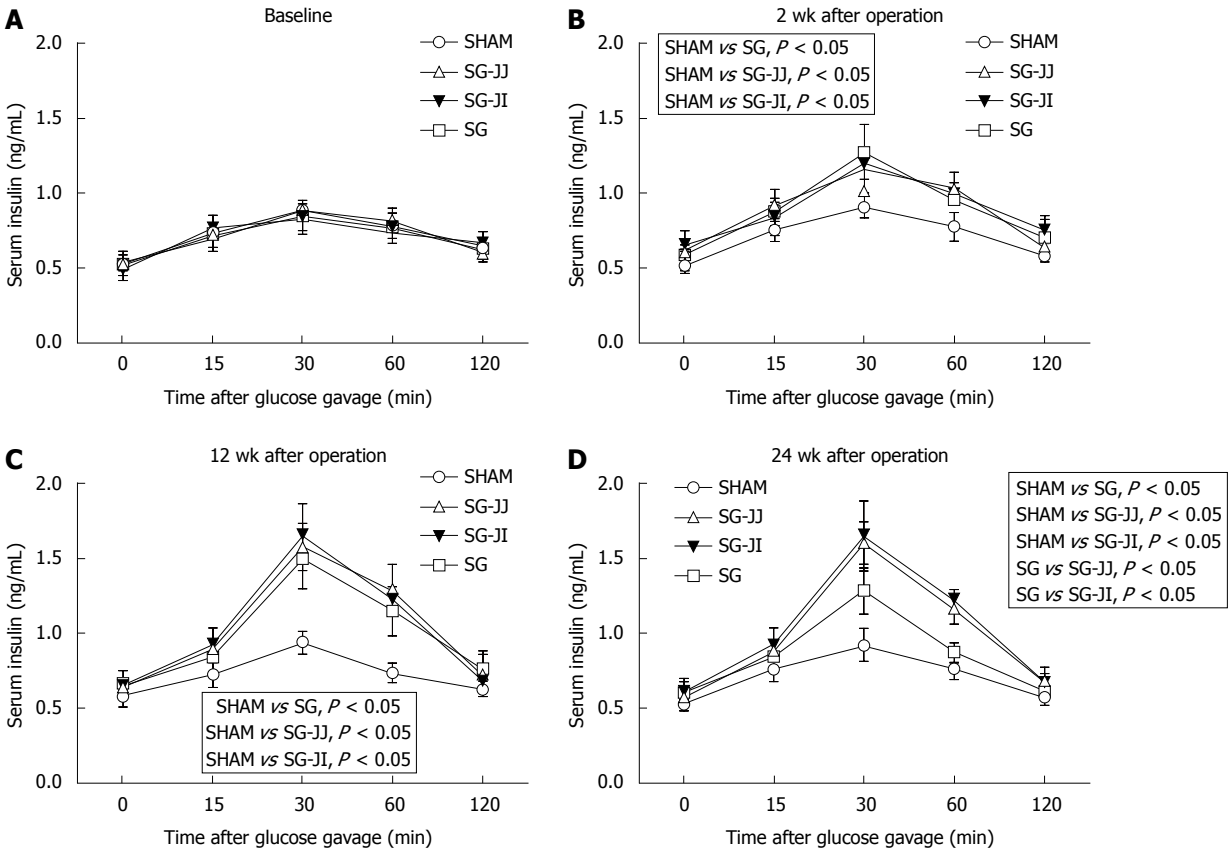

Figure 4 Insulin secretion curves after gavage at baseline (A), and 2 wk (B), 12 wk (C) and 24 wk (D) after surgery. Analysis was carried out by mixed model ANOVA followed by Bonferroni *post hoc* comparison. The *P* value is shown in the rectangular frame. $P < 0.05$ was considered statistically significant. SHAM: Sham-operated; SG: Sleeve gastrectomy; SG-JJ: SG with jejuno-jejunal loop; SG-JI: SG with jejuno-ileal loop.

in the SG-JI group ($P < 0.05$) at 24 wk postoperatively.

Fasting serum ghrelin

No significant differences were seen for fasting serum ghrelin at baseline in any of the rats (Figure 6) or for any other time points for the SG, SG-JJ and SG-JI groups. The SG, SG-JJ and SG-JI groups showed lower fasting serum ghrelin than those in the SHAM group at all postoperative times ($P < 0.05$).

DISCUSSION

Diabetes commonly threatens human health, with an estimated 422 million cases worldwide, according to the data reported by the World Health Organization in 2014; moreover, the prevalence of diabetes shows an increasing growth rate in countries with low-middle incomes[20].

Metabolic surgery has become increasingly common worldwide because of its efficacy in improving management of diabetes, especially for cases inadequately controlled by optimal treatment[1]. Metabolic surgery is classified as restrictive, malabsorptive and mixed operation. SG used to be considered as a pure restrictive operation, but in present thought is more than that. Compared with other restrictive techniques (*e.g.,* adjustable gastric banding), performance of SG can achieve more excellent efficacy, producing more efficient gastric emptying and intestinal transit as well as higher GLP-1 levels and lower ghrelin levels, similar to RYGB[21]. Hence, SG is the most popular method of metabolic surgery in the United States/Canada and Asia/Pacific regions, in addition to its advantages of faster operation, fewer technical requirements, lower complication rate, and fewer postoperative nutritional problems[2,3]. SG, as a novel surgery approach, has unclear long-term effect on diabetes, with a reported inadequate remission rate compared with RYGB[6]. In previous clinical study, single-anastomosis duodeno-jejunal bypass with SG[7], gastroileostomy loop with SG[8], jejuno-ileal bypass with SG[9] and duodeno-ileal bypass with SG[10] were performed, with the aim to achieve improvement in controlling diabetes of SG. These studies demonstrated that SG with different intestinal bypass showed excellent antidiabetic effects. However, there have been limited prospective RCTs. In this study, we performed a RCT to compare the effect of SG, SG-JJ and SG-JI on diabetes control, and showed that all of the three surgeries have excellent antidiabetic effects shortly after surgery. What's more, this study demonstrated that SG-JJ and SG-JI could provide better glycolipid metabolism than SG, despite a maintained HFD chow administered until 24 wk after surgery.

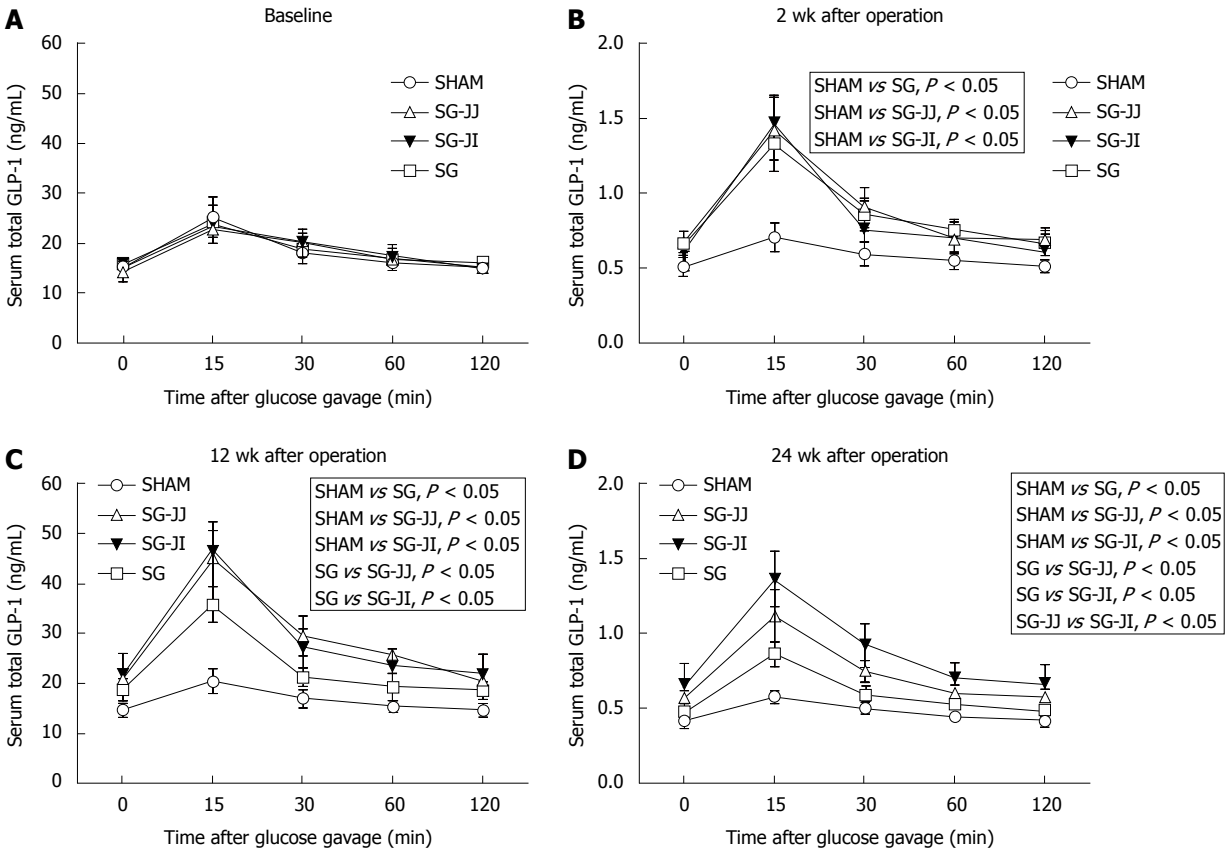

Figure 5 Glucagon-like peptide-1 secretion curves after gavage at baseline (A), 2 wk (B), 12 wk (C) and 24 wk (D) after surgery. Analysis was carried out by mixed model ANOVA followed by Bonferroni *post hoc* comparison. The *P* value is shown in the rectangular frame. $P < 0.05$ was considered statistically significant. GLP-1: Glucagon-like peptide-1; SHAM: Sham-operated; SG: Sleeve gastrectomy; SG-JJ: SG with jejuno-jejunal loop; SG-JI: SG with jejuno-ileal loop.

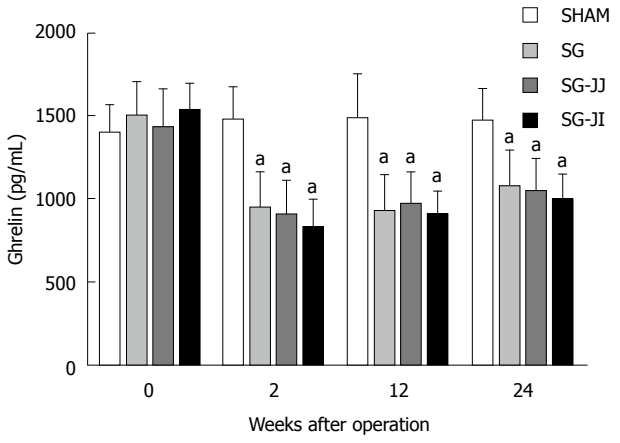

Figure 6 Fasting serum ghrelin at baseline, and 2, 12 and 24 wk after surgery. Analysis was carried out by one-way ANOVA followed by Bonferroni *post hoc* comparison. Sleeve gastrectomy (SG), SG with jejuno-jejunal loop (SG-JJ) and SG with jejuno-ileal loop (SG-JI) groups showed lower fasting serum ghrelin at all postoperative time points, and no difference in fasting serum ghrelin among the three groups was discovered during this study ($^aP < 0.05$ *vs* SHAM). SHAM: Sham-operated group.

Excellent postoperative management contributes much to the antidiabetic effect of metabolic surgery, suggesting that good postoperative diet control is necessary for SG[22]. In this study, we used post-operative HFD to simulate undesirable dietary control. Although antidiabetic activity decreased over time, the rats with SG still manifested better diabetes control than the SHAM rats. Compared with SG, SG-JJ and SG-JI did not show an improved antidiabetic effect at 12 wk after surgery. Nevertheless, an improved antidiabetic effect of SG-JJ and SG-JI was observed at 24 wk after surgery. That finding demonstrates that SG-JJ and SG-JI can enhance improvement in glucose metabolism after SG. We did not observe any difference in AUC_{OGTT} between the SG-JJ and SG-JI groups.

Lipid metabolism demonstrated a similar trend to glucose metabolism. SG-JI and SG-JJ rats showed lower fasting triglyceride and cholesterol level than SG rats. This may partly be because some chyme entered the distal intestine through the jejuno-jejunal or jejuno-ileal anastomotic stoma. At 24 wk after surgery, the rats with SG-JI demonstrated lower fasting triglyceride and cholesterol level than the SG-JJ group. We suggest that SG can improve glucose and lipid metabolism, SG-JI and SG-JJ can enhance these improvements, and SG-JI improves lipid metabolism more effectively than SG-JJ does.

At 12 wk postoperatively, SG-JI and SG-JJ were observed to achieve better weight control. Calorie intake, however, was not statistically different among the three groups. Similarly, since 12 wk after surgery,

rats that underwent SG-JI had better weight control than those that underwent SG-JJ, but with comparable calorie intake. We speculate that body weight is affected by digestion or absorption. We regret that the caloric content in feces was not measured. It has been determined that weight loss can lead to increased insulin level, and improved glucose homeostasis and inflammation[23]. Our study showed that SG-JJ and SG-JI could lead to a higher insulin secretion level and lower HOMA-IR (an index for insulin resistance assessment) than SG, which can contribute to better diabetes control by the greater weight reduction of SG-JJ and SG-JI compared with SG.

Besides weight loss, other independent mechanisms of metabolic surgery have been explored, such as the foregut hypothesis, which demonstrates an independent beneficial effect, irrelevant to caloric intake, body weight or delivery of nutrients to the hindgut, in controlling type 2 diabetes through exclusion of the proximal small intestine[24], and the hindgut hypothesis which proposes an improvement of gastric bypass surgery in delivering nutrients and the elevated postprandial levels of gut hormones (*i.e.* GLP-1 and peptide YY)[25]. Our previous study compared different portions of small intestine, performing duodeno-jejunal bypass, ileal and sub-ileal interposition, and duodeno-jejunal bypass with ileal interposition, and concluded that small intestine contributes to enhancing glucose homeostasis[26].

Outcomes in the present research partly conformed to the hindgut hypothesis. We discovered that both rats with SG-JJ and SG-JI showed higher GLP-1 secretion than SG rats showed, and GLP-1 secretion in the SG-JI group was the highest one among the three metabolic surgery groups. The higher GLP-1 secretion observed in the SG-JJ and SG-JI groups may have been because of undigested chyme arriving at the hindgut through the intestinal loops. Compared with the SG-JJ group, the undigested chyme was delivered to hindgut more quickly in the SG-JI group, so the latter group demonstrated higher GLP-1 secretion. GLP-1 can regulate glucose homeostasis by stimulating insulin secretion, suppressing glucagon secretion, and promoting proliferation and inhibiting apoptosis of β cells[27]. Exenatide is a GLP-1 analogue that has been used clinically as an antidiabetic drug[28]. In this study, GLP-1 secretion showed the opposite trend to AUC_{OGTT}, except at 24 wk after surgery. At 24 wk after surgery, GLP-1 secretion in the SG-JI group was higher than that in the SG-JJ group; however, there was no significant difference in AUC_{OGTT} between the two groups.

Researchers have verified that serum ghrelin levels are reduced remarkably after SG, as shown in multiple clinical studies[29,30], and they believe that body weight loss and diabetes control after SG are associated with the reduced serum ghrelin levels. However, Chambers *et al*[31] performed SG on both ghrelin-deficient and wild-type mice, and did not find any difference in diabetes control between the two types of mice. We suggest that this finding demonstrates that the effect of SG on diabetes control is ghrelin independent, but it does not prove that ghrelin makes no contribution to diabetes control. Our research group has demonstrated that rats from which only the glandular stomach has been removed secrete ghrelin and show significant improvement of diabetes[32]. In our study, rats in the SG, SG-JJ and SG-JI groups showed lower fasting ghrelin levels than rats in the SHAM group. Nevertheless, there was no difference among the three metabolic surgery groups, indicating that ghrelin plays an important role in diabetes control after SG, SG-JJ and SG-JI, but it did not enhance diabetes control of SG after addition of jejuno-jejunal or jejuno-ileal loop.

There are some limitations in this study. First, the calorie content in feces was not measured, so the calorie absorption from food could not be calculated. Second, we demonstrated that SG-JJ and SG-JI enhanced diabetes control and lipid metabolism compared with SG alone. However, the results of animal experiments cannot be transferred into humans directly. We suggest that further clinical studies should be performed, to explore the optimal procedures (SG, SG-JJ, or SG-JI) for individual patients, after controlling for differences in BMI, age, duration and severity of T2DM, serum lipid, compliance with postoperative administration and so forth.

In conclusion, SG-JJ and SG-JI demonstrate better glycolipid metabolism than SG, which may result from better body weight control, enhanced insulin and GLP-1 secretion, and improved insulin resistance. Compared with SG-JJ, the improvement in lipid metabolism after SG-JI is more apparent and SG-JI induces further enhancement of GLP-1 secretion.

COMMENTS

Background

Metabolic surgery, a novel algorithm for treatment of type 2 diabetes mellitus, has been increasingly performed worldwide, the majority of which is sleeve gastrectomy (SG) with its long-term effect, however, being controversial. Moreover, SG has been reported as an independent predictor for the recurrence of diabetes, and SG seems to require urgent postoperative dietary control.

Research frontiers

To improve the effect of SG on diabetes control, SG with duodeno-jejunal bypass, loop gastro-ileostomy, jejuno-ileal bypass, and duodeno-ileal bypass has been performed sporadically by surgeons. These additional surgical procedures to SG were not clearly elaborated and lacked a theoretical foundation.

Innovations and breakthroughs

The authors used postoperative high-fat diet to simulate undesirable dietary control and performed SG, jejuno-jejunal loop (SG-JJ) and jejuno-ileal loop (SG-JI) on diabetic rats. SG-JJ and SG-JI were superior to SG in improving glycolipid metabolism. Compared with SG-JJ, the improvement in lipid metabolism after SG-JI was more apparent.

Applications

The findings in this study suggest that if a patient is thought to have undesirable

postoperative dietary control, SG-JJ and SG-JI may be considered as preferential surgical procedures. SG-JI may be selected for patients with hyperlipidemia. These discoveries might help surgeons to select surgical procedures for individual patients.

Terminology

SG, a novel procedure of metabolic surgery to remove 70%-80% of the stomach from the greater curvature, has demonstrated short- and medium-term roles in improving glycolipid metabolism in obese patients with/without type 2 diabetes mellitus, but its long-term beneficial effect remains controversial. In SG-JJ and SG-JI surgery, a jejuno-jejunal or jejuno-ileal side-by-side anastomosis is made based on SG surgery.

Peer-review

The study is very interesting. Regarding new guidelines, there is a need for the surgical methods dedicated for diabetic patients. The authors explored the effect of SG with jejuno-jejunal or jejuno-ileal loop on glycolipid metabolism in diabetic rats.

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ORIGINAL ARTICLE

Basic Study

Synergistic anticancer effect of exogenous wild-type *p53* gene combined with 5-FU in human colon cancer resistant to 5-FU *in vivo*

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Author contributions: Xie Q designed the study; Wu MY, Zhang DX and Yang YM performed the research; Zhang J and Xu J collected and analyzed the data; Wang BS performed the MRI; Zhong WD conducted the statistical analysis; Xie Q and Zhang DX wrote the manuscript;Hu JN revised the manuscript.

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Abstract

AIM

To investigate the anticancer effect of a recombinant adenovirus-mediated p53 (rAd-p53) combined with 5-fluorouracil (5-FU) in human colon cancer resistant to 5-FU *in vivo* and the mechanism of rAd-p53 in reversal

of 5-FU resistance.

METHODS

Nude mice bearing human colon cancer SW480/5-FU (5-FU resistant) were randomly assigned to four groups (n = 25 each): control group, 5-FU group, rAd-p53 group, and rAd-p53 + 5-FU group. At 24 h, 48 h, 72 h, 120 h and 168 h after treatment, 5 mice were randomly selected from each group and sacrificed using an overdose of anesthetics. The tumors were removed and the protein expressions of p53, protein kinase C (PKC), permeability-glycoprotein (P-gp) and multidrug resistance-associated protein 1 (MRP1) (Western blot) and apoptosis (TUNEL) were determined.

RESULTS

The area ratios of tumor cell apoptosis were larger in the rAd/p53 + 5-FU group than that in the control, 5-FU and rAd/p53 groups ($P < 0.05$), and were larger in the rAd/p53 group than that of the control group ($P < 0.05$) and the 5-FU group at more than 48 h ($P < 0.05$). The p53 expression was higher in the rAd/p53 and the rAd/p53 + 5-FU groups than that of the control and 5-FU groups ($P < 0.05$), and were higher in the rAd/p53 + 5-FU group than that of the rAd/p53 group ($P < 0.05$). Overexpression of PKC, P-gp and MRP1 was observed in the 5-FU and control groups. In the rAd/p53 + 5-FU group, the expression of P-gp and MRP1 was lower that of the control and 5-FU groups ($P < 0.05$), and the expression of PKC was lower than that of the control, 5-FU and rAd/p53 groups at more than 48 h ($P < 0.05$). In the rAd/p53 group, the expression of P-gp and MRP1 was lower that of the control and 5-FU groups at more than 48 h ($P < 0.05$), and the expression of PKC was lower than that of the control and 5-FU groups at more than 120 h ($P < 0.05$).

CONCLUSION

5-FU combined with rAd-p53 has a synergistic anticancer effect in SW480/5-FU (5-FU resistance), which contributes to reversal of 5-FU resistance.

Key words: Human colon cancer; Multidrug resistance; 5-Fluorouracil; Recombinant adenovirus-mediated p53; Xenografts in nude mice

Core tip: To observe anticancer action of a recombinant adenovirus-mediated p53 (rAd-p53) combined with 5-fluorouracil (5-FU) in human colon cancer with resistance to 5-FU *in vivo* to investigate the potential and mechanism of rAd-p53 in the reversal of resistance to 5-FU in human colon cancer. Our previous results revealed that exogenous wild-type *p53* gene from rAd-p53 can decrease expression of PKC, P-gp and MRP1 in SW480/5-FU (5-FU resistance) and promote apoptosis of tumor cell, which contributes to reversing 5-FU resistance *in vivo*. 5-FU can increase the expression of exogenous wild-type p53, so 5-FU combined with rAd-p53 has a synergistic anticancer effect for colon cancer of 5-FU resistance *in vivo*.

Xie Q, Wu MY, Zhang DX, Yang YM, Wang BS, Zhang J, Xu J, Zhong WD, Hu JN. Synergistic anticancer effect of exogenous wild-type *p53* gene combined with 5-FU in human colon cancer resistant to 5-FU *in vivo*. *World J Gastroenterol* 2016; 22(32): 7342-7352 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i32/7342.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i32.7342

INTRODUCTION

Colorectal cancer (CRC) is one of the most common gastrointestinal cancers. In 2013, there were 1.6 million incident cases of CRC worldwide, with 56% occurring in developing countries and 44% in developed countries, which caused 771000 deaths[1]. Most patients are usually at an advanced stage at the time of diagnosis.

To date, 5-fluorouracil (5-FU) remains a widely used chemotherapeutic drug in the treatment of advanced CRC; however, response rates are only 10% to 15%, due to severe side effects and resistance[2]. The anticancer efficacy of 5-FU is thought to be partly attributed to its ability to induce p53-dependent cell growth arrest and apoptosis; consequently, mutations or deletions of p53 can cause cells to become resistant to 5-FU[3-6]. Therefore, overcoming 5-FU resistance caused by mutations or deletions of p53 will be a key issue in the design of more effective individualized therapeutic strategies.

Gene replacement therapy for a mutated p53 gene using a recombinant adenovirus-mediated p53 (rAd-p53) gene reportedly increases apoptosis after administration[7-12]. Our previous results revealed that exogenous wild-type *p53* (wt-*p53*) from rAd-p53 increased tumor necrosis in human colon cancer SW480 (5-FU responsive) harboring mutant p53, and 5-FU combined with rAd-p53 had a synergistic anticancer effect *in vivo*[13]. Therefore, rAd-p53 may contribute to the reversal of resistance to 5-FU in colon cancer.

The present study determined the early therapeutic effectiveness of rAd-p53 alone or in combination with 5-FU for the treatment of human colon cancer SW480/5-FU (5-FU resistant) in a nude mouse model. The potential and mechanism of rAd-p53 in the reversal of resistance to 5-FU in human colon cancer *in vivo* was also investigated.

MATERIALS AND METHODS

The present study strictly complied with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Sun Yat-sen University (2011-0702) and Guangzhou Medical University, Guangzhou, China.

Cell culture

The human colon cancer cell line SW480 was purchased from the Cell Bank of Sun Yat-sen University. The cells were cultured in RPMI 1640 with 10% fetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin, and grown at 37 ℃ in a 5% CO_2 humidified atmosphere. 5-FU resistant SW480 (SW480/5-FU) cells were generated by continuous exposure to increasing concentrations of 5-FU for more than 5 mo. SW480/5-FU cells were able to survive in 6 μg/mL of 5-FU. The IC50 of 5-FU, based on the results of a [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT)] assay, was 23.593 μg/mL for parental cells (SW480) and 140.642 μg/mL for resistant cells (SW480/5-FU). The resistance index (RI) was 5.93. The IC50 of 5-FU in SW480 and SW480/5-FU cells was assayed with MTT.

Animal model

BALB/c nude mice were purchased from the Animal Center of Sun Yat-sen University. A total of 100 4-wk-old BALB/c nude mice, weighing 16-18 g regardless of sex, were subcutaneously implanted with SW480/5-FU tissues in the rear flank to generate xenograft models as described previously[13,14]. All surgical procedures were performed under anesthesia induced by chloral hydrate (4.5% chloral hydrate, 2 mL/100 g body weight, intraperitoneal injection), and all efforts were made to minimize suffering. Mice were fed in a specific pathogen-free (SPF) laboratory. One month after implantation, the mice were randomly assigned to four groups (25 per group): control group (medical-grade saline), 5-FU group, rAd-p53 group (Gendicine, SibionoGeneTech Co., Ltd, Shenzhen, China), and rAd-p53 + 5-FU group. The above-mentioned therapeutic agents were administered by intratumoral injection. The dose of rAd-p53 administered was 1×10^7 VIP/mm^3 tumor for each group. The dose of 5-FU was 25 mg for tumors 0.5-0.9 cm, 50 mg for tumors 1.0-1.4 cm, and 75 mg for tumors more than 1.5 cm[13].

Assessment of tumor response

At 24, 48, 72, 120 and 168 h after treatment, 5 mice were randomly selected from each group and euthanized with an overdose of anesthetics. The tumors were removed and divided into equal halves. One half was immediately frozen at -30 ℃ for Western blot analysis. The other half was fixed in phosphate-buffered saline (pH 7.3) containing 4% formaldehyde and 0.2% glutaraldehyde, embedded in paraffin, and sectioned for TUNEL assay.

Measurement of apoptosis

Pathological sections were stained using the TUNEL apoptosis *in situ* detection reagent kit (Keygen, Nanjing, China) according to the manufacturer's instructions. The area ratio of tumor cell apoptosis was calculated as the percentage of positively stained cell nuclei (dark brown) at magnification × 100. The average of the evaluations by the pathologists (Zhang & Xu) was used for analysis.

Western blot analysis

Western blot analysis was used to detect the protein expression of p53, protein kinase C (PKC), permeability-glycoprotein (P-gp) and multidrug resistance-associated protein 1 (MRP1) as described in the instruction manual (Phototope®-HRP Western blot kit; Cell Signaling Technology, United States). Cell extracts were obtained from frozen tumor tissues (-30 ℃). Immunoblot analysis was performed using anti-p53 monoclonal antibodies (Santa Cruz Biotech, United States), anti-PKC and multidrug resistance protein 1 (MDR1) and MRP1 monoclonal antibodies (Santa Cruz Biotech). Subsequent protein detection was performed using an enhanced chemiluminescence (ECL) detection system (Hitachi, Japan).

The band intensities (IOD) of protein expression stated above were scanned into the computer and analyzed with Image-Pro Plus 6.0 software. The relative IOD (RIOD) of protein expression was calculated as the IOD of the protein in the control group and therapeutic groups at each time point divided by the corresponding IOD of GAPDH (internal control).

Statistical analysis

The SPSS 13.1 statistical package (SPSS Inc., United States) was used for all calculations. The tumor responses of the entire sample set (ANOVA repeated data) and between groups (SNK test) were compared, and the correlations between parameters were evaluated with Pearson's correlation at a significance level of 0.05. Data are presented as mean ± SD of a representative of at least three independently performed experiments.

RESULTS

Tumor cell IC50

The IC50 of 5-FU based on the results of the MTT assay was 23.593 μg/mL for parental cells (SW480) and 140.642 μg/mL for resistant cells (SW480/5-FU). The resistance index (RI) was 5.93.

Tumor cell apoptosis

Tumor cell apoptosis was detected in sections from the 5-FU group and the control group at the observed time points (Figure 1); however, there were no significant differences in tumor cell apoptosis between the two groups (SNK test, $P > 0.05$; Table 1).

The area ratio of tumor cell apoptosis in the rAd-p53 + 5-FU group was significantly larger than

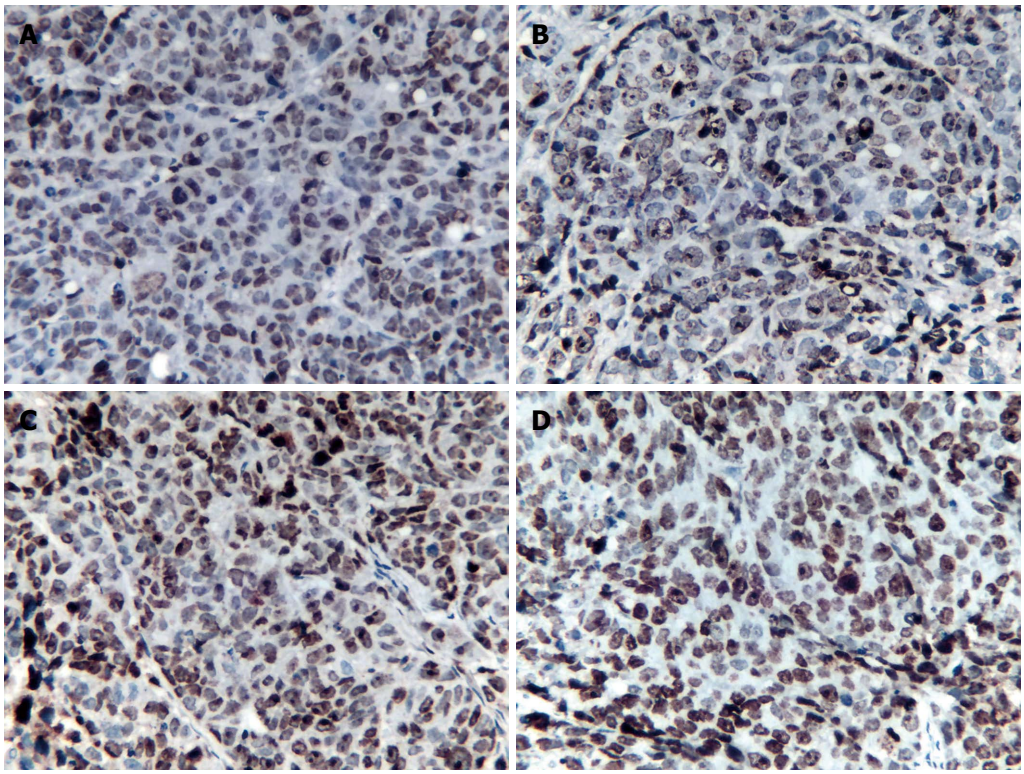

Figure 1 Tumor cell apoptosis. Tumor cell apoptosis at 72 h (magnification × 400) in the control group (A), 5-FU group (B), rAd-p53 group (C) and rAd-p53 + 5-FU group (D).

Table 1 Tumor apoptosis ratios in experimental groups and control group (mean ± SD)

Group	24 h	48 h	72 h	120 h	168 h
Control	0.25 ± 0.02	0.28 ± 0.01	0.27 ± 0.02	0.29 ± 0.02	0.24 ± 0.04
5-FU	0.27 ± 0.03	0.31 ± 0.03	0.31 ± 0.05	0.30 ± 0.04	0.31 ± 0.02
rAd-p53	0.29 ± 0.02	0.35 ± 0.03	0.43 ± 0.08	0.42 ± 0.06	0.46 ± 0.06
5-FU + rAd-p53	0.33 ± 0.03	0.44 ± 0.08	0.58 ± 0.07	0.59 ± 0.05	0.62 ± 0.07
F	13.235	41.487	53.812	61.676	80.755
P value	0	0	0	0	0
P value	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05
5-FU *vs* Control					
P value	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
rAd-p53 *vs* Control					
P value	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
rAd-p53 + 5-FU *vs* Control					
P value	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
rAd-p53 + 5-FU *vs* rAd-p53					
P value	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
rAd-p53 + 5-FU *vs* 5-FU					
P value	>0.05	< 0.05	< 0.05	< 0.05	< 0.05
rAd-p53 *vs* 5-FU					

that in the control group, 5-FU group and the rAd-p53 group (SNK test, $P < 0.05$; Table 1). The area ratio of tumor cell apoptosis in the rAd-p53 group was significantly larger than that in the control group (SNK test, $P < 0.05$). After > 48 h of treatment, the area ratio of tumor cell apoptosis in the rAd-p53 group was significantly larger than that in the 5-FU group (SNK test, $P < 0.05$).

The area ratio of tumor cell apoptosis in the rAd-p53 group and the rAd-p53 + 5-FU group tended to increase with time (ANOVA, P <0.05).

Protein expression

P53 expression: P53 protein showed weak expression in the 5-FU group and the control group at the observed time points (Figure 2A). There were no significant differences in the RIOD of p53 expression between the two groups (SNK test, $P > 0.05$; Table 2). P53 expression level in the rAd-p53 and the rAd-p53 + 5-FU group was higher than that in the control and 5-FU groups (SNK test, $P < 0.05$) and increased in a time-dependent manner (ANOVA, $P < 0.05$; Table 2), with peak expression at 120 h. P53 expression in the

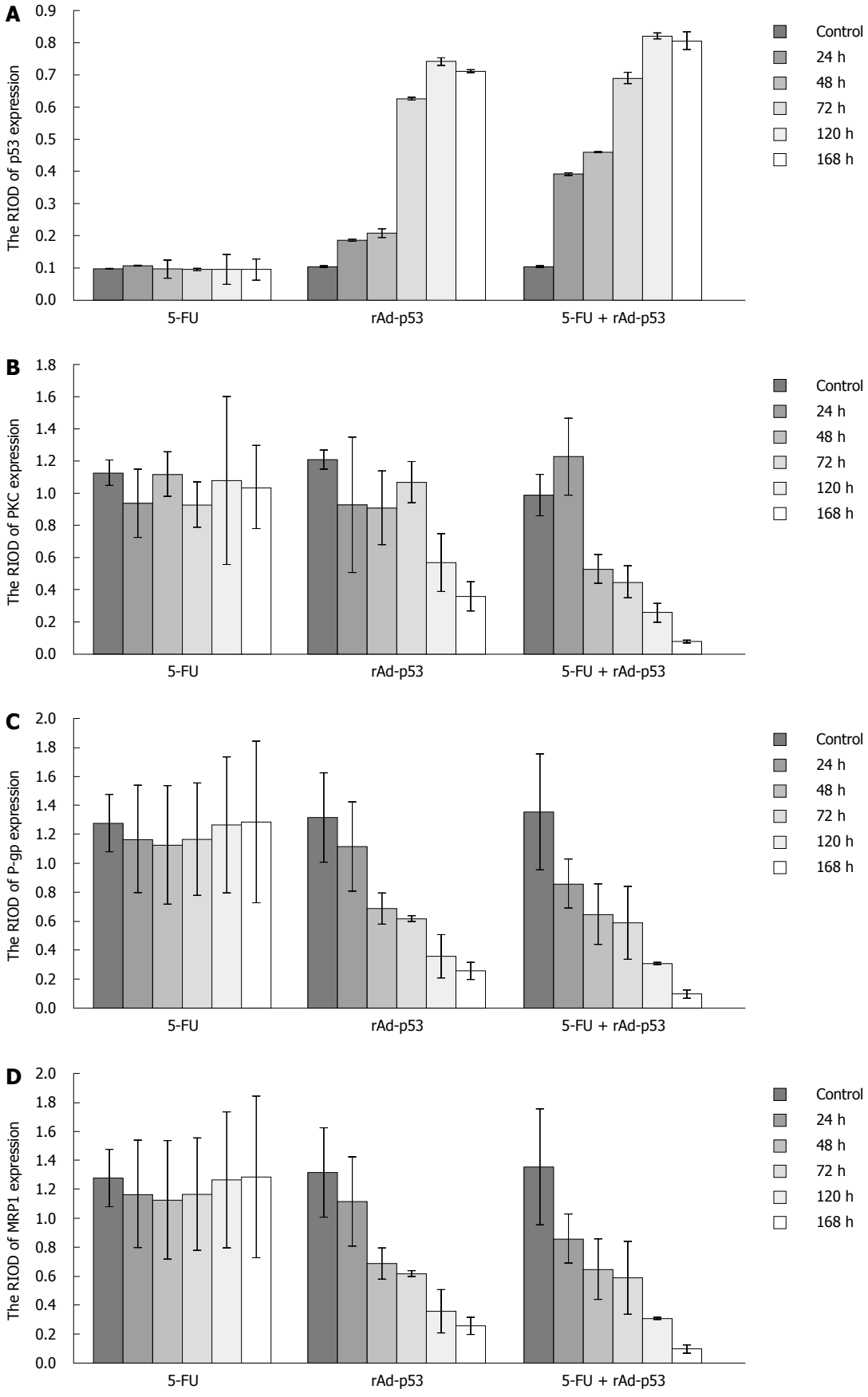

Figure 2 Relative band intensities of p53 expression (A), protein kinase C expression (B), permeability-glycoprotein expression (C) and MRP1 expression (D) in the three experimental groups.

Table 2 Relative IOD of p53 expression in experimental groups and control group (mean ± SD)

Group	24 h	48 h	72 h	120 h	168 h
Control	0.099 ± 0.001	0.105 ± 0.003	0.101 ± 0.007	0.105 ± 0.015	0.103 ± 0.001
5-FU	0.108 ± 0.002	0.098 ± 0.028	0.096 ± 0.004	0.097 ± 0.047	0.096 ± 0.033
rAd-p53	0.187 ± 0.003	0.209 ± 0.014	0.627 ± 0.005	0.743 ± 0.012	0.713 ± 0.005
5-FU + rAd-p53	0.393 ± 0.004	0.461 ± 0.002	0.691 ± 0.018	0.822 ± 0.009	0.808 ± 0.027
F	221.565	270.392	3983.173	9639.595	3867.703
P value	0	0	0	0	0
P value	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05
5-FU *vs* Control					
P value	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
rAd-p53 *vs* Control					
P value	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
rAd-p53 + 5-FU *vs* Control					
P value	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
rAd-p53 + 5-FU *vs* rAd-p53					
P value	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
rAd-p53 + 5-FU *vs* 5-FU					
P value	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
rAd-p53 *vs* 5-FU					

Table 3 Relative IOD of protein kinase C expression in experimental groups and control group (mean ± SD)

Group	24 h	48 h	72 h	120 h	168 h
Control	1.13 ± 0.08	1.21 ± 0.06	0.99 ± 0.13	1.26 ± 0.42	1.08 ± 0.20
5-FU	0.94 ± 0.21	1.12 ± 0.14	0.93 ± 0.14	1.08 ± 0.52	1.04 ± 0.26
rAd-p53	0.93 ± 0.42	0.91 ± 0.23	1.07 ± 0.13	0.57 ± 0.18	0.36 ± 0.09
5-FU + rAd-p53	1.23 ± 0.24	0.53 ± 0.09	0.45 ± 0.10	0.26 ± 0.06	0.08 ± 0.01
F	1.036	33.972	14.348	24.072	71.559
P value	0.427	0	0.01	0	0
P value	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05
5-FU *vs* Control					
P value	> 0.05	< 0.05	> 0.05	< 0.05	< 0.05
rAd-p53 *vs* Control					
P value	> 0.05	< 0.05	< 0.05	< 0.05	< 0.05
rAd/p53 + 5- FU *vs* Control					
P value	> 0.05	< 0.05	< 0.05	< 0.05	< 0.05
rAd-p53 + 5-FU *vs* rAd-p53					
P value	> 0.05	< 0.05	< 0.05	< 0.05	< 0.05
rAd-p53 + 5-FU *vs* 5-FU					
P value	> 0.05	< 0.05	> 0.05	< 0.05	< 0.05
rAd-p53 *vs* 5-FU					

rAd-p53 + 5-FU group was significantly higher than that in the rAd-p53 group (SNK test, $P < 0.05$; Table 2).

PKC, P-gp and MRP1 expression: Overexpression of PKC, P-gp and MRP1 was observed in the 5-FU group and the control group (Figure 2B-D). There were no significant differences in the RIOD of the expression of these proteins between the two groups (SNK test, $P > 0.05$; Tables 3-5).

At the observed time points, the expression of PKC, P-gp and MRP1 in the rAd/p53 + 5-FU group gradually decreased in a time-dependent manner (ANOVA, $P < 0.05$; Tables 3-5). The expression of P-gp and MRP1 was significantly lower than that in the control group and the 5-FU group (SNK test, $P < 0.05$). More than 48 h after treatment, the expression of PKC in the rAd-p53 + 5-FU group was significantly lower than that in the control, 5-FU and rAd-p53 groups.

In the rAd-p53 group, the expression of P-gp and MRP1 was significantly lower than that in the control group and the 5-FU group at > 48 h of treatment (SNK test, $P < 0.05$). The expression of PKC was significantly lower than that in the control group and the 5-FU group at > 120 h of treatment (SNK test, $P < 0.05$).

Pearson's correlation test

The RIOD of p53 expression was positively correlated with the area ratio of tumor cell apoptosis (the correlation coefficient and *P* value were 0.545 and 0.000, respectively).

The RIOD of PKC, P-gp and MRP1 expression was negatively correlated with the area ratio of tumor cell apoptosis (correlation coefficients were -0.322, 0.012 and -0.335 and *P* values were 0.009, -0.541 and 0.000, respectively).

Table 4 Relative IOD of P-gp expression in experimental groups and control group (mean ± SD)

Group	24 h	48 h	72 h	120 h	168 h
Control	1.28 ± 0.20	1.32 ± 0.31	1.36 ± 0.40	1.29 ± 0.43	1.30 ± 0.56
5-FU	1.17 ± 0.37	1.13 ± 0.41	1.17 ± 0.39	1.27 ± 0.47	1.29 ± 0.56
rAd-p53	1.12 ± 0.31	0.69 ± 0.11	0.62 ± 0.02	0.36 ± 0.15	0.26 ± 0.06
5-FU + rAd-p53	0.86 ± 0.17	0.65 ± 0.21	0.59 ± 0.25	0.31 ± 0.01	0.10 ± 0.03
F	5.670	7.301	14.645	9.844	7.971
P value	0.022	0.011	0.001	0.005	0.009
P value	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05
5-FU *vs* Control					
P value	> 0.05	< 0.05	< 0.05	< 0.05	< 0.05
rAd-p53 *vs* Control					
P value	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
rAd-p53 + 5-FU *vs* Control					
P value	< 0.05	> 0.05	> 0.05	> 0.05	> 0.05
rAd-p53 + 5-FU *vs* rAd-p53					
P value	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
rAd-p53 + 5-FU *vs* 5-FU					
P value	> 0.05	< 0.05	< 0.05	< 0.05	< 0.05
rAd-p53 *vs* 5-FU					

Table 5 Relative IOD of MRP1 expression in experimental groups and control group (mean ± SD)

Group	24 h	48 h	72 h	120 h	168 h
Control	1.31 ± 0.36	1.33 ± 0.08	1.09 ± 0.24	1.22 ± 0.35	1.07 ± 0.22
5-FU	1.27 ± 0.10	1.31 ± 0.11	1.14 ± 0.16	1.20 ± 0.31	1.13 ± 0.14
rAd-p53	0.98 ± 0.09	0.74 ± 0.11	0.58 ± 0.18	0.87 ± 0.31	0.31 ± 0.08
5-FU + rAd-p53	0.71 ± 0.17	0.62 ± 0.02	0.51 ± 0.10	0.42 ± 0.08	0.13 ± 0.10
F	6.438	123.754	20.567	15.512	36.073
P value	0.016	0	0	0.001	0
P value	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05
5-FU *vs* Control					
P value	> 0.05	< 0.05	< 0.05	< 0.05	< 0.05
rAd-p53 *vs* Control					
P value	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
rAd-p53 + 5-FU *vs* Control					
P value	> 0.05	< 0.05	> 0.05	< 0.05	> 0.05
rAd-p53 + 5-FU *vs* rAd-p53					
P value	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
rAd-p53 + 5-FU *vs* 5-FU					
P value	> 0.05	< 0.05	< 0.05	< 0.05	< 0.05
rAd-p53 *vs* 5-FU					

The RIOD of p53 expression and the RIOD of PKC, P-gp and MRP1 expression showed a negative correlation (correlation coefficients were -0.366, 0.004 and -0.406 and *P* values were 0.001, -0.488 and 0.000, respectively).

DISCUSSION

5-FU is still widely used as a major anticancer drug in the treatment of colon cancer[3]. However, a major impediment to the success of colon cancer chemotherapy is the development of cancer variants exhibiting multidrug resistance (MDR)[2-6,15,16].

MDR usually presents as cross-resistance to multiple chemotherapeutic drugs with different structures[16,17]. Anti-cancer drug resistance in colon cancer cells can be caused by various factors, including alterations in drug influx and efflux, enhancement of drug inactivation and mutation of the drug target induced by various proteins[18-21]. To date, multiple factors have been reported to lead to resistance to chemotherapeutic drugs[16-23]. P-gp, PKC and the multidrug resistance-associated proteins (MRPs) contribute to chemotherapy resistance[17-23]. In our previous studies, overexpression of P-gp, PKC and MRP1 was observed in human colon cancer SW480/5-FU cells (5-FU resistant) and weak expression of these proteins was seen in parental human colon cancer SW480 cells (5-FU responsive)[14].

Various mechanisms contribute to MDR, including the overexpression of drug efflux pumps (pump resistance) and the up-regulation of cellular antiapoptotic defense systems (non-pump resistance)[23,24]. P-gp encoded by the MDR1 gene and MRPs belong to the ATP-binding cassette (ABC) superfamily. These transporter proteins (responsible for pump resistance) mediate the efflux of drugs in the MDR spectrum, such as anthracyclines, out

of cells, thus reducing drug efficacy[24]. Generally, there are two approaches used to reverse ABC superfamily-mediated MDR: blocking its drug-pump function and inhibiting its expression[25-27].

PKC is one of the signaling enzymes that is positively regulated by reactive oxygen species (ROS) and plays a crucial role in a variety of pathophysiological states, including tumor progression. PKC contains multiple cysteine residues that can be activated by ROS oxidatively[28,29]. PKC represents a family of serine/threonine kinases that are involved in the regulation of cell growth, cell death and stress responsiveness[30]. Generally, the PKCs are classified into three subfamilies based on their structural and activation characteristics: the conventional or classic (α, βI, βⅡ, and γ), the novel or non-classic (δ, ε, η and θ), and the atypical PKC isoenzymes (ζ, ι and λ)[30]. Different PKC isoenzymes may exert similar or opposite cellular effects by differential coupling of signaling pathways[31]. Cancer cells survive by evading apoptosis or promoting proliferation, invasion and metastasis. PKC may act as a downstream effector of the signaling protein phosphatidylinositol 3-kinase (PI3K). The PI3K-mediated signaling cascade regulates cell proliferation, cell survival, differentiation and apoptosis[32-34]. Phosphorylation of the regulatory subunit p85a is linked to increased survival of cancer cells. The p85 subunit regulates the catalytic subunit p110 by stabilization and inactivation of its kinase activity in the basal state as well as by recruitment of PI3K to phospho-tyrosine residues of the activated receptors[34,35].

PKCα and PKCβ may promote ABCB1 function by phosphorylation[18,33,34]. Notably, the effects of PKC signaling on ABCB1 phosphorylation and function appear to be cell type-dependent. In ovarian carcinoma cells, antisense oligomers directed against PKCα and PKCβ reversed ABCB1-mediated drug resistance[36]. In contrast, PKCβ was not detectable in some reports, and siRNAs targeting PKCα interfered with PKC signaling, but not with ABCB1 function[18]. Moreover, p53 was shown to suppress PKCα-mediated ABCB1 activation in leiomyosarcoma, fibrosarcoma, and osteosarcoma cells[18,34].

Mutations or deletions of suppressor gene *p53* are the most common genetic abnormalities that occur during cancerogenesis in the majority of human neoplasms. The *p53* gene, localized on the short arm of chromosome 17 (17p13), encodes nucleic phosphoproteins, and affects several cell functions (induction of many genes, regulation of the cell cycle and apoptosis control)[37]. Under the condition of p53 gene mutation, cancer cells remain intact and survive[38].

Evasion from chemotherapy-induced apoptosis due to *p53* loss strongly contributes to drug resistance[3-6,16,38]. Wild-type *p53* is a key tumor suppressor in preventing tumorigenesis and cancer progression; however, mutant p53, detected in over 50% of all human tumors and in approximately 70% of colorectal cancers[39-43], promotes tumor progression and resistance to therapies[3-6,38,44], and such mutants have become the most common prognostic indicators for both tumor recurrence and cancer death[40,43,45,46]. Prevention of *p53* mutation to restore wild-type *p53* activity is an attractive anticancer therapy to reverse 5-FU resistance in colon cancer.

Infection with Ad-p53 can significantly down-regulate MDR1 transcription and P-gp expression in breast cancer cell lines and reverse resistance to adriamycin[47]. Treatment with rAd-p53 alone, oxaliplatin alone or combined treatment led to a decrease in Bcl-2 expression and an increase in Bax expression in gastric cancer cells, and induced apoptosis of gastric cancer cells, which was accompanied by increased expression of caspase-3[12]. Therefore, rAd-p53 may enhance the sensitivity of gastric cancer cells to chemotherapy by promoting apoptosis. In the present study, exogenous wild-type *p53* gene from rAd-p53 decreased the expression of PKC, P-gp and MRP1 and promoted apoptosis of colon carcinoma cells in nude mice implanted with human colon carcinoma SW480/5-FU (5-FU resistant), which contributed to the reversal of 5-FU resistance.

The antimetabolite, 5-FU, is an analogue of uracil with a fluorine atom at the C5 position of the pyrimidine ring. 5-FU is converted in cells to different active metabolites, including fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). These metabolites have been implicated in both global RNA metabolism due to incorporation of the ribonucleotide FUMP into RNA, and DNA metabolism due to thymidylate synthase (TS) inhibition or direct incorporation of FdUMP into DNA, leading to a wide range of biological effects which can act as triggers for apoptotic cell death[3,4,15,16]. Therefore, 5-FU can be regarded as a genotoxic agent.

P53 protein in the production of wild-type *p53* expression plays a key role in cell cycle regulation and in the cellular response to cytotoxic stress and DNA damage[48-51]. P53 protein is maintained at low levels by MDM2, an E3-ligase that binds p53 and promotes its degradation[52-54]. DNA damage and other stresses, including gamma and UV irradiation, chemotherapeutic agents, hypoxia, heat or alterations in intracellular nucleotide pools, disrupt p53-MDM2 binding, causing p53 levels to increase[49-51,55]. Wild-type p53 is induced in response to a host of genotoxic and environmental stresses, a host of target genes are then transcriptionally activated, including p21, GADD45, Bax and Bcl-2. Induction of p21, in turn, leads to cell cycle arrest at both G1 and G2 checkpoints. This function is thought to be essential in preserving the integrity of the cellular genome in response to treatment with cytotoxic agents. In addition to mediating cell cycle arrest, p53 is a potent inducer of apoptosis and programmed cell death[49-51].

Increased p53 protein in response to genotoxic stress also occurs in cancer cells[56-59]. Treatment of

human colon cancer RKO cells and tetraploid cancer cells with 5-FU resulted in a significant increase in the levels of the endogenous p53 protein family *in vitro* and enhanced tumor suppression[59]. The p53 protein family forms an interacting network of proteins[60]. Cancer cell responses to 5-FU treatment are determined by the total activity of the entire p53 family rather than p53 alone[60]. Suppressor p53 is one of the molecular targets of 5-FU. With regard to 5-FU, translational regulation is an important process for controlling endogenous p53 expression[59-61]. Our previous study demonstrated that the expression of exogenous wild-type *p53* gene in colon cancer cells in nude mice bearing human colon carcinoma SW480 (5-FU responsive) treated with rAd-p53 + 5-FU was significantly higher than that with rAd-p53 alone, and tumor necrosis was positively correlated with p53 expression *in vivo*[13]. 5-FU also increased the anticancer effect of rAd/p53 *in vivo*.

In the present study, p53 expression in colon cancer SW480/5-FU in the rAd/p53 group and rAd-p53 + 5-FU group was higher than in the control and 5-FU groups and increased in a time-dependent manner. P53 expression in the rAd-p53 + 5-FU group was significantly higher than in the rAd-p53 group. These results suggest that 5-FU increased the expression of exogenous wild-type *p53* gene in colon cancer (resistant to 5-FU) *in vivo*. Exogenous wild-type p53 is also induced in response to genotoxic stress in chemotherapy-resistant cancer cells.

In summary, exogenous wild-type *p53* gene from rAd-p53 can decrease the expression of PKC, P-gp and MRP1 in SW480/5-FU (5-FU resistant) and promote apoptosis of tumor cells, which contributes to the reversal of 5-FU resistance *in vivo*. 5-FU can increase the expression of exogenous wild-type p53, thus 5-FU combined with rAd-p53 has a synergistic anticancer effect in colon cancer resistant to 5-FU *in vivo*. Therefore, the DNA-damaging agent 5-FU combined with exogenous wild-type p53 provides a potential therapeutic strategy and can enhance the sensitivity and reduce the toxicity of chemotherapy and improve the clinical efficacy of colon cancer chemotherapy.

ACKNOWLEDGMENTS

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COMMENTS

Background

5-fluorouracil (5-FU) is a widely used chemotherapeutic drug in the treatment of advanced colorectal cancer (CRC); however, response rates are only 10% to 15%, due to severe side effects and resistance. Mutations or deletions of p53 can cause cells to become resistant to 5-FU. Therefore, gene replacement therapy for a mutated p53 gene using recombinant adenovirus-mediated p53 (rAd-p53) may overcome 5-FU resistance caused by mutations or deletions of p53. Therefore, rAd-p53 may contribute to the reversal of 5-FU resistance in colon cancer.

Research frontiers

Exogenous wild-type p53 from rAd-p53 increased tumor necrosis in human colon cancer SW480 (5-FU responsive) harboring mutant p53, and 5-FU combined with rAd-p53 had a synergistic anticancer effect *in vivo*.

Innovations and breakthroughs

This is the first study to evaluate 5-FU combined with rAd-p53 in the treatment of colon cancer SW480/5-FU (5-FU resistant) compared with controls *in vivo*.

Applications

This study first showed that exogenous wild-type p53 gene from rAd-p53 can decrease the expression of PKC, P-gp and MRP1 in colon cancer SW480/5-FU (5-FU resistant) and promote apoptosis of tumor cells, which contributes to the reversal of 5-FU resistance *in vivo*. 5-FU can increase the expression of exogenous wild-type p53; thus, 5-FU combined with rAd-p53 had a synergistic anticancer effect in 5-FU resistant colon cancer *in vivo*.

Terminology

5-FU increased the expression of exogenous wild-type p53 gene in colon cancer (5-FU resistant) *in vivo*. Exogenous wild-type p53 is also induced in response to genotoxic stress in chemotherapy-resistant cancer cells.

Peer-review

The authors demonstrated that 5-FU increased the expression of exogenous wild-type p53 gene, and exogenous wild-type p53 is induced in response to genotoxic stress by 5-FU in colon cancer (resistant to 5-FU) *in vivo*. These results are interesting. Previous studies have established that increased p53 protein in response to genotoxic stress occurs in cancer cells *in vitro*. In this study, the authors showed for the first time that 5-FU combined with rAd-p53 has a synergistic anticancer effect for colon cancer resistant to 5-FU *in vivo*.

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ORIGINAL ARTICLE

Basic Study

Effects of different diets on intestinal microbiota and nonalcoholic fatty liver disease development

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Abstract

AIM

To study the effects of different diets on intestinal microbiota and nonalcoholic fatty liver disease (NAFLD) development at the same caloric intake.

METHODS

Thirty male Sprague-Dawley rats were randomized into five groups (six rats each). The control diet (CON) group and free high-fat diet (FFAT) group were allowed *ad libitum* access to a normal chow diet and a high-fat diet, respectively. The restrictive high-fat diet (RFAT) group, restrictive high-sugar diet (RSUG) group, and high-protein diet (PRO) group were fed a high-fat diet, a high-sugar diet, and a high-protein diet, respectively, in an isocaloric way. All rats were killed at 12 wk. Body weight, visceral fat index (visceral fat/body weight), liver index (liver/body weight), insulin resistance, portal lipopolysaccharide (LPS), serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), and liver triglycerides were measured. The intestinal microbiota in the different groups of rats was sequenced using high-throughput sequencing technology.

RESULTS

The FFAT group had higher body weight, visceral fat index, liver index, peripheral insulin resistance, portal LPS, serum ALT, serum AST, and liver triglycerides compared with all other groups ($P < 0.05$). Taking the same calories, the RFAT and RSUG groups demonstrated increased body weight, visceral fat index, peripheral insulin resistance and liver triglycerides compared with the PRO group ($P < 0.05$). The RFAT group also showed increased portal LPS compared with the PRO group ($P < 0.05$). Unweighted UniFrac principal coordinates analysis of the sequencing data revealed that the intestinal microbiota structures of the CON, FFAT, RSUG and PRO groups were roughly separated away from each other. Taxon-based analysis showed that, compared with the CON group, the FFAT group had an increased abundance of *Firmicutes*, *Roseburia* and *Oscillospira* bacteria, a higher ratio of *Firmicutes* to *Bacteroidetes*, and a decreased abundance of *Bacteroidetes*, *Bacteroides* and *Parabacteroides* bacteria ($P < 0.05$). The RFAT group showed an increased abundance of *Firmicutes* and decreased abundance of *Parabacteroides* bacteria ($P < 0.05$). The RSUG group showed an increased abundance of *Bacteroidetes* and *Sutterella* bacteria, higher ratio of *Bacteroidetes* to *Firmicutes*, and a decreased abundance of *Firmicutes* ($P < 0.05$). The PRO group showed an increased abundance of *Bacteroidetes*, *Prevotella*, *Oscillospira* and *Sutterella* bacteria, and a decreased abundance of *Firmicutes* ($P < 0.05$). Compared with the FFAT group, the RFAT group had an increased abundance of *Bacteroidetes*, higher ratio of *Bacteroidetes* to *Firmicutes*, and decreased abundance of *Firmicutes* and *Oscillospira* bacteria ($P < 0.05$).

CONCLUSION

Compared with the high-protein diet, the NAFLD-inducing effects of high-fat and high-sugar diets are independent from calories, and may be associated with changed intestinal microbiota.

Key words: Nonalcoholic fatty liver disease; High-fat diet; Restrictive high-fat diet; Restrictive high-sugar diet; High-protein diet; Intestinal microbiota

Core tip: Diet plays an important role in development of nonalcoholic fatty liver disease (NAFLD), and can shape intestinal microbiota, which is closely linked to NAFLD. We studied the effects of high-fat, high-sugar and high-protein diets on intestinal microbiota and NAFLD development in an isocaloric way. NAFLD-inducing effects of high-fat and high-sugar diets, compared with high-protein diet, are independent from calories, and these diets can alter intestinal microbiota independently from calories. The effects of these diets on NAFLD development at the same caloric intake may be associated with changes in intestinal microbiota. These findings are meaningful for appropriate dietary therapy for NAFLD.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a chronic liver disease in which triglycerides accumulate within the hepatocytes of patients with minimal or no alcohol intake and without any other known cause. It comprises a wide spectrum of liver damage ranging from steatosis to nonalcoholic steatohepatitis (NASH), advanced fibrosis and cirrhosis[1,2]. With the increasing incidence of obesity and diabetes mellitus, NAFLD has been recognized as a burgeoning health burden which affects 10%-24% of the general population and 70% of obese patients[3,4]. Approximately 30%-40% of individuals with simple steatosis progress to NASH, and NASH can progress to cirrhosis, which is a major risk factor for hepatocellular carcinoma[5,6]. Studies also have reported that NAFLD is a strong independent risk factor for cardiovascular disease[7].

Although many genetic and environmental factors contribute to the development of NAFLD, diet is an important environmental factor that can affect the development of NAFLD. High-fat diet is a widely-studied diet that can induce NAFLD[8] and is often used to induce animal models of the disease[9]. Recently, high-sugar diet, mainly as high-fructose diet, has been found to play an important role in the development of NAFLD[10-12]. However, unlike high-fat and high-sugar diets, high-protein diet can ameliorate NAFLD[13]. The results of these former studies suggest that different types of diets may have different effects on the development of NAFLD, and high-fat and high-sugar diets are NAFLD-inducing diets.

To understand the effects of diet on the development of NAFLD, the intestinal microbiota must be considered because it is the interface between diet and the liver. Germ-free mice are resistant to NAFLD induced by high-fat diet[14]. However, when the intestinal microbiota was introduced into germ-free mice, the mice showed a rapid increase in body fat content and liver triglycerides. When a high-fat diet induces NAFLD, it also causes dysbiosis of the intestinal microbiota[15]. Moreover, small bowel bacterial overgrowth is associated with NAFLD, and patients with NASH have a lower percentage of *Bacteroidetes*[16,17]. These studies show a close link between the intestinal microbiota and NAFLD. As diet can affect the development of NAFLD through

Table 1 Percentage of energy and energy density of the four diets

	Normal	High protein	High sugar	High fat
Protein (casein)	19.2%	60.0%	19.2%	19.2%
Sugar (fructose)	10.5%	10.5%	60.0%	10.5%
Fat (lard)	16.5%	16.5%	16.5%	60.0%
Energy density (kcal/kg)	3810	3810	3810	5179

the intestinal microbiota, it may be useful for us to understand the relationship between diet, intestinal microbiota, and NAFLD in order to prevent or treat this disease.

High-fat and high-sugar diets are associated with hyperphagia; however, high-protein diet can reduce caloric intake[18-20]. In order to understand the effects of different diets on the development of NAFLD more clearly, we restricted the caloric intake of rats in the high-fat and high-sugar diet groups to the same levels as rats in the high-protein diet group, to exclude caloric intake as a confounder. Given the important role that the intestinal microbiota plays in the pathogenesis of NAFLD, and that the intestinal microbiota is greatly influenced by diet, we examined the dietary effects on the intestinal microbiota.

MATERIALS AND METHODS

Animals, diets and experimental design

Thirty 5-wk-old male Sprague-Dawley rats (Hunan SJA Laboratory Animal Co. Ltd., China) were housed at a regulated temperature (21 ± 1.6 ℃), humidity (45% ± 10%), and an alternating 12-h light and dark cycle. After 1 wk of acclimation, rats were randomized into five groups (six rats each). The control diet (CON) group was allowed *ad libitum* access to a normal chow diet and the free high-fat diet (FFAT) group was allowed *ad libitum* access to a high-fat diet. The restrictive high-fat diet (RFAT) group, restrictive high-sugar diet (RSUG) group and high-protein diet (PRO) group had access to a high-fat diet, a high-sugar diet and a high-protein diet, respectively. The caloric intake of the RFAT and RSUG groups was calibrated to the same caloric intake as the PRO group. The energy density and percentage of energy derived from protein, sugar and fat in the different diets are shown in Table 1. Dietary foods were irradiated by Co^{60} and stored at 4 ℃, protected from air. Fresh diets were administered daily and any remaining food was weighed and discarded. Food intake was measured every day. The study was approved by the Animal Ethics Committee of Central South University and all efforts were made to minimize animal suffering.

Oral glucose tolerance test

Two days before the rats were sacrificed, Oral glucose tolerance test (OGTT) was performed. Briefly, 12-h fasted rats were administered 2 g/kg glucose orally. Blood samples were taken from the tail to measure blood glucose levels before and 30, 60, 90 and 120 min after glucose administration by using an OneTouch-UltraSmart Blood Glucose Monitoring System (LifeScan, Milpitas, United States).

Liver index and visceral fat index assay

After the rats were killed, the livers were isolated and weighed. Liver index was calculated as the ratio of liver to body weight. Mesenteric, retroperitoneal, and epididymal fat was isolated and weighed as visceral fat mass. The visceral fat index was calculated as the ratio of visceral fat to body weight.

Biochemical analysis

At the end of the experiment, all the animals were killed after a 12-h overnight fast. Blood samples were collected *via* cardiac puncture and centrifuged to obtain serum. The concentrations of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured by standard procedures. The level of serum insulin was assayed with an ELISA kit (Mercodia, Uppsala, Sweden). Insulin resistance (IR) was determined by the homeostasis model assessment (HOMA) formula: [HOMA-IR = fasting insulin (μU/mL) × plasma glucose (mmol/L)/22.5)][21]. One milliliter of portal blood was collected into an apyrogenic tube for the lipopolysaccharide (LPS) assay. The level of portal LPS was measured with a chromogenic limulus amoebocyte lysate test kit (Bokang, Zhanjiang, China).

Liver lipid measurement

Liver lipids were extracted using a modified chloroform/methanol method[22,23]. A small fragment of snap-frozen liver tissue was pulverized to a fine powder in liquid nitrogen. Approximately 50 mg of liver tissue was extracted twice in 1 mL of 2:1 (v/v) chloroform: methanol solution. The organic extracts were air dried and resuspended in 1 mL of 2% Triton X-100 solution. Liver triglycerides were measured colorimetrically using a Triglyceride Reagent Kit (Dongou, Wenzhou, China). Frozen sections stained with Oil Red O were also used for liver lipid detection.

High-throughput sequencing of bacterial 16S rRNA gene and bioinformatic analysis

Before the rats were killed, fecal samples were collected and stored at -80 ℃. 16S rRNA genes of V4 hypervariable region were amplified using specific primers (515F: 5'-GTGCCAGCMGCCGCGGTAA-3', 806R: 5'-GGACTACHVGGGTWTCTAAT-3') with the barcode. All polymerase chain reactions (PCRs) were carried out with Phusion High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, United States). PCR products were mixed in equidensity ratios. The mixture of PCR products was purified using a Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany). Sequencing

libraries were generated using the TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, United States). Lastly, the library was sequenced on an Illumina HiSeq2500 platform and 250 bp paired-end reads were generated.

QIIME pipeline (1.9.1) was used to process and analyze the 16S raw data for all samples[24]. Paired-end reads were merged using join_paired_ends.py script, and then the raw reads were demultiplexed and quality filtered (at Phred ⩾ Q20) using split_libraries_fastq.py script. The high quality reads were clustered into operational taxonomic units (OTUs) at 97% similarity using pick_open_reference_otus.py script. This script also picked a representative sequence for each OTU, and all representative sequences were taxonomically classified using the Greengene database gg_13_8. Sequence alignment was conducted using the PyNAST method and a phylogenetic tree was constructed. The phylogenetic tree was then used for Unweighted UniFrac principal coordinates analysis (PCoA). The chimera sequences were removed with pick_open_reference_otus.py script. To remove sampling depth heterogeneity, OTU abundance information was normalized using a standard sequence number corresponding to the sample with the least sequences. Subsequent analyses were all performed based on these normalized data.

Statistical analysis

Data are presented as mean ± SE. The differences in quantitative data were statistically analyzed using one-way analysis of variance. When differences were significant, *post hoc* comparisons were made using a Bonferroni multiple-comparison test. The relative abundance of different phyla and genera was compared between groups using the Mann-Whitney test. SPSS version 20.0 software was used for statistical analyses of the data. The results were considered significant at $P < 0.05$.

RESULTS

Caloric intake, body weight, and liver index

Figure 1A shows the mean caloric intake of the different groups of rats. The FFAT group, which was fed a diet containing 60% of calories from fat, consumed more calories than the other groups. The RFAT, RSUG and PRO groups, which were restricted to the same caloric intake, consumed fewer calories than the CON group. Figure 1B shows the body weight of each group of rats over time. At the end of the experiment, the FFAT group had the highest body weight, visceral fat index, and liver index among all the groups ($P < 0.05$, Figure 1C-E). The RFAT and RSUG groups displayed no significant differences in body weight and visceral fat index compared with the CON group ($P > 0.05$); however, the body weight and visceral fat index of these two groups were higher than those in the PRO group ($P < 0.05$, Figure 1C and D). Compared with the CON group, the PRO group showed a decrease in body weight ($P < 0.05$) but not visceral fat index ($P > 0.05$). There were no significant differences in liver index among the other groups ($P > 0.05$, Figure 1E).

Fasting serum insulin, IR, and OGTT

There were no significant differences in fasting blood glucose levels among the different groups (data not shown). However, compared with other groups, the FFAT group exhibited elevated fasting serum insulin and HOMA-IR values ($P < 0.05$; Figure 2A and B). Although the RFAT, RSUG and PRO groups showed no significant differences in fasting serum insulin and HOMA-IR values compared with the CON group ($P > 0.05$), the PRO group showed decreased fasting serum insulin and HOMA-IR values compared with the RFAT and RSUG groups ($P < 0.05$; Figure 2A and B). The OGTT confirmed the impaired glucose tolerance of the FFAT group (Figure 2C and D). The area under the curve (AUC) of the OGTT showed that the PRO group had decreased AUC_{OGTT} when compared with the RFAT and RSUG groups ($P < 0.05$), but these three groups showed no significant differences in AUC_{OGTT} values when compared with the CON group ($P > 0.05$; Figure 2D).

Portal LPS and liver function

At the end of the trial, portal LPS was measured. The FFAT group had higher levels of portal LPS compared with the other groups ($P < 0.05$; Figure 3A). Taking the same calories, the RFAT group showed higher levels of portal LPS than the PRO group ($P < 0.05$; Figure 3A). Serum ALT and AST are biomarkers of liver function, which can reflect liver injury. Only the FFAT group had higher serum ALT and AST than the other groups ($P < 0.05$), and other groups had the same level of serum ALT and AST (Figure 3B and C).

Liver triglycerides

The results of oil red O staining showed that the FFAT group had obvious accumulation of triglycerides in the liver (Figure 4A). However, the RFAT and RSUG groups had significantly less triglyceride accumulation in the liver than the FFAT group. The CON and PRO groups had no detectable triglyceride accumulation in the liver by oil red O staining. Colorimetric measurement confirmed the oil red O staining findings. Using colorimetric measurement, the FFAT group also showed increased liver triglyceride levels compared with the other groups ($P < 0.05$; Figure 4B). Among the three isocaloric groups, the level of liver triglycerides was higher in the RFAT and RSUG groups than in the PRO group ($P < 0.05$); however, when compared with the CON group, no significant differences were found ($P > 0.05$).

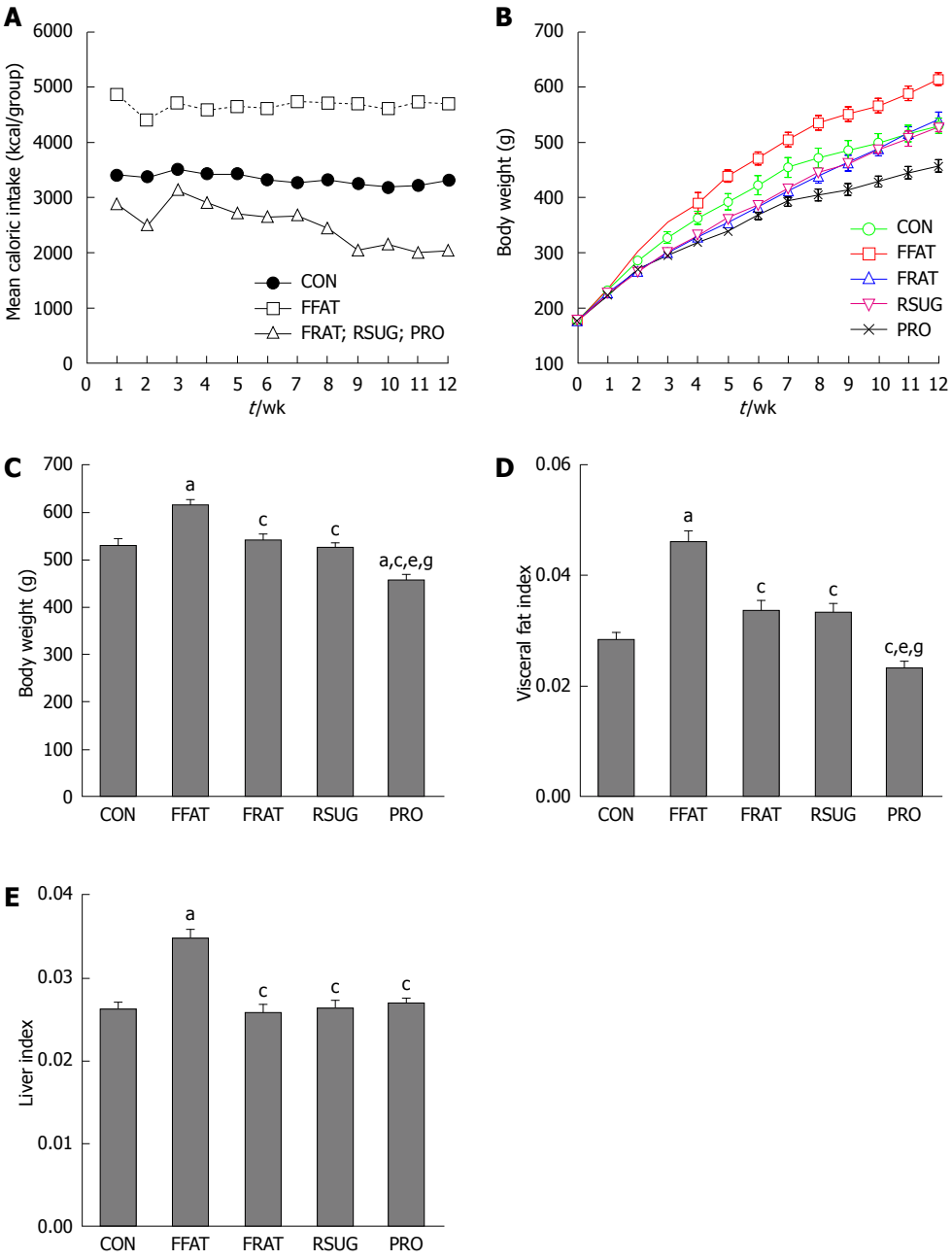

Figure 1 Caloric intake, body weight, visceral fat index, and liver index in rats. A: Mean caloric intake; B: Body weight at different experimental periods; C: Body weight at 12 wk; D: Visceral fat index, calculated as visceral fat weight/body weight; E: Liver index, calculated as liver weight/body weight. Differences were denoted as follows: $^{a}P < 0.05$ *vs* CON group rats, $^{c}P < 0.05$ *vs* FFAT group rats, $^{e}P < 0.05$ *vs* RFAT group rats, $^{g}P < 0.05$ *vs* restrictive high-sugar diet (RSUG) group rats.

Intestinal bacterial composition

A total of 1705045 high-quality reads (average of 56 835 sequences per sample) were delineated into OTUs at the 97% similarity level. Rarefaction curves revealed that all the samples had a similar pattern, which indicates that some new OTU would be expected with additional sequencing (Figure 5A). However, the good-coverage index, an indicator of sequencing depth, of all samples ranged from 0.968 to 0.977. Combining the results of the rarefaction curves and the good-coverage index, we demonstrated that the sequencing depth in the present study was sufficient to reflect the bacterial composition of different group samples.

Unweighted UniFrac PCoA revealed that despite inter-individual variation, the intestinal microbiota structures of the CON, FFAT, RSUG and PRO groups were roughly separated away from each other (Figure 5B). However, the intestinal microbiota structure of the RFAT group was not well separated.

For taxon-based analysis, a total of 15 phyla were detected and the most abundant phyla included *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Tenericutes* (Figure 5C). Compared with the CON group samples, the FFAT group samples showed an increased abundance of *Firmicutes*, decreased abundance of *Bacteroidetes*, and lower ratio of *Bacteroidetes* to

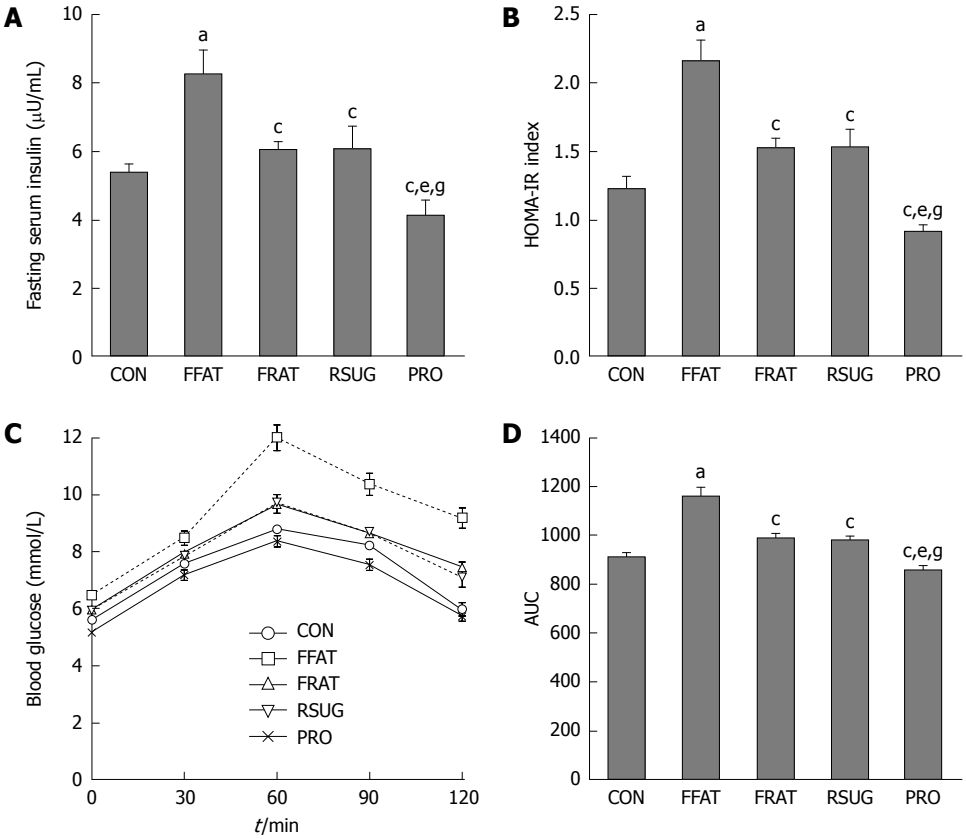

Figure 2 Fasting serum insulin, insulin resistance, and oral glucose tolerance test in rats. A: Fasting serum insulin; B: HOMA-IR, calculated according to the formula fasting insulin (μU/mL) × fasting glucose (mmol/L)/22.5; C: Oral glucose tolerance test (OGTT); D: Area under the curve (AUC) of OGTT. Differences were denoted as follows: $^{a}P < 0.05$ *vs* CON group rats, $^{c}P < 0.05$ *vs* free high-fat diet (FFAT) group rats, $^{e}P < 0.05$ *vs* RFAT group rats, $^{g}P < 0.05$ *vs* restrictive high-sugar diet (RSUG) group rats.

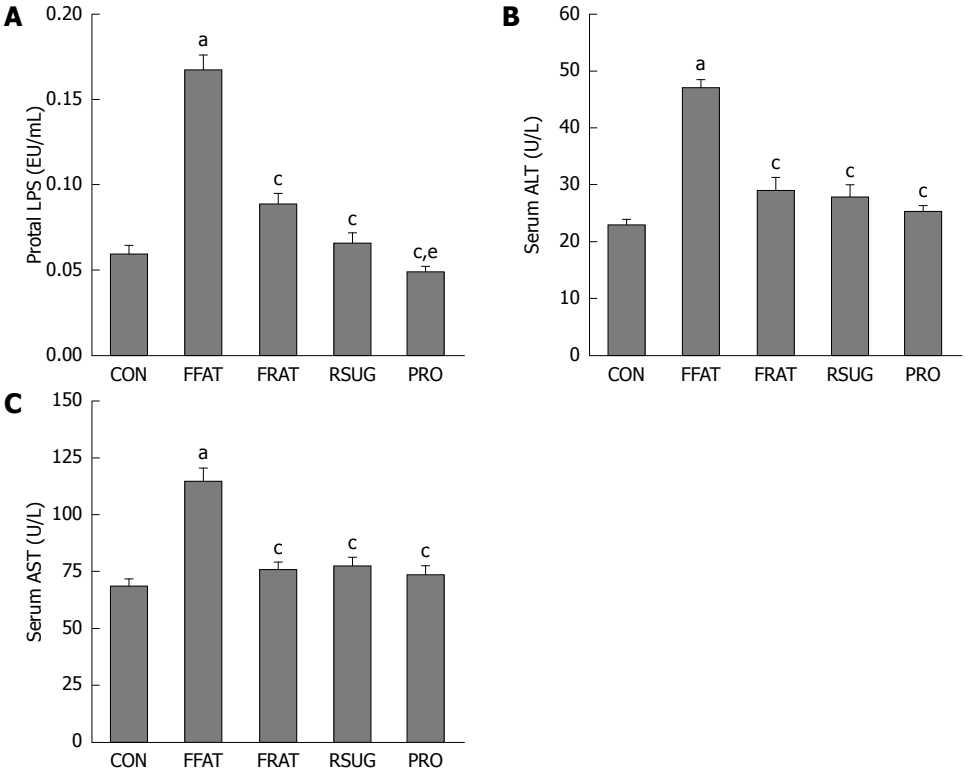

Figure 3 Portal lipopolysaccharide and liver function in rats. A: Portal LPS; B: Serum ALT; C: Serum AST. Values are expressed as mean ± SE. Differences were denoted as follows: $^{a}P < 0.05$ *vs* CON group rats, $^{c}P < 0.05$ *vs* FFAT group rats, $^{e}P < 0.05$ *vs* RFAT group rats. LPS: Lipopolysaccharide; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

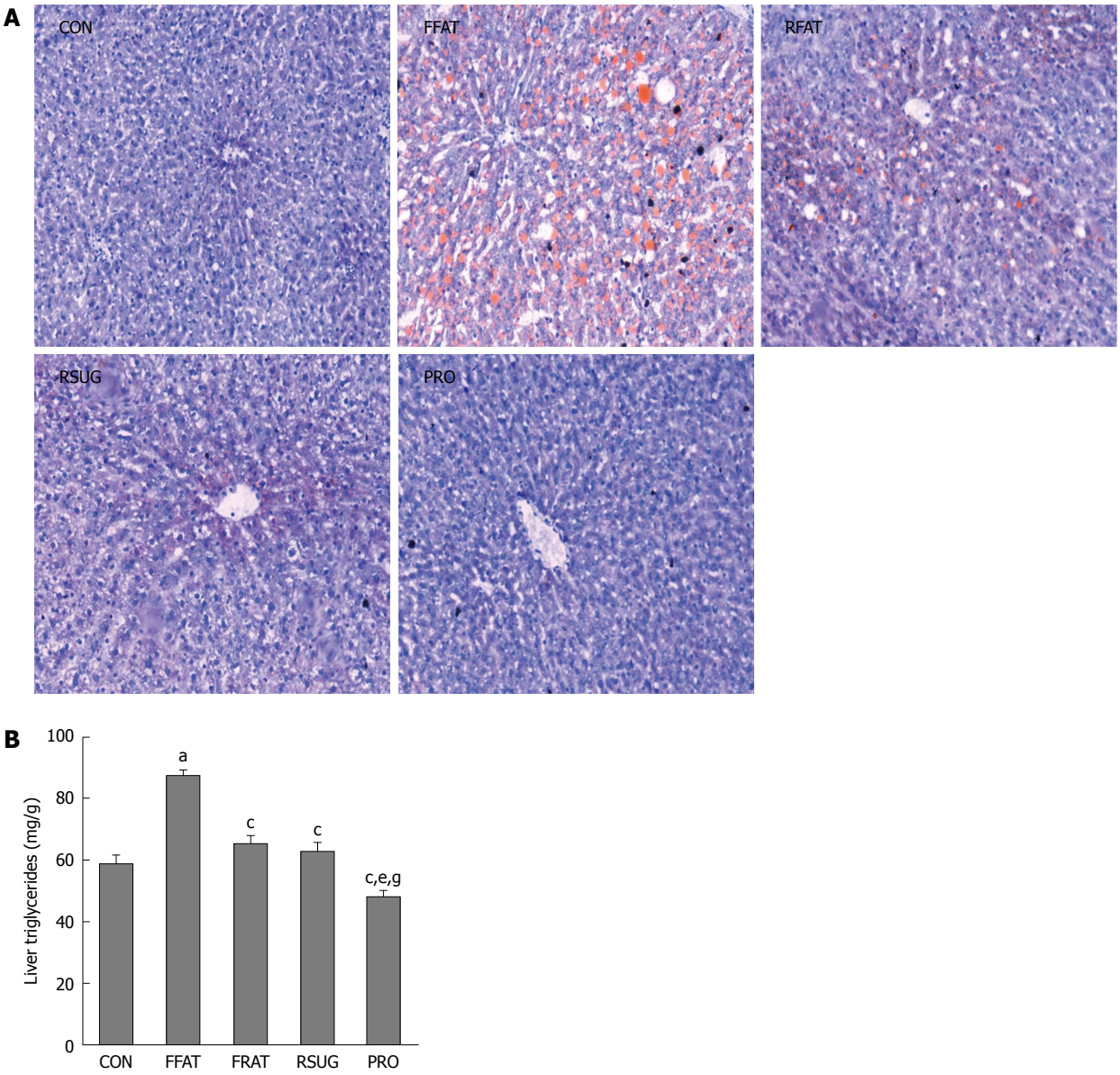

Figure 4 Liver triglycerides in rats. A: Liver sections stained with oil red O; representative photomicrographs were captured at magnification × 200; B: Liver triglycerides. Differences were denoted as follows: $^{a}P < 0.05$ *vs* CON group rats, $^{c}P < 0.05$ *vs* FFAT group rats, $^{e}P < 0.05$ *vs* RFAT group rats, $^{g}P < 0.05$ *vs* RSUG group rats. CON: Control diet; FFAT: Free high-fat diet; RSUG: Restrictive high-sugar diet; RFAT: Restrictive high-fat diet.

Firmicutes ($P < 0.05$; Figure 6A). The RFAT group samples showed only an increased abundance of *Firmicutes* ($P < 0.05$; Figure 6A). The RSUG group samples showed an increased abundance of *Bacteroidetes*, higher ratio of *Bacteroidetes* to *Firmicutes*, and decreased abundance of *Firmicutes* ($P < 0.05$; Figure 6A). The PRO group samples showed an increased abundance of *Bacteroidetes* and decreased abundance of *Firmicutes* ($P < 0.05$; Figure 6A). The FFAT and RFAT groups had the same high-fat diet, and the only difference between these two groups was the total caloric intake. However, compared with the FFAT group samples, the RFAT group samples showed an increased abundance of *Bacteroidetes*, higher ratio of *Bacteroidetes* to *Firmicutes*, and decreased abundance of *Firmicutes* ($P < 0.05$; Figure 6A).

At the genus level, a total of 109 genera were found, and we only selected the genera with a relative abundance > 1% for analysis. Compared with the CON group samples, the FFAT group samples showed an increased abundance of *Roseburia* and *Oscillospira*, and a decreased abundance of *Bacteroides* and *Parabacterioides* ($P < 0.05$; Figure 6B). The RFAT group samples showed a decreased abundance of *Parabacterioides* ($P < 0.05$; Figure 6B); the RSUG group samples showed an increased abundance of *Sutterella* ($P < 0.05$; Figure 6B); the PRO group samples showed an increased abundance of *Prevotella*, *Oscillospira,* and *Sutterella* ($P < 0.05$; Figure 6B). Compared with the FFAT group samples, the RFAT group samples showed a decreased

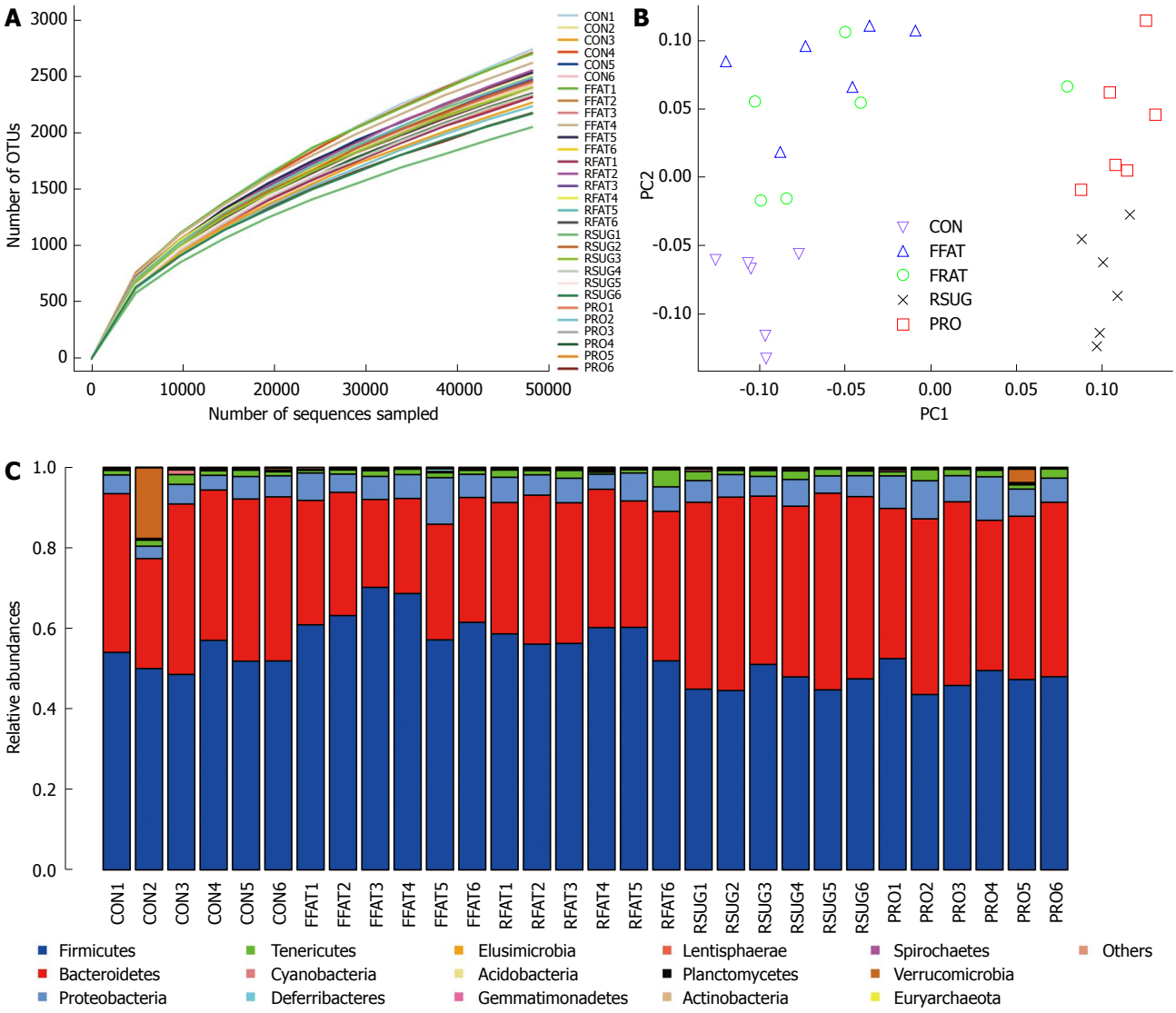

Figure 5 Bacterial composition of different group samples. A: Rarefaction curves (at a 97% similarity level); B: Unweighted UniFrac PCoA; C: Intestinal microbiota composition at the phylum level. FFAT: Free high-fat diet; RSUG: Restrictive high-sugar diet; RFAT: Restrictive high-fat diet; CON: Control diet.

abundance of *Oscillospira* ($P < 0.05$; Figure 6B).

DISCUSSION

We investigated the effects of different diets on the development of NAFLD, excluding caloric intake as a confounder. We demonstrated that high-fat and high-sugar diets in rats increased body weight, IR, and liver triglycerides than a high-protein diet, and that these effects were independent of caloric intake. We also found that different diets changed the intestinal microbiota composition independently from caloric intake. The different effects of these diets on NAFLD development, at the same caloric intake, may be associated with changes in the intestinal microbiota.

Obesity and IR are recognized as risk factors for NAFLD[25,26]. In most cases, NAFLD is a complication of obesity[4]. Peripheral IR may cause accumulation of liver triglycerides *via* three main mechanisms: increased peripheral lipolysis (which increases free fatty acids in the liver), hyperinsulinemia (which stimulates activity of the key lipogenic transcription factors) and hyperglycemia (which increases glucose concentration in the liver and provides substrates for lipogenesis)[27]. Free availability of a high-fat or high-sugar diet can cause rodent obesity, IR, and accumulation of liver triglycerides[28,29]. However, free availability of a high-protein diet has the opposite effect[14]. In the present study, we restricted the caloric intake of these three diets to the same level, and found that high-fat and high-sugar diets caused experimental rats to have greater body weight, peripheral IR, and accumulation of liver triglycerides than a high-protein diet. This suggests that, compared with a high-protein diet, the NAFLD-inducing effects of high-fat and high-sugar diets are independent from caloric intake. The results of the present study showed no significant difference in body weight, peripheral IR, and accumulation of liver triglycerides between the RFAT and CON groups. However, de Meijer *et al*[30] found that mice with a

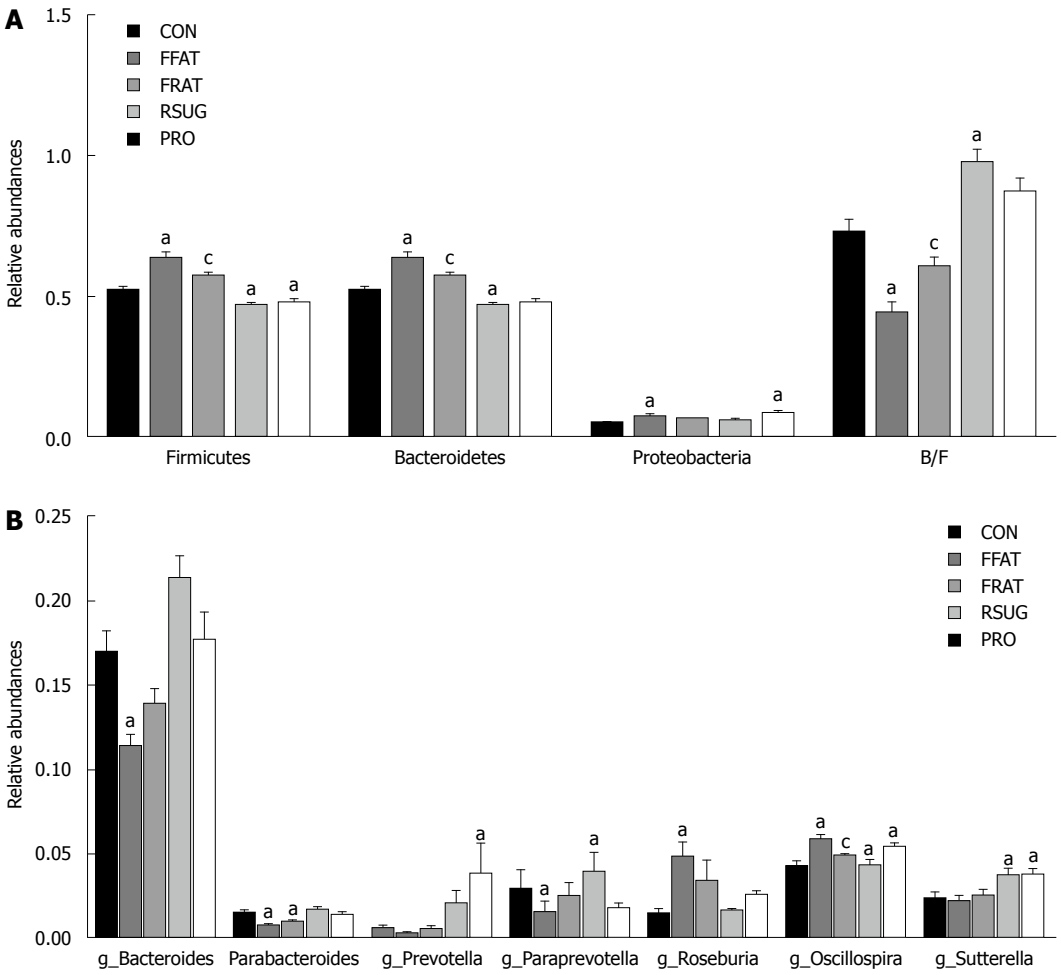

Figure 6 Comparison of relative abundance at the phylum and genus levels. A: Comparison of relative abundance at the phylum level; B: Comparison of relative abundance at the genus level. $^{a}P < 0.05$ *vs* CON group rats; $^{c}P < 0.05$, FFAT group rats *vs* RFAT group rats. B/F: Ratio of *Bacteroidetes* to *Firmicutes*; FFAT: Free high-fat diet; RSUG: Restrictive high-sugar diet; RFAT: Restrictive high-fat diet; CON: Control diet.

restrictive high-fat diet showed higher body weight, peripheral IR, and accumulation of liver triglycerides than the control diet mice. The main reason for this discrepancy may be the different restrictive levels that we chose. The study by de Meijer *et al*[30] restricted the caloric intake of the high-fat diet group to the same level as the control diet group; however, we restricted the caloric intake of the high-fat diet group to the same level as the high-protein diet group, which consumed fewer calories than the control diet group. This discrepancy may reflect the role that calories play in the development of NAFLD. Compared with the FFAT group, the RFAT group showed decreased body weight, peripheral IR, and accumulation of liver triglycerides. This confirms the role of calories in the development of NAFLD. We conclude that dietary composition and caloric intake are two independent factors that can affect the development of NAFLD.

LPS is a constituent of Gram-negative bacteria and can trigger the secretion of proinflammatory cytokines, such as tumor necrosis factor-α, when it binds to Toll-like receptor (TLR) 4 on the membrane surface of monocytes, macrophages, adipocytes and hepatocytes. After continuous subcutaneous infusion of LPS for 4 wk, wild-type mice exhibited obesity, adipose tissue weight gain, steatosis, and fasting hyperglycemia[31]. However, mice mutant for CD14 (a key multifunctional receptor that mediates the combination of LPS and TLR4) resisted most of the LPS-induced features of metabolic diseases. Therefore, LPS is considered a triggering factor in the development of metabolic disorders. In NAFLD patients, elevated LPS levels have been observed[32]. However, the relationship between different diets and LPS is unclear. In the present study, with the same caloric intake, rats fed a high-fat diet showed higher portal LPS levels than those fed a high-protein diet, suggesting that compared with a high-protein diet, a high-fat diet can elevate portal LPS level independently from caloric intake. Unexpectedly, although the RFAT group showed increased portal LPS levels, serum ALT and AST levels in the RFAT and PRO groups did not differ significantly. A possible explanatory hypothesis is that there is a threshold level of LPS concentration in the liver that induces obvious injury, and the liver can handle the elevated LPS which does not reach the threshold level. The higher levels of

portal LPS and serum ALT and AST in the FFAT group compared with the RFAT group partly confirm this hypothesis.

The human intestine harbors > 2000 species of commensal bacteria that define the intestinal microbiota. This community consists of 10 times more bacteria than human cells and encodes 100-200-fold more genes than our own genome[33,34]. The intestinal microbiota provides the host with enhanced metabolic capabilities (fermentation of nondigestible dietary residue, production of vitamin K, and absorption of ions), protection against pathogens, education of the immune system, and modulation of gastrointestinal development. So the intestinal microbiota can be viewed as a "metabolic organ". This organ can affect host metabolic processes[35,36]. Although the composition of the intestinal microbiota is influenced by multiple factors, diet plays an important role in shaping it[37,38]. In the present study, Unweighted UniFrac PCoA results showed that the intestinal microbiota structures of the CON, FFAT, RSUG and PRO groups were roughly separated away from each other; however, the intestinal microbiota of the RFAT group was not well separated. However, taxon-based analysis found that intestinal microbiota composition of the RFAT, RSUG and PRO groups differed from that of the CON group. These results suggest that different diets can affect the composition of the intestinal microbiota independently from caloric intake. This important finding may be helpful in better understanding the effect of diet on the composition of the intestinal microbiota. In the present study, the RSUG group showed an increased abundance of *Bacteroidetes*. Some species of bacteria in this phylum, such as *Bacteroides thetaiotaomicron*, can encode adequate carbohydrate active enzymes for carbohydrate metabolism of food[39]. This enables the host to extract more energy from the diet, which will be deposited in the liver in the form of triglycerides. Unlike the RSUG group, the RFAT group showed an increased abundance of *Firmicutes*; however, how the bacteria in this phylum affect NAFLD development is not clear. The effect of a high-protein diet on the composition of the intestinal microbiota is not well studied. We found that the PRO group had an increased abundance of *Bacteroidetes* and *Sutterella* bacteria, and decreased abundance of *Firmicutes*. This change in intestinal microbiota was similar to that in the RSUG group; however, the PRO group also had an increased abundance of *Prevotella* and *Oscillospira*. Kovatcheva-Datchary *et al*[40] found that *Prevotella* is associated with improvement in glucose metabolism. *Oscillospira* has never been cultured, so it is an enigmatic bacterial genus and little is known of it role in the intestinal tract. However, recent studies found that *Oscillospira* is positively associated with leanness, and is reduced in pediatric NASH[41,42]. We conclude that the beneficial effects of high-protein diet on NAFLD may be closely associated with *Prevotella* and *Oscillospira*, which requires further study. Changes in intestinal microbiota are one mechanism by which diet affects NAFLD development, so we can conclude that the different effects of these diets on NAFLD development, at the same caloric intake, may be associated with changes in intestinal microbiota. However, the exact role that these different bacteria play in the development of NAFLD remains unclear and needs further study. Previous studies have found that high-fat diet reduces *Bacteroides* and increases *Firmicutes* and *Proteobacteria*. The ratio of *Bacteroidetes* to *Firmicutes* was also decreased by a high-fat diet[43,44]. The results of the present study confirmed these findings. Moreover, we found that the high-fat diet increased *Roseburia* and *Oscillospira* spp. However, Neyrinck *et al*[45] have reported that the number of *Roseburia* spp. was decreased when mice were fed a high-fat diet. As host genotype is also an important factor that can affect the intestinal microbiota, the reason for this contrary result may be mainly due to the different animals that were used[21]. Taxon-based analysis also found that intestinal microbiota composition of the RFAT group was different from that of the FFAT group, suggesting that, except for different dietary composition, caloric intake is an independent factor that can shape the intestinal microbiota.

Because of the limitations of the bacterial 16S rRNA gene sequencing technique that we adopted, we could not classify sequences at the species level, and the relationship between intestinal bacteria and portal LPS levels was undefined. It should be pointed out that the results of the present study were obtained from rats and host genotype also can affect intestinal microbiota composition, so it may not be appropriate to apply our results directly to humans.

In conclusion, our present study found that compared with the high-protein diet, the NAFLD-inducing effect of the high-fat and high-sugar diets is independent from caloric intake. This helps in understanding the effects of diet on the development of NAFLD. In addition, the effects of diet on the intestinal microbiota in the present study extend our knowledge of the relationship between diet and the intestinal microbiota. In the future, we can manipulate the intestinal microbiota by diet to prevent or treat NAFLD. Overall, our findings shed some light on the desirability of dietary therapy for NAFLD.

COMMENTS

Background

Although several studies have elaborated the relationship between different diets and nonalcoholic fatty liver disease (NAFLD) development, those studies had the confounder of caloric intake. The intestinal microbiota is the interface between diet and the liver; however, the relationship between diet, intestinal microbiota and NAFLD is unclear.

Research frontiers

Previous studies have shown that high-fat and high-sugar diets can induce NAFLD; however, a high-protein diet can ameliorate it. Diet is also an important factor that can shape the intestinal microbiota, and NAFLD patients are always

associated with changes in intestinal microbiota.

Innovations and breakthroughs

To the best of the authors' knowledge, this is the first study to evaluate the effects of different diets on the intestinal microbiota and NAFLD development at the same caloric intake. This study suggests that compared with a high-protein diet, the NAFLD-inducing effect of high-fat and high-sugar diets is independent from caloric intake. The different effects of these diets on NAFLD development, at the same caloric intake, may be associated with changes in the intestinal microbiota.

Applications

This study extends the authors' knowledge of the relationship between diet, intestinal microbiota, and NAFLD. It sheds some light on the desirability of dietary therapy for NAFLD.

Terminology

Unweighted UniFrac principal coordinates analysis is a method used to discriminate the microbiota composition of the different groups based on evolutionary distance.

Peer-review

In this paper, the authors examined the effects of different diets on NAFLD and intestinal microiota. They found that high-fat diet and high-sugar diet but not high-protein diet induced NAFLD independently from calories. Also diet affects the microbiota.

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ORIGINAL ARTICLE

Observational Study

Prevalence of colorectal neoplasms in young, average risk individuals: A turning tide between East and West

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Abstract

AIM

To determine the prevalence of colorectal neoplasia in average risk persons 40-59 years of age in Israel and to compare the results with other populations.

METHODS

We reviewed the results of asymptomatic average-risk subjects, aged 40 to 59 years, undergoing their first screening colonoscopy between April 1994 and January 2014. The detection rates of adenoma, advanced adenoma (AA) and colorectal cancer (CRC) were determined in the 40's and 50's age groups by gender. The prevalence of lesions was compared between age groups. After meticulous review of the literature, these

results were compared to published studies addressing the prevalence of colorectal neoplasia in similar patient groups, in a variety of geographical locations.

RESULTS

We included first screening colonoscopy results of 1750 individuals. The prevalence of adenomas, AA and CRC was 8.3%, 1.0% and 0.2% in the 40-49 age group and 13.7%, 2.4% and 0.2% in the 50-59 age group, respectively. Age-dependent differences in adenoma and AA rates were significant only among men (P < 0.005). Literature review disclosed 17 relevant studies. As expected, in both Asian and Western populations, the risks for overall adenoma and advanced adenoma was significantly higher in the 50's age group as compared to the 40's age group in a similar fashion. The result of the current study were similar to previous studies on Western populations. A substantially higher rate of adenoma, was observed in studies conducted among Asian populations in both age groups.

CONCLUSION

The higher rate of colorectal neoplasia in Asian populations requires further investigation and reconsideration as to the starting age of screening in that population.

Key words: Colonoscopy; Adenoma; Colorectal cancer; Average risk; Young; Asian; Western

Core tip: This research focuses on evaluating detection rates of colorectal neoplasia among average risk individuals aged 40-59 years and comparing the detection rates between the fifth and sixth decades. In this prospective study of first screening colonoscopy from 1750 consecutive average risk subjects aged 40-59, we found that the prevalence of colorectal neoplasia is age and gender dependent. In addition we did an extensive search of the literature that revealed a markedly higher adenoma detection rate among Asians, and in particular Koreans compares to Western populations.

Leshno A, Moshkowitz M, David M, Galazan L, Neugut AI, Arber N, Santo E. Prevalence of colorectal neoplasms in young, average risk individuals: A turning tide between East and West. *World J Gastroenterol* 2016; 22(32): 7365-7372 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i32/7365.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i32.7365

INTRODUCTION

Colorectal cancer (CRC) is the third leading cause of cancer mortality in the Western world[1,2], with an estimated lifetime risk of 5%-6%. Approximately 70% of patients who develop CRC are considered "average risk"[3]. The incidence and mortality rates of CRC increase after 50 years of age, the time that screening should be initiated. Only a small number of studies focusing on the prevalence of adenomas among persons younger than 50 years have been published and revealed variable results.

The aim of this study was to determine the prevalence of colorectal neoplasia in average risk persons in the fifth and sixth decades and to compare it to other populations from various geographical areas.

MATERIALS AND METHODS

Study design

Between April 1994 and June 2014, we have been prospectively collecting and documenting the results of screening colonoscopies carried out by several senior gastroenterologists from the Division of Gastroenterology at the Tel-Aviv Medical Center (TASMC). In addition to endoscopic reports, all participants were interviewed by the physician regarding symptoms, details of recent laboratory tests, and any personal or family history. The collected data were entered into a computerized database. Consecutive first screening colonoscopy of asymptomatic average-risk (*i.e.*, no personal or family history of colorectal CRC or CRC related syndrome) subjects aged 40-59 years old at the time of colonoscopy were included.

Individuals were excluded if they reported a personal history of colorectal adenoma or carcinoma at any time; if they had a family history of colorectal adenoma or carcinoma (one first-degree relative aged < 70, or two or more family relatives at any age); if they reported symptoms suggestive of neoplasia (rectal bleeding, change in bowel habit, abdominal pain, or unexplained weight loss during the previous 6 mo); or if they had a positive fecal occult blood test (FOBT), or laboratory abnormalities, such as iron-deficiency anemia. Other exclusion criteria were inflammatory bowel disease (IBD) or severe co-morbidity (*e.g.*, malignancy, significant cardio-pulmonary, renal or hepatic diseases). Mild complaints (such as chronic constipation without a recent change in bowel habit, or minor anal or abdominal discomfort, such as flatulence or bloating) were not regarded as neoplasia-related symptoms and were not considered to be exclusion criteria. Written informed consent was obtained from all participants. The study was approved by the TASMC Helsinki Committee. Colonoscopy was performed according to the usual protocol in our institute, as previously described[4].

Literature review

English language medical literature searches for human studies were performed through June 2015 using "screening", "colonoscopy", and "average risk" as keywords. Articles describing the prevalence of

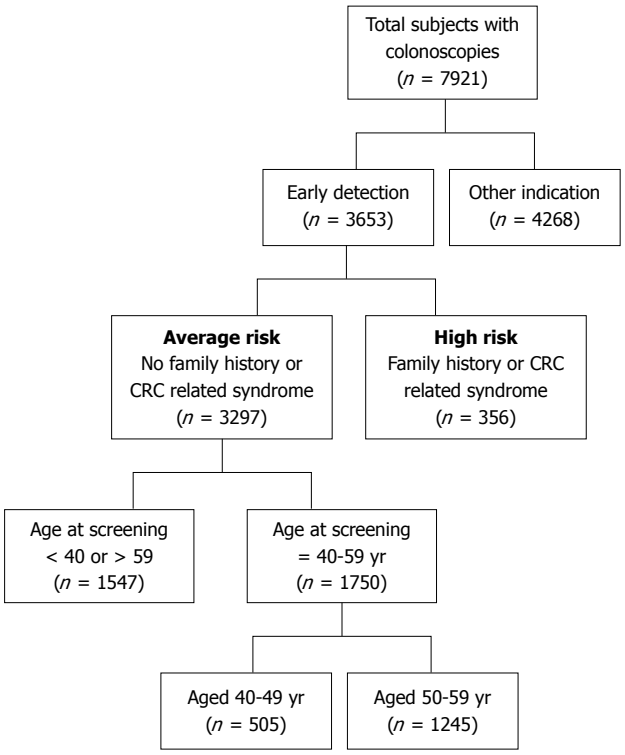

Figure 1 Flow chart for selection of study population.

colorectal neoplasia during screening colonoscopy among asymptomatic persons in their fifth and sixth decades were used to compare the current report.

Statistical analysis

Statistical analyses were performed using IBM SPSS 21.0 software. The statistical significance level was set to 0.05. The prevalence of lesions was presented as proportions with 95%CI. Age was considered as a categorical variable, divided into two groups (40-49, 50-59 years). Pearson's χ^2 tests and student's *t*-test were used for comparison of lesion prevalence according to sex and age group.

RESULTS

Data were collected from a total of 7921 subjects who underwent colonoscopy of which 3653 were primary screening procedures. After exclusion of subjects aged above 59 or below 40 years at the time of the procedure, there were 1750 eligible subjects for final analysis (Figure 1).

The mean age of the subjects was 51.9 ± 5.1 years and men comprised 52.3% of the study population. There were 505 subjects aged 40-49 (mean age 45.3 ± 2.8 years) of whom 278 (55.0%) were men. In the 50-59 age group (mean age 51.9 ± 5.09), there were 1245 screenees, of whom 916 (51.2%) were men. Demographic features are shown in Table 1.

All lesions were sampled and sent for pathological examination. If more than one lesion was detected, the colonoscopic findings were classified according to the most advanced lesion, *e.g.*, adenocarcinoma, adenoma, hyperplastic or inflammatory polyp. Advanced neoplasia was defined as any adenoma measuring more than 10 mm, or with villous or tubulovillous histology, adenoma with high-grade dysplasia, or cancer[5,6]. Of the 1750 study subjects, 212 (12.1%) were found to have one or more colorectal neoplasia (non-advanced adenoma 10.4%; advanced 2.0%; invasive cancer 0.2%). The prevalence of colorectal neoplasia in the two age brackets according to gender is illustrated in Table 2. Among the 40-49 year age group, 43 (8.5%) individuals had adenomas (7.5% non-advanced, 1% advanced). Among the 50-59 year age group, 174 (13.7%) individuals had adenomas (11.6% non-advanced, 2.4% advanced, P = 0.01 and 0.06 respectively). A total of four (0.2%) cases of adenomas with high-grade dysplasia were detected all of which were in the 50's age group. Four (0.2%) CRC cases were detected (three in the 40's and one in the 50's year groups). The increased prevalence of neoplastic findings among the 50's age group was significant only among men for both adenomatous polyps and advanced adenoma (P = 0.001 and 0.005, respectively). No differences were observed for women (P = 0.435 and 0.744, respectively). The difference in CRC prevalence rate between the age and gender groups didn't reach statistical significance most probably due to the small sample size.

Table 1 Group size, mean age and male percentage according to age study group

Age study group	40-49 yr	50-59 yr	Total
Number of subjects	505	1245	1750
Mean age (yr) at colonoscopy (SD)	45.30 (2.81)	54.64 (2.83)	51.94 (5.09)
Male percentage (%)	55	51.2	52.3

Literature review disclosed 17 relevant studies (Table 3). The adenoma detection rate (ADR) and advanced lesion in each study is depicted in Figures 2 and 3, respectively. As expected, in both Asian and Western populations, the risks for overall adenoma and advanced adenoma was significantly higher in the 50's age group as compared to the 40's age group in a similar fashion (Table 4).

The most prominent observation is the markedly higher ADR among Asians, and in particular Koreans for both age groups (OR = 2.56, 95%CI: 2.04-2.51; and OR = 2.64, 95%CI: 2.09-2.23, in the 40's and 50's age groups, respectively, P < 0.0001).This difference between Western and Asian studies is significantly diminished in both age groups with regard to advanced adenoma and CRC rates. The prevalence of advanced lesions had a narrower range between studies (range between 1.1% to 3.7% in the 40's group and between 1.5% to 7.5% in the 50's age group), and in contrast to ADR, the detection rate of advanced lesions was significantly lower in the Asian

Table 2 Comparison of detection rates of neoplastic findings on primary screening between subjects aged 40-49 and subjects aged 50-59 years, according to gender *n* (%)

Gender finding type	40-49 yr	50-59 yr	Total	*P* value[1]	OR (95%CI)
All					
Total colonoscopies	505	1245	1750		
Adenomatous polyps	42 (8.3)	170 (13.7)	212 (12.1)	0.002	1.74 (1.22-2.49)
Advanced adenoma	5 (1.0)	30 (2.4)	35 (2.0)	0.060	2.47 (0.95-6.40)
Cancer	1 (0.2)	3 (0.2)	4 (0.2)	1.000	1.22 (0.13-11.73)
Men					
Total colonoscopies	278	638	916		
Adenomatous polyps	23 (8.3)	106 (16.6)	129 (14.1)	0.001	2.21 (1.37-3.55)
Advanced adenoma	1 (0.4)	22 (3.4)	23 (2.5)	0.005	9.89 (1.33-73.76)
Cancer	1 (0.4)	2 (0.3)	3 (0.3)	1.000	0.87 (0.08-9.65)
Women					
Total colonoscopies	227	607	834		
Adenomatous polyps	19 (8.4)	64 (10.5)	83 (10.0)	0.435	1.29 (0.76-2.21)
Advanced adenoma	4 (1.8)	8 (1.3)	12 (1.4)	0.744	0.75 (0.22-2.50)
Cancer	0 (0.0)	1 (0.2)	1 (0.1)	1.000	1

[1]χ^2 statistical comparison of distribution of selected findings.

Table 3 Studies reporting detection rates of adenoma and advanced lesion among average risk individuals aged 40-49 and 50-59 years

Ref.	Country of origin	Year published	Number of subjects in age group	
			40-49 yr	50-59 yr
Western origin				
de Jong *et al*[17]	the Netherlands	2005	90	36
Regula *et al*[8]	Poland	2006	2392	37313
Boursi *et al*[18]	Israel	2009	262	672
Current study	Israel	-	505	1245
Strul *et al*[4]	Israel	2006	183	409
Imperiale *et al*[6]	United States	2002	906	1533
Thoma *et al*[19]	United States	2010	247	747
Rundle *et al*[20]	United States	2008	553	352
Studies of Western origin (8)			5138	40774
Asian origin				
Hemmasi *et al*[21]	Iran	2014	333	407
Chiu *et al*[22]	Taiwan (China)	2005	654	592
Chiu *et al*[23]	Taiwan (China)	2008	4161	4211
Liu *et al*[24]	Taiwan (China)	2005	2656	1903
Choe *et al*[25]	South Korea	2006	1875	1587
Hong *et al*[26]	South Korea	2010	1049	712
Kim *et al*[27]	South Korea	2013	4550	4436
Chung *et al*[28]	South Korea	2009	1930	2716
Park *et al*[29]	South Korea	2009	1057	1207
Ko *et al*[30]	South Korea	2012	1200	1038
Studies of Asian origin (9)			19465	18807
All studies (17)			24603	59581

Table 4 Odds ratio for colorectal neoplasia detection among individuals aged 50-59 years compared to those aged 40-49 according to country of origin

Finding	OR (95%CI)	*P* value[1]
Studies of Western origin		
Overall adenomas	1.7 (1.53-1.88)	< 0.0001
Advanced adenoma	2.34 (1.95-2.81)	< 0.0001
Cancer	2.33 (1.45-3.75)	< 0.0001
Studies of Asian origin		
Overall adenomas	1.99 (1.89-2.09)	< 0.0001
Advanced adenoma	2.36 (2.05-2.72)	< 0.0001
Cancer	3.88 (2.41-6.23)	< 0.0001
Total		
Overall adenomas	1.25 (1.20-1.30)	< 0.0001
Advanced adenoma	2.65 (2.38-2.94)	< 0.0001
Cancer	3.39 (2.45-4.68)	< 0.0001

[1]χ^2 statistical comparison of distribution of selected findings.

population (OR = 0.79, 95%CI: 0.72-0.86, $P < 0.0001$; and OR = 0.78, 95%CI: 0.63-0.97, $P = 0.027$). The rate of CRC ranged from 0% to 0.7% in the 40's age group and 0.1% to 0.9% in the 50's age group. The differences between Asian and Western populations in CRC rate were not statistically significant.

DISCUSSION

Overall, the 50 year age group had higher rate of colorectal neoplasia as compared to the 40 year age group, similar to all studies across the world. There is a significant increased risk for colorectal neoplasia, in both age groups, between Western and Asian origins.

The differences between the two age brackets are statistically significant among men only. It is consistent with previous studies which found that women have a lower risk for colorectal neoplasia across all age groups[7]. Regula *et al*[8] and Ferlitsch *et al*[9] observed that men tend to develop advanced adenomas a decade earlier than women. The current study is limited to subjects younger than 60 years which can explain the lack of difference among women.

The findings in an Israeli population are comparable with observations from previous studies conducted in the West, indicating that age and male gender are the most important risk factors for the development of colorectal adenomas. A review of studies from Western

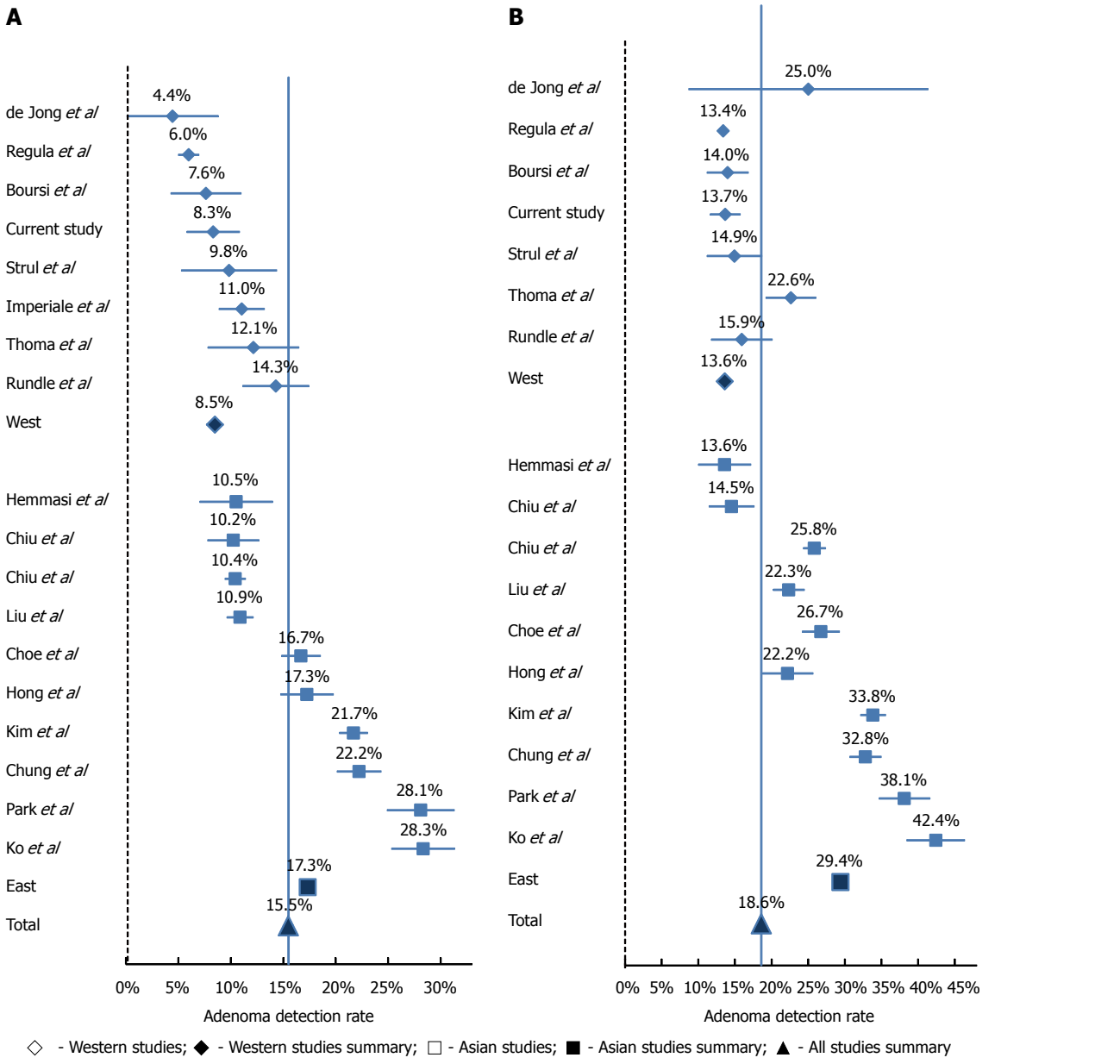

Figure 2 Overall adenoma detection rate in 40's (A) and 50's (B) age groups.

countries, including Israel, revealed heterogeneity in the prevalence rates of adenomas and advanced adenomas. ADR and the detection of advanced adenomas were slightly lower in Israel than in other countries. Various factors might explain these discrepancies, mainly differences in genetic and environmental backgrounds. The use of different study design is another possible explanation.

The current study included real average population as they were all asymptomatic subjects with no family history of CRC. Other studies, like the landmark study of Regula *et al*[8], included patients with a family history of CRC.

Herein, it can be confirmed that CRC screening in the West should begin earlier than the age of 50.

During our review of the literature we detected a secondary important finding regarding a significantly increased ADR among Asians, and in particular from the Far East, as compared to those from Western countries. This observation is consistent with reports showing an increase in CRC in eastern countries[10] which is considered the result of rapid economic development and life style modification. Shin *et al*[11] reported a rapid increase in CRC incidence in Korea between 1999-2009 in both men and women. Although in part attributed to the introduction of colorectal cancer screening, they concluded that transition in risk factors (*e.g.*, alcohol consumption, obesity and increased meat consumption) also has a significant role in the increase of CRC incidence.

Interestingly, despite the increased overall adenoma rate in Asian countries, the prevalence of advanced adenomas did not increase in similar proportions, and were even lower than in the West. This observation

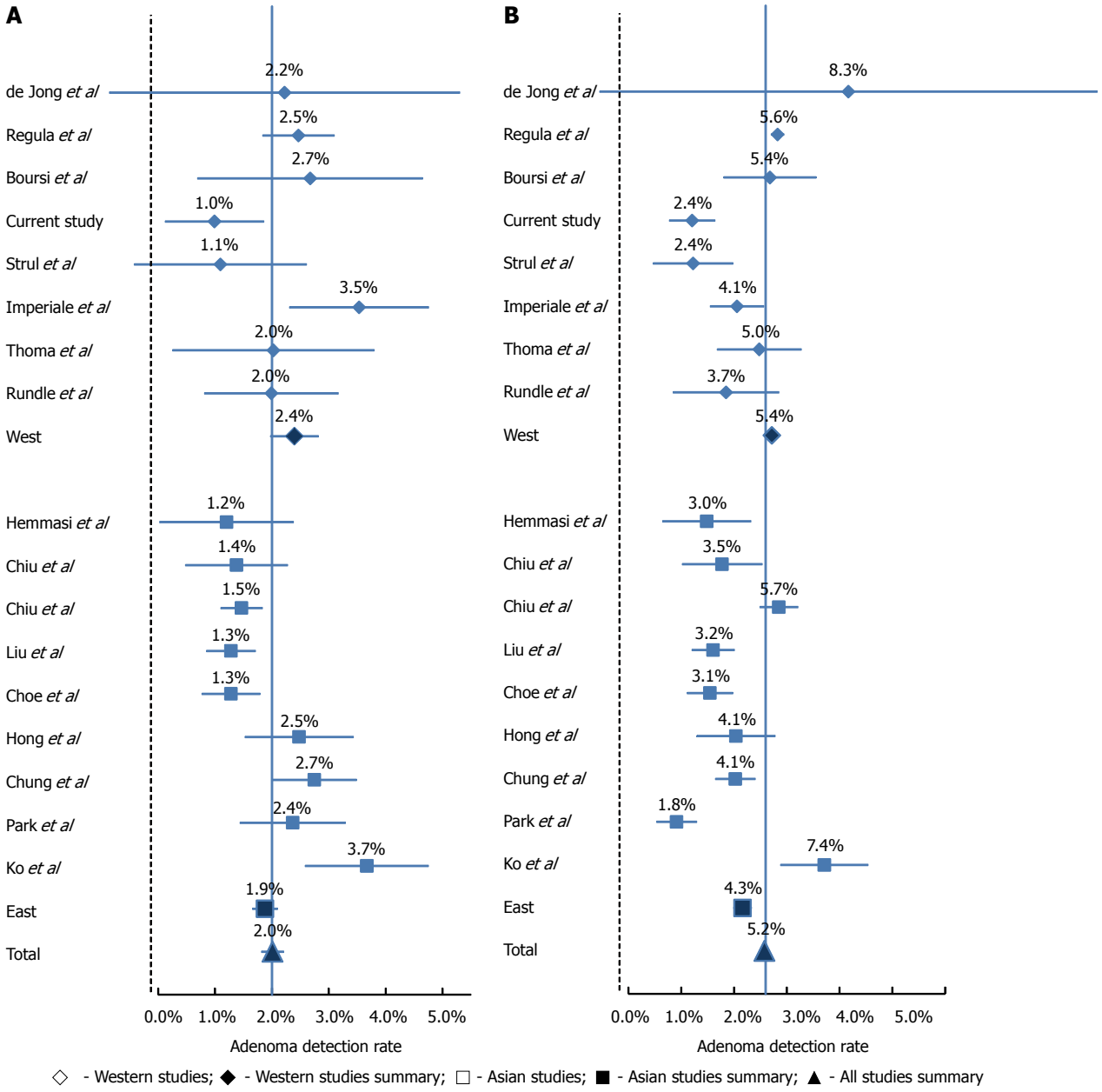

Figure 3 Advanced adenoma detection rate in 40's (A) and 50's (B) age groups.

suggests a slower rate of adenoma progression in Eastern Asia which may be due to genetic and/or environmental factors.

The high rates of colorectal neoplasia in East Asia population, is of great significance regarding immigrants from these countries to the West and specifically the United States. Ladabaum and colleagues[12] calculated CRC incidence rates in foreign-born Asian ethnic subgroups in California and found significant impacts of nativity and residence in an ethnic enclave on CRC incidence, suggesting a substantial effect of acquired environmental factors. In contrast to other minorities, such as African Americans, studies regarding the rate of polyps among East Asian immigrants aged 40-59 in the United States is limited, while there is a lot of information about the poor adherence of this population to CRC screening programs. In fact, Asian Americans have been described as one of the underrepresented and hard-to-reach populations[13]. Several studies have found that Asian Americans have lower CRC screening rates than non-Hispanic white Americans[14,15]. Moreover, Oh *et al*[16] described lower rates of CRC screening knowledge and uptake among Korean Americans compared to Asian Americans as a whole.

In conclusion, the detection rates of colorectal neoplasms among asymptomatic average-risk subjects in this study were compatible with previous results of Western origin, confirming the current guidelines to begin screening for CRC at the age of 50. The substantial differences between Western and East Asian populations in terms of overall adenoma and advanced adenoma

detection rates mandate further investigation and imply the need for specific screening recommendation for this population.

COMMENTS

Background

Colorectal cancer (CRC) is the third leading cause of cancer mortality in the Western world and approximately 70% of patients who develop CRC are considered "average risk". According to previous studies, the incidence and mortality rates of CRC increase after 50 years of age, and therefore screening should be initiated at this age. However, only few studies focused on the prevalence of adenomas among persons younger than 50 years and those that have been published revealed variable results.

Research frontiers

As the rate of CRC increases over time, it is important to re-evaluate and examine the detection rates of screening colonoscopy among average-risk individuals to better determine the the recommended age to begin screening. It is of no less importance to search for CRC risk factors including gender and origin.

Innovations and breakthroughs

The present study confirms the current guidelines to begin screening for CRC at the age of 50. Environmental factors such as diet and lifestyle are known to have a significant effect on risk for CRC. The substantial differences between Western and East Asian populations in terms of overall adenoma and advanced adenoma detection rates observed in this study is a clear proof that such factors should be taken into consideration.

Applications

The results of this study mandate further investigation and imply the need for specific screening recommendation for the Asian population especially among immigrants to western countries.

Terminology

CRC is the third leading cause of cancer mortality in the Western world. As in most cancers the best treatment is detection and removal of premalignant lesions by colonoscopy. Currently the recommended age for screening is 50 years in which these lesion start to develop.

Peer-review

This is an interesting and well-designed study, addressing the issue of CRC screening in two year groups, 40-50 and 50-59, in an Israeli population. The results are comparable with studies conducted in the West, but interestingly with a slightly lower prevalence rate of adenomas and advanced adenomas in Israel.

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ORIGINAL ARTICLE

Randomized Controlled Trial

Carbon dioxide insufflation in esophageal endoscopic submucosal dissection reduces mediastinal emphysema: A randomized, double-blind, controlled trial

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Author contributions: Maeda Y and Hirasawa D conceived of and designed this study; Maeda Y, Hirasawa D, Ohira T, Harada Y, Yamagata T, Suzuki K and Koike Y acquired the data; Maeda Y and Hirasawa D performed analysis and interpretation of the data; Maeda Y drafted the manuscript and performed statistical analysis; Hirasawa D and Fujita N provided administrative, technical and material support and supervised the study.

Institutional review board statement: This study was approved by the institutional review board of Sendai City Medical Center.

Clinical trial registration statement: The trial was registered with the UMIN Clinical Trials Registry (No. UMIN000006441).

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Conflict-of-interest statement: None of the authors have any conflicts of interest concerning this clinical research.

Data sharing statement: No additional data are available.

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Abstract

AIM

To assess the efficacy of CO_2 insufflation for reduction of mediastinal emphysema (ME) immediately after endoscopic submucosal dissection (ESD).

METHODS

A total of 46 patients who were to undergo esophageal ESD were randomly assigned to receive either CO_2 insufflation (CO_2 group, n = 24) or air insufflation (Air group, n = 22). Computed tomography (CT) was carried out immediately after ESD and the next morning. Pain and abdominal distention were chronologically recorded using a 100-mm visual analogue scale (VAS). The volume of residual gas in the digestive tract was measured using CT imaging.

RESULTS

The incidence of ME immediately after ESD in the CO_2 group was significantly lower than that in the Air group (17% *vs* 55%, P = 0.012). The incidence of ME the next morning was 8.3% *vs* 32% respectively (P

= 0.066). There were no differences in pain scores or distention scores at any post-procedure time points. The volume of residual gas in the digestive tract immediately after ESD was significantly smaller in the CO_2 group than that in the Air group (808 mL *vs* 1173 mL, P = 0.013).

CONCLUSION

CO_2 insufflation during esophageal ESD significantly reduced postprocedural ME. CO_2 insufflation also reduced the volume of residual gas in the digestive tract immediately after ESD, but not the VAS scores of pain and distention.

Key words: Endoscopic submucosal dissection; Carbon dioxide insufflation; Mediastinal emphysema; Superficial esophageal cancer; Complication

Core tip: This randomized, double-blind, controlled trial assessed the efficacy of CO_2 insufflation for reduction of mediastinal emphysema immediately after endoscopic submucosal dissection (ESD). This study showed that CO_2 insufflation during esophageal ESD significantly reduced postprocedural mediastinal emphysema. CO_2 insufflation also reduced the volume of residual gas in the digestive tract immediately after ESD, but not the visual analogue scale scores of pain and distention.

Maeda Y, Hirasawa D, Fujita N, Ohira T, Harada Y, Yamagata T, Koike Y, Suzuki K. Carbon dioxide insufflation in esophageal endoscopic submucosal dissection reduces mediastinal emphysema: A randomized, double-blind, controlled trial. *World J Gastroenterol* 2016; 22(32): 7373-7382 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i32/7373.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i32.7373

INTRODUCTION

Carbon dioxide (CO_2) is rapidly cleared from the gastrointestinal (GI) tract by passive absorption and subsequently exhaled from the lungs. In several studies, CO_2 insufflation during diagnostic or therapeutic endoscopy has been shown to be safe and effective in reducing procedure-related pain and discomfort[1-5].

The safety of CO_2 insufflation for endoscopic submucosal dissection (ESD) has also been shown in several studies[6-8]. As for esophageal ESD, it is known that mediastinal emphysema (ME) can develop even if no perforation is recognized during or at the end of the procedure because the esophagus has no serosa[9-11]. CO_2 insufflation during esophageal ESD is expected to reduce the incidence of ME. We have previously reported the results of a pilot study concerning ME after esophageal ESD with CO_2 insufflation[8]. To further assess the efficacy of CO_2 insufflation for reduction of post-ESD ME, we conducted a prospective, double-blind, randomized controlled trial, the results of which are reported herein.

MATERIALS AND METHODS

Study design

This study was a single-center, randomized, double-blind, controlled trial in Japan. This study was approved by the institutional review board of Sendai City Medical Center and met all criteria of the Declaration of Helsinki. The trial was registered with the UMIN Clinical Trials Registry (No. UMIN000006441).

Participants

Between February 2011 and May 2012, all consecutive patients undergoing esophageal ESD at the center were screened for recruitment. The inclusion criterion was all consecutive patients undergoing esophageal ESD. The following patients were excluded: those who had severe chronic obstructive pulmonary disease (COPD) resulting in less than 50% of the predicted values of the forced expiratory volume in 1 s (FEV1.0) or less than 70% of FEV1.0/FVC (forced vital capacity)[12], those who had experienced CO_2 retention, those who had multiple synchronous esophageal lesions treated at one time, those who were to undergo esophageal ESD under general anesthesia with positive pressure ventilation, and those who refused to participate. All participants provided written informed consent prior to enrollment in the study.

Randomization and blinding

Participants were randomly assigned to either the CO_2 insufflation group (CO_2 group) or the air insufflation group (Air group). Randomization took place immediately before the ESD procedure. Individual randomization to the two treatment groups (1:1) was performed by using computer-generated random numbers. A sequentially numbered, opaque, sealed envelope containing a random number was opened sequentially by an endoscopy nurse after participant details were written on the envelope. When the number was even, the patient was allocated to the CO_2 group and administration of CO_2 was started. When the number was odd, the endoscopy nurse pretended to start administration of CO_2. Both the CO_2 regulator and the air inlet button on the processor were concealed from the endoscopists, so that the patients and the endoscopists were all blind with regard to the type of gas used. The endoscopy nurse was responsible for switching the CO_2 device on and off. The CO_2 delivery system was set in the endoscopy room and attached to the endoscopic air-water auxiliary system throughout the study period, regardless of its use.

Procedure of ESD

ESD was performed as described by Oyama *et al*[10], using a HookKnife, GIF-Q260J Gastroscope (Olympus Medical System Corp., Tokyo, Japan) and an electrocautery unit (ICC200; ERBE, Tübingen, Germany). The modes of electric power used were the 50 W autocut mode and the 50 W spray-coagulation mode[8,9]. Ten percent glycerin with 0.007% epinephrine was used for local injection into the submucosal layer. The ESD procedures in this study were performed by three endoscopists who had at least 5 years' experience in endoscopy and experience in more than 20 cases of gastric ESD. The procedures were performed on an inpatient basis.

Intraoperative management

In the CO_2 group, CO_2 was administered by using a commercially available CO_2 regulation unit (OLYMPUS UCR; Olympus), which was connected to a CO_2 bottle. A CO_2 nasal sampling set with O_2 tubing (CapnoLine H O2; ORIDON MEDICAL 1987 Ltd., Israel) was used to monitor end-tidal CO_2 pressure ($EtCO_2$). Standard monitors including electrocardiography, an oscillometric blood pressure cuff and a pulse oximeter were employed.

The sedation technique was standardized for all patients. No premedication was given. Propofol was administered slowly as a drip infusion approximately 10 mg/kg per hour initially, with monitoring of the patient's level of consciousness and movement. The level of sedation was evaluated following the American Society of Anesthesiologists classification and maintained at a moderate to deep level[13,14]. After achieving a suitable sedation level for ESD, drip infusion (1-5 mg/kg per hour) of propofol using a syringe pump was continued and adjusted to maintain an adequate depth of sedation. An analgesic (pentazocine, 7.5-15 mg) was given intravenously at the beginning of sedation and further injection was performed depending on the patient's condition. When the combination of propofol and pentazocine could not achieve or maintain an adequate level of sedation, droperidol was added[15].

Periprocedural patient management

On the day of ESD and the day after, the patient was kept fasting. An antibiotic (Cefamezin 1 g × 2/d) was administered intravenously for 3 d after the procedure. When a patient suffered from a fever of over 38 ℃ and/or from post-sternal pain, fasting and administration of antibiotics were prolonged until symptoms improved.

Outcome measurement

The primary outcome was the incidence of ME evaluated on computed tomography (CT) immediately after ESD. The secondary outcome measurements were as follows: incidence of ME the next morning, severity of pain and bowel distention, volume of residual gas in the GI tract, amount of sedative drugs, procedure time, $EtCO_2$ pressure, oxygen saturation, rate of en-bloc resection and R0-resection, and clinical course.

Low-dose plain CT was carried out immediately after ESD and the next morning. A 64-detector row helical CT (Aquilion 64 TSX-101A; Toshiba Medical Systems Co., Tochigi, Japan) with automatic exposure control (AEC) (Volume EC; Toshiba Medical Systems Co.), which adjusts tube current automatically to achieve consistent image quality and to reduce the radiation dose, was employed[16-18]. To further reduce the radiation dose, targeted SD of CT values in the setting of CT-AEC in this study was set at 30 as a low-dose protocol, which is much higher than that of 7.5 in the standard protocol. All other parameters were the same as those of the standard protocol of CT scanning with a constant voltage of 120 kV. For a CT scan with a scanned length of approximately 400 mm, an effective dose based on the effective weighted CT dose index was expected to be approximately 1.9 mGy in the low-dose technique used in this study, which is much lower than that of 30 mGy in the standard protocol.

Four grades of ME were employed: Grade-0, no ME; Grade-Ⅰ, bubbles around the esophagus; Grade-Ⅱ, ME around the thoracic aorta; Grade-Ⅲ, ME extending around the heart and/or beyond the mediastinum into the neck; and Grade-Ⅳ, ME with pneumothorax and/or subcutaneous emphysema (Figure 1)[9].

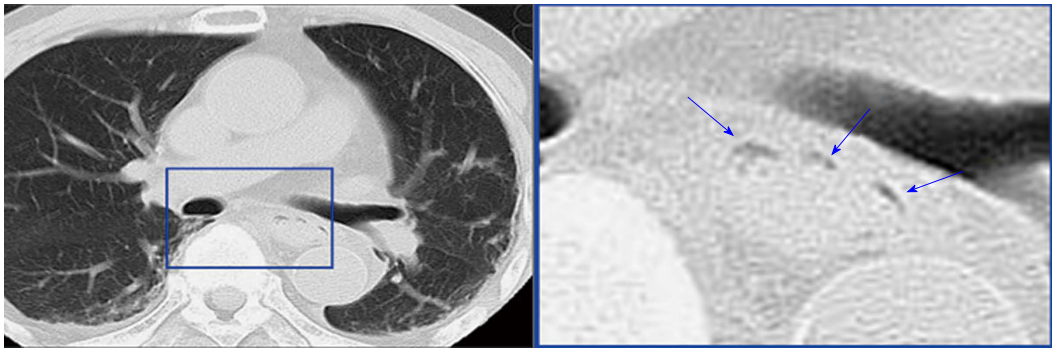
The CT data were transferred to a workstation running a software program (Ziosstation; Ziosoft Inc. Tokyo, Japan) for volume rendering. The volume of residual gas was calculated from the volume-rendering image of the GI tract. Figure 2 shows a rendering image of the residual gas in the GI tract after completion of ESD with CO_2 insufflation immediately after ESD (Figure 2A) and the next morning (Figure 2B), the volume of residual gas being 517 mL and 217 mL respectively. The case shown in Figure 3 received air insufflation during ESD, the volume of residual gas being 1638 mL immediately after ESD (Figure 3A) and 224 mL the next morning (Figure 3B).

The degrees of pain and bowel distention were recorded using a 100-mm visual analogue scale (VAS) immediately after the procedure, 1 and 3 h after the procedure and the next morning. The amount of sedative drugs (propofol, pentazocine and droperidol), procedure time, $EtCO_2$ pressure, oxygen saturation, rate of *en bloc* resection and R0-resection, and clinical courses were recorded.

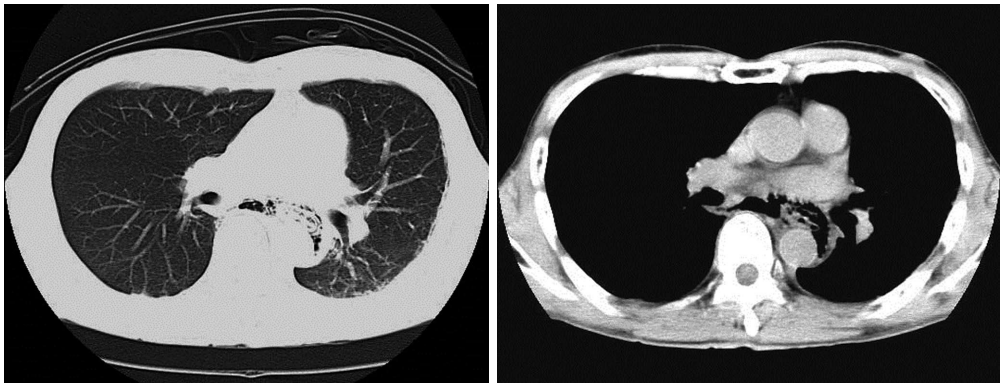
Statistical analysis

Sample size was determined by power calculation using Fisher's exact test. Based on the results of a pilot study[8], the incidence of ME with air insufflation was 63% and that of CO_2 was 30%. To detect this difference with a power of 0.7 and alpha of 0.05, 22 patients per group would be required. Assuming dropout, we set our recruitment goal as 46 patients total.

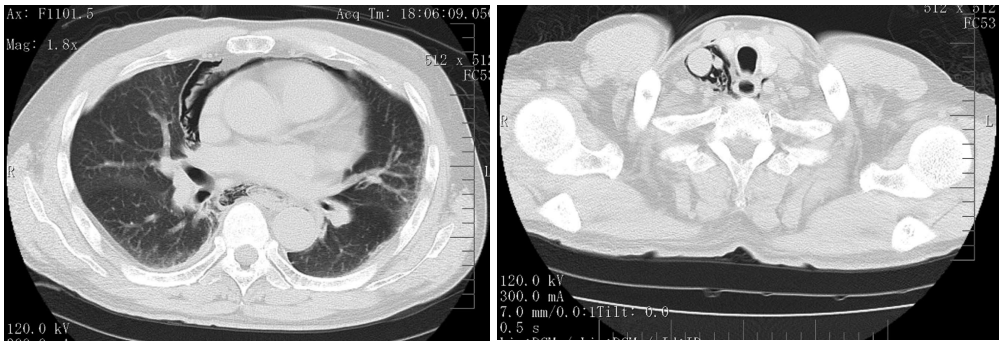
Grade-0	None	No ME
Grade-I	Bubble	Bubbles around the esophagus



Grade-Ⅱ	Localized	ME around the thoracic aorta



Grade-Ⅲ	Diffuse	ME extending around the heart or beyond the mediastinum



Grade-Ⅳ	Extensive	ME extending to pneumothorax or subcutaneous emphysema

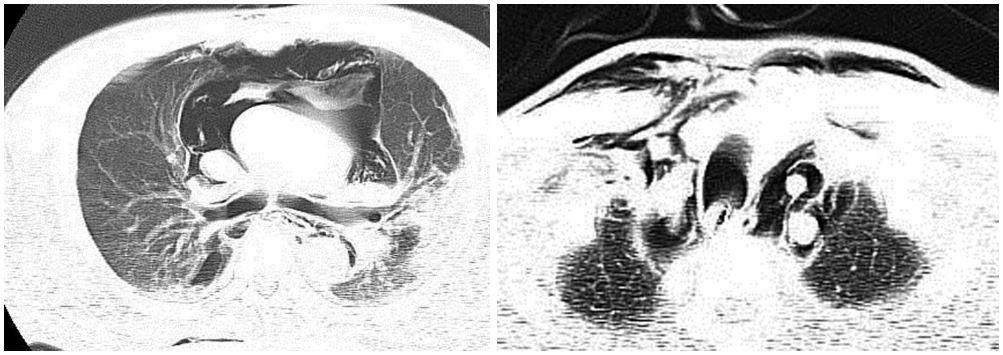


Figure 1 Grade of mediastinal emphysema on computed tomography[9].

Analyses were performed on an intention-to-treat basis for patients who underwent the treatment. Continuous variables (*e.g.*, VAS) were compared by using the *t*-test, and categorical variables (*e.g.*, incidence of ME) were compared by using the χ^2 test (or Fisher's exact test, when appropriate). A two-sided *P* value of < 0.05 was considered statistically significant for all tests.

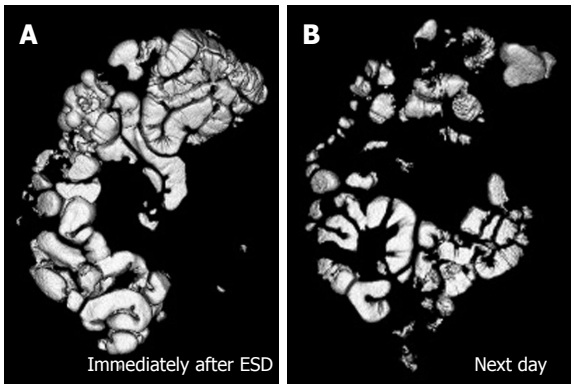


Figure 2 Volume-rendering image of bowel gas immediately after endoscopic submucosal dissection with CO_2 insufflation (A) and that of the next day (B).

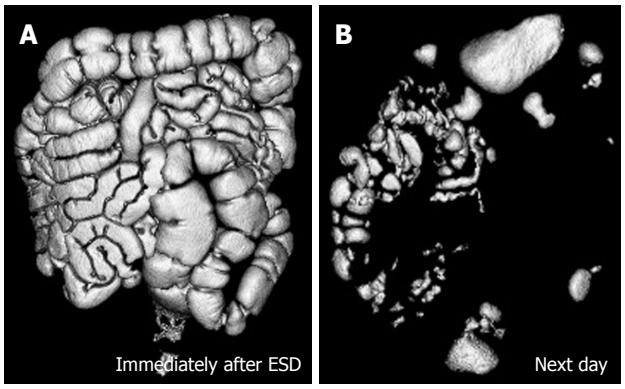


Figure 3 Volume-rendering image of bowel gas immediately after endoscopic submucosal dissection with air insufflation (A) and that of next day (B).

RESULTS

Details of subjects

Between February 2011 and May 2012, 53 patients underwent esophageal ESD in our department. Figure 4 shows the flow of these patients. After exclusion of those who were to undergo ESD under general anesthesia with positive pressure ventilation (n = 2) and those who refused to participate (n = 5), a total of 46 patients consented to take part in the trial and were randomized: 24 to receive CO_2 insufflation (CO_2 group) and 22 to receive air insufflation (Air group).

The demographic data of patients are shown in Table 1; the two groups did not differ at baseline. The mean procedure time was 69.2 min in the CO_2 group and 65.0 min in the Air group, with no statistically significant difference (NS).

Although most patients in both groups had squamous cell carcinoma, 1 patient in the CO_2 group and 3 patients in the Air group had Barrett's adenocarcinoma.

The average size of the resected specimen was 40.0 mm *vs* 42.3 mm, respectively (NS). The rate of R0 resection was 92% *vs* 95%, respectively (NS).

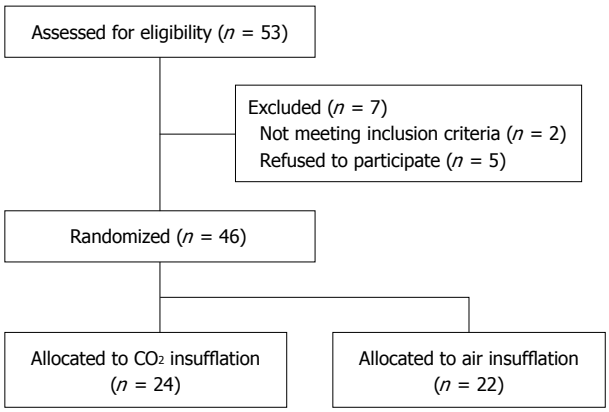


Figure 4 Patient flow chart.

Table 1 Patient characteristics

	CO_2 group	Air group	*P* value
Total No. of patients	24	22	
Sex, M/F	21/3	19/3	0.7460
Age (yr, mean ± SD)	67.5 ± 5.8	72.0 ± 7.2	0.7718
Location1			
Cervical esophagus (Ce)	0	0	0.6015
Upper thoracic esophagus (Ut)	2	4	
Middle thoracic esophagus (Mt)	17	11	
Lower thoracic esophagus (Lt)	4	4	
Abdominal esophagus (Ae)	1	3	
Histology[1]			
Squamous cell carcinoma	23	19	0.3364
Barrett's adenocarcinoma	1	3	
Histological depth[1]			
EP	5	6	0.1734
LPM	11	9	
MM	4	6	
SM1	0	1	
SM2	4	0	
Tumor size (mm, mean ± SD)	26.6 ± 14.4	27.4 ± 22.9	0.8955
Resection size (mm, mean ± SD)	40.0 ± 14.1	42.3 ± 21.2	0.6620
En-bloc resection	24	22	-
R0 resection	22	20	0.9378
HM+	1	1	0.5087
VM+	1	0	0.9649
Ly+	0	0	-
V+	1	3	0.3364
Procedure time (min, mean ± SD)	69.2 ± 28.1	65.0 ± 39.2	0.6847

[1]Based on the Japanese Classification of Esophageal Cancer[19]. EP: Carcinoma *in situ*; LPM: Tumor invades lamina propria mucosa; MM: Tumor invades muscularis mucosa; SM1: Tumor invades upper third of the submucosal layer; SM2: Tumor invades middle third of the submucosal layer or deeper.

Incidence and severity of ME

In the CO_2 group, the incidence of ME immediately after ESD was significantly less compared with that in the Air group (17% *vs* 55%, P = 0.012) (Figure 5A). As for the grade of ME immediately after ESD, Grade-I was 13% in the CO_2 group *vs* 36% in the Air group. Grade-Ⅱ was 4.2% *vs* 18%, and Grade-Ⅲ and Grade Ⅳ were 0% in both groups. The CO_2 group tended to have a lower grade of ME (P = 0.065) (Figure 5A). The

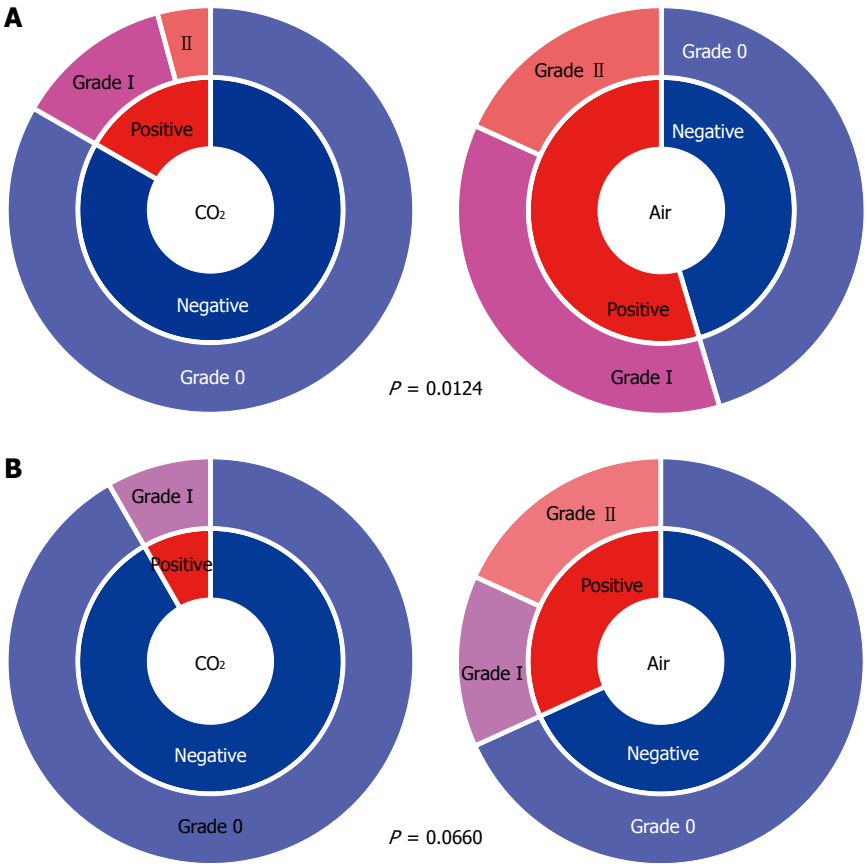


Figure 5 Incidence and degree of mediastinal emphysema immediately after endoscopic submucosal dissection (A) and on the day after endoscopic submucosal dissection (B). *P* value for the incidence of ME. Grade-0 means negative for ME. ME: Mediastinal emphysema.

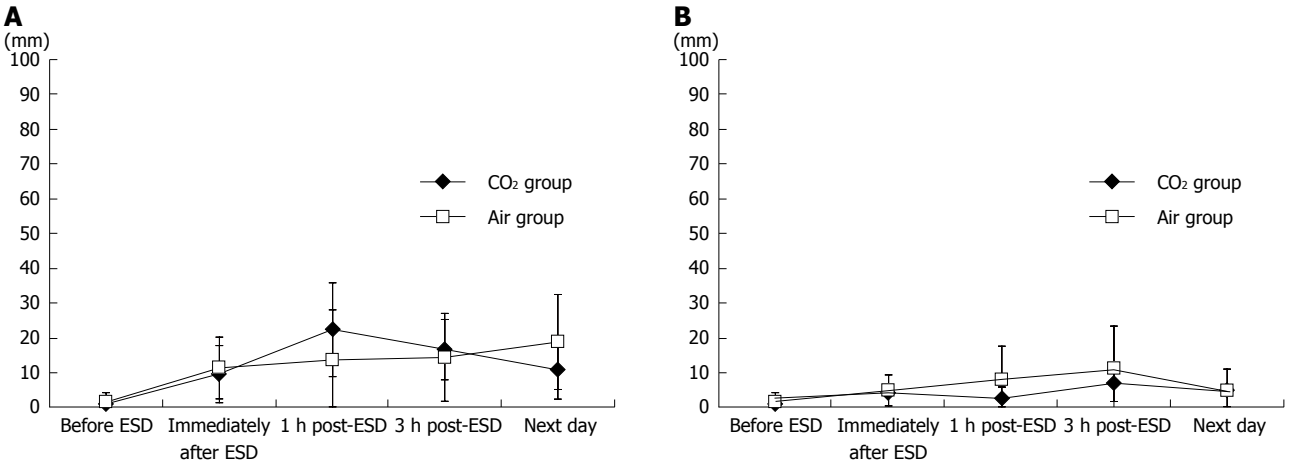


Figure 6 Mean pain score (A) and distension score (B) on the 100-mm visual analogue scale at different time points before and after endoscopic submucosal dissection in the CO_2 and Air groups. ESD: Endoscopic submucosal dissection.

incidence of ME the next morning tended to be lower in the CO_2 group compared with that in the Air group (8.3% *vs* 32%, *P* = 0.066) (Figure 5B). About half of Grade-I ME observed immediately after ESD had disappeared by the next morning (Figure 5).

Pain and distention

The mean severity of pain on a 100-mm VAS in the CO_2 group compared that in the Air group was 9.6 mm *vs* 11.1 mm immediately after ESD, 22.4 *vs* 13.9 at 1-h after the procedure, 16.7 *vs* 14.3 at 3-h after the procedure, and 10.9 *vs* 18.8 the next morning, showing no difference between the groups (Figure 6A). There were no differences in the mean severity of abdominal distension at any post-procedure point of time either (Figure 6B).

Table 2 Effect of CO_2 insufflation *vs* air insufflation (mean ± SD)

	CO_2 group	Air group	*P* value
Gas volume in the GI tract			
Immediately after ESD (mL)	803 ± 371	1173 ± 580	0.0128
Next day (mL)	300 ± 136	304 ± 215	0.9449
End-tidal carbon dioxide partial pressure ($EtCO_2$) measurements			
Baseline $EtCO_2$ level (mmHg)	38.2 ± 3.6	38.1 ± 4.1	0.7543
Maximum $EtCO_2$ level (mmHg)	45.9 ± 4.1	44.3 ± 6.7	0.8562
Oxygen saturation (SpO_2) measurements			
Baseline SpO_2 level (%)	98.9 ± 1.3	98.4 ± 1.0	0.2762
Minimum SpO_2 level (%)	93.7 ± 3.4	93.9 ± 2.3	0.8198
Sedative drugs			
Propofol dose (mg)	537 ± 258	610 ± 533	0.5655
Pentazocine hydrochloride dose (mg)	27.2 ± 4.5	27.1 ± 5.8	0.9658
Droperidol used, No. of patients	5	2	0.4859
Droperidol dose (mg)	3.0 ± 1.1	2.5 ± 0.0	0.5761

Residual gas

The mean volume of residual gas in the GI tract immediately after ESD was significantly smaller in the CO_2 group than that in the Air group (803 mL *vs* 1173 mL, $P = 0.013$) (Table 2). On the day after the procedure, gas volume in the GI tract was reduced in both groups without a significant difference between the groups ($P = 0.945$).

Respiratory depression

Concerning maximum $EtCO_2$ pressure levels during ESD, there was no difference between the CO_2 and the Air groups (45.9 mmHg *vs* 44.3 mmHg). Minimum oxygen saturation levels by pulse oximeter (SpO_2) did not differ by group either (93.7% *vs* 93.9%) (Table 2).

Sedative use

The impact of CO_2 insufflation on the dosages of sedative drugs administered during the procedures was assessed. The mean dosage of propofol used was 537 mg in the CO_2 group and 610 mg in the Air group, with no statistically significant difference (Table 2). The mean dosage of pentazocine did not differ either. The number of the cases using droperidol was 5 in the CO_2 group and 2 in the Air group. The mean dosage of droperidol in those cases was 3.0 mg in the CO_2 group and 2.5 mg in the Air group (Table 2).

Clinical course

No perforation or postprocedural bleeding was encountered in either of the groups. The incidence of fever of over 38 ℃ was infrequent and similar in both groups (8.3% *vs* 9.1%, NS). The mean duration of fever over 38 ℃ was 1.5 d *vs* 2.0 d, respectively (NS) (Table 3). The mean duration of fasting did not differ by group. The incidence of adverse events was infrequent and did not differ between the two groups. All cases recovered with conservative treatment such as prolonged fasting and administration of antibiotics (Table 3).

Table 3 Clinical course

	CO_2 group	Air group	*P* value
Fever ⩾ 38 ℃	8.3%	9.1%	0.6652
Duration of fever ⩾ 38 ℃ (d)	1.5 ± 0.7	2.0 ± 1.4	0.6984
Duration of fasting, d	2.4 ± 0.8	2.1 ± 0.2	0.1639
Complications			
Perforation	0%	0%	-
Post-procedure hemorrhage	0%	0%	-
Esophageal stricture with dysphagia	8.3%	9.1%	0.6652
Pneumonia	0%	0%	-
Death	0%	0%	-

DISCUSSION

CO_2 is rapidly absorbed from the GI tract into the bloodstream and subsequently excreted through expiration. The usefulness and safety of CO_2 as an alternative to air in patients who undergo diagnostic or therapeutic endoscopy under conscious or intravenously sedated conditions have been demonstrated in several randomized controlled studies[1-6]. No pulmonary complications or CO_2 retention have reportedly occurred from CO_2 insufflation in patients without some type of pulmonary dysfunction, and no adverse event related to CO_2 insufflation developed in the present study either. Neither elevation of $EtCO_2$ levels nor depression of SpO_2 levels occurred due to CO_2 insufflation, compared with air insufflation. These results indicate that CO_2 insufflation is safe to use during esophageal ESD.

ME can develop after esophageal ESD even without perforation because the esophagus has no serosa. In contrast, no free air without perforation after gastric ESD was observed in a previously reported randomized controlled study[6]. During ESD, it is mandatory to maintain an adequate endoscopic view with insufflation of gas to achieve a safe procedure. In cases with exposure of the muscular layer, leakage of the insufflated gas into the mediastinum *via* the gap of the muscle fibers is considered to be a mechanism for the development of ME during ESD. However, ME can develop even in cases without exposure of the muscular layer[9], indicating that preservation of the submucosa is not a perfect barrier against leakage of insufflated gas. This randomized controlled study demonstrated that CO_2 insufflation during esophageal ESD can significantly reduce postprocedural ME as compared with air insufflation. CO_2 insufflation may restrain the increase in the inner pressure of the esophagus as a result of rapid absorption into the bloodstream. ME itself may also rapidly disappear because leaking CO_2 in the mediastinum is also more quickly absorbed into the bloodstream than air. ME detected by X-ray is not so common though CT immediately after ESD revealed a certain prevalence of post-ESD ME[9-11]. Patients

with high-grade ME are more likely to develop severe inflammatory changes and to experience a longer febrile period[9]. CT evaluation of the mediastinum for early recognition of extensive ME will lead to prompt careful observations and timely treatments, resulting in avoidance of severe complications. Meanwhile, low-grade ME is asymptomatic. Evaluation of ME on CT is not always necessary for patients who have undergone esophageal ESD. Based on the results of this study, we now evaluate ME on CT only for suspected cases of severe ME. The incidences of ME in both groups were lower in this study than those in the prior pilot study[8]. Improvement of ESD techniques might decrease the incidence of ME. CO_2 insufflation may be more effective for beginners.

The volume of residual gas in the GI tract immediately after ESD was significantly smaller in the CO_2 group than in the Air group. The gas volume in the GI tract on the day after the procedure decreased in both groups to about the same level. Scores of pain and distention on 100-mm VAS at any post-procedure points of time were consistently low and similar in both groups. Neither the dosage of sedative drugs required during ESD nor the clinical course differed. These results were similar to those of a trial performed in patients undergoing gastric ESD[6], namely, CO_2 insufflation reduces bowel gas volume but not procedure-related pain and discomfort. Although most trials concerning CO_2 insufflation during various kinds of endoscopic procedures have demonstrated a reduction of pain and discomfort[2-5], some randomized trials in endoscopic retrograde cholangiopancreatography have reported that CO_2 insufflation was not effective in reducing procedure-related pain[20,21], the same as in this trial. Most of the patients in this trial had no pain after the procedure and the mean VAS scores of pain and distension were consistently low not only in the CO_2 group but also, unexpectedly, in the Air group. One of the reasons for this result may be that sufficiently deep sedation with propofol and pentazocine during ESD may have provided palliation of pain and discomfort of the patients. The half-life of the sedative drugs used, propofol and pentazocine, is 2.6 and 43.8 min, respectively, though pentazocine is reported to provide analgesia as long as 3 to 4 h[22]. The effectiveness of CO_2 insufflation in ESD for the reduction of pain and discomfort remains in question.

Another possible advantage of CO_2 insufflation is fewer adverse events. Air insufflation is associated with rare but serious adverse events of endoscopic procedures, such as air embolism and tension pneumothorax[23-27]. As a matter of fact, several fatal air embolisms caused by endoscopic procedures have been reported. CO_2 is expected to reduce the incidence and severity of such adverse events because CO_2 in the vessels is also more rapidly absorbed into bloodstream than air.

In this study protocol, low-dose CT (approximately 1.9 mGy, which is much lower than 30 mGy in the standard technique) was performed immediately after ESD and the next morning. In view of the inherently high contrast between air and the soft tissue density of body organs, a low-dose protocol was employed for CT, without a loss of diagnostic accuracy. Low-dose protocols for CT have been used in many studies, such as the CT colonography and for detection of occult colonic perforation after colonoscopy[28-30]. Low-dose CT is considered to be a standard technique for the evaluation of ME and measurement of the residual gas in the GI tract.

The present study has some limitations. This trial was conducted at a single center. Clinical significance in consequence of a reduction of ME was not demonstrated. In spite of these limitations, the use of CO_2 for insufflation during esophageal ESD is recommended due to the above-mentioned reasons.

In conclusion, insufflation of CO_2 during esophageal ESD, as compared with that of air, significantly reduced postprocedural ME. CO_2 insufflation can be recommended for esophageal ESD.

COMMENTS

Background

Carbon dioxide (CO_2) is rapidly cleared from the GI tract by passive absorption and subsequently exhaled from the lungs. In several studies, CO_2 insufflation during diagnostic or therapeutic endoscopy has been shown to be safe and effective in reducing procedure-related pain and discomfort. As for esophageal endoscopic submucosal dissection (ESD), it is known that mediastinal emphysema can develop after ESD even without perforation because the esophagus has no serosa. CO_2 insufflation during esophageal ESD is expected to reduce the incidence of mediastinal emphysema.

Research frontiers

The authors have previously reported the results of a pilot study concerning mediastinal emphysema after esophageal ESD with CO_2 insufflation. To further assess the efficacy of CO_2 insufflation for reduction of post-ESD mediastinal emphysema, they conducted a prospective, double-blind, randomized controlled trial.

Innovations and breakthroughs

This randomized controlled study demonstrated that CO_2 insufflation during esophageal ESD can significantly reduce postprocedural mediastinal emphysema as compared with air insufflation. CO_2 insufflation was also shown to reduce the volume of residual gas in the digestive tract immediately after ESD.

Applications

Insufflation of CO_2 during esophageal ESD, as compared with that of air, significantly reduced postprocedural mediastinal emphysema. CO_2 insufflation can be recommended for esophageal ESD.

Terminology

Mediastinal emphysema sometimes develops following esophageal ESD without perforation because the esophagus has no serosa. In cases with exposure of the muscular layer during ESD, leakage of the insufflated gas into the mediastinum *via* the gap of the muscle fibers is considered to be a mechanism for the development of mediastinal emphysema. However, mediastinal emphysema can develop even in cases without exposure of the muscular layer, indicating that preservation of the submucosa is not a perfect barrier against leakage of insufflated gas. Mediastinal emphysema detected by X-ray is not so common, although CT immediately after ESD revealed a

certain prevalence of post-ESD mediastinal emphysema. Patients with high-grade mediastinal emphysema are more likely to develop severe inflammatory changes and to experience a longer febrile period. Endoscopists should strive to avoid mediastinal emphysema in esophageal ESD.

Peer-review

The work is well-done, well-written, documented and structured. The information included is interesting and the number of cases presented is very valuable. This study provides interesting results on the efficacy of CO_2 insufflation for reduction of ME immediately after ESD.

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CASE REPORT

Does massive intraabdominal free gas require surgical intervention?

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Author contributions: Furihata T served as an attending doctor for the patient, designed the report, and wrote the manuscript; Furihata M contributed equally to this manuscript; Ishikawa K, Kosaka M and Satoh N clinically managed the present case; and Kubota K comprehensively revised the manuscript.

Institutional review board statement: This manuscript was reviewed and approved by the institutional review board of Kyouwa Chuo Hospital.

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Abstract

We describe a rare case of an 81-year-old man who presented with severe epigastralgia. A chest radiograph showed massive free gas bilaterally in the diaphragmatic spaces. Computed tomography (CT) scan also showed massive free gas in the peritoneal cavity with portal venous gas. We used a wait-and-see approach and carefully considered surgery again when the time was appropriate. The patient received conservative therapy with fasting, an intravenous infusion of antibiotics, and nasogastric intubation. The patient soon recovered and was able to start eating meals 4 d after treatment; thus, surgical intervention was avoided. Thereafter, colonoscopy examination showed pneumatosis cystoides intestinalis in the ascending colon. On retrospective review, CT scan demonstrated sporadic air-filled cysts in the ascending colon. The present case taught us a lesson: the presence of massive intraabdominal free gas with portal venous gas does not necessarily require surgical intervention. Pneumatosis cystoides intestinalis should be considered as a potential causative factor of free gas with portal venous gas when making the differential diagnosis.

Key words: Case reports; Portal vein; Pneumatosis cystoides intestinalis; Colonoscopy; General surgery

Core tip: We describe a rare case of an 81-year-old man with pneumatosis cystoides intestinalis (PCI).

PCI is characterized by free gas in the submucosal or subserosal layer of the gastrointestinal tract, and its etiology is unknown. The patient presented with massive intraabdominal free gas and portal venous gas (PVG) due to PCI, and he was successfully treated without surgical intervention. When clinicians encounter free gas with PVG, PCI should be considered.

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INTRODUCTION

Pneumatosis cystoides intestinalis (PCI) is a rare clinical entity with an unknown etiology, and it is characterized by free gas in the submucosal or subserosal layer of the gastrointestinal tract[1-3]. PCI has been associated with various underlying etiologies to explain the pathogenic mechanisms causally involved in the accumulation of intramural gas. There are four main theories: (1) the mechanical theory; (2) pulmonary theory; (3) bacterial theory; and (4) chemical theory or nutrition deficiency theory. Currently, chemotherapy, hormonal therapy, steroids, immunosuppression, and connective tissue disease have been reported as causative factors of PCI[2,4-8]. Yet, the exact theory has not been determined. Symptoms usually stem from a secondary underlying disease, including abdominal discomfort, diarrhea, constipation, rectal bleeding, tenesmus, or weight loss. About 3% of patients with PCI develop complications, including tension pneumoperitoneum, intestinal volvulus, obstruction, hemorrhage, and intestinal perforation[9]. One study reported a higher rate of complications with PCI (16.3%), including intestinal obstruction (51.3%), intestinal perforation (35.9%), atypical hyperplasia and canceration (10.2%), and intussusceptions and intestinal necrosis (2.6%)[2]. The most important issue for physicians is recognizing the entity of PCI so that they do not misdiagnose or mismanage it as another disease such as a malignancy or polyposis. It is also important to differentiate the harmless type from the life-threatening type, for which immediate surgery is required[8]. Thus, the conditions of patients with PCI can be confusing, and the decision to treat PCI should be carefully made in consideration of the risks. We herein emphasize how all physicians in the field of gastroenterology can recognize PCI by describing a patient who presented with massive intraabdominal free gas and portal venous gas (PVG) who was successfully treated without surgical intervention.

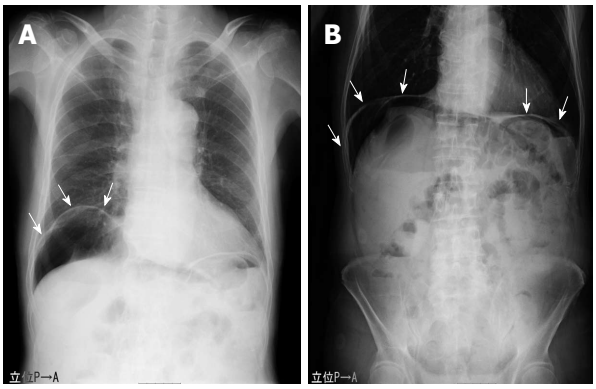

Figure 1 Plain radiograph demonstrating massive intraabdominal free gas bilaterally in the subdiaphragmatic spaces (white arrows). Grape-like clusters or honeycomb-shaped shadows, which are characteristic of pneumatosis cystoides intestinalis, were not observed.

CASE REPORT

An 81-year-old man who presented at our emergency department with acute severe epigastralgia, periumbilical pain, and progressive abdominal distention was admitted to the Department of General Surgery at our institution. The patient visited our institution for an old myocardial and cerebral infarction, hypertension, and chronic obstipation, and he received a variety of medications. He had a mildly increased body temperature (37.2 ℃). His cramping but persistent pain started abruptly. Physical examination showed severe epigastric tenderness and distension without any peritoneal signs. His facial appearance expressed agony, and he presented with excessive sweating. His blood pressure slightly decreased to 12.7/8.65 kPa, which was his usual hypertensive state, and his pulse rate was 61 beats/min. Regarding the hematological parameters, the white blood cell count and C-reactive protein level were mildly increased (10, 500/μL and 0.91 mg/dL, respectively). Serum levels of blood urea nitrogen and creatinine were also increased (26.3 mg/dL and 1.29 mg/dL, respectively), and the levels were slightly different compared to those of his blood samples collected during his normal state (18.5 mg/dL and 0.89 mg/dL, respectively). On radiologic examination, a chest and abdominal plain radiograph showed massive free gas bilaterally in the subdiaphragmatic spaces (Figure 1). Computed tomography (CT) scan also showed that massive free gas was widespread bilaterally under the diaphragm. Moreover, PVG was identified, but ascites was not (Figure 2). Consequently, we performed arterial blood gas analysis, which showed no abnormality (pH = 7.46). First, we considered perforation of the digestive tract. Then, we assumed that the pathological digestive tract would have been accompanied by irreversible ischemia; however, we could not establish a definitive preoperative diagnosis. We used a wait-and-see approach, because the phy-

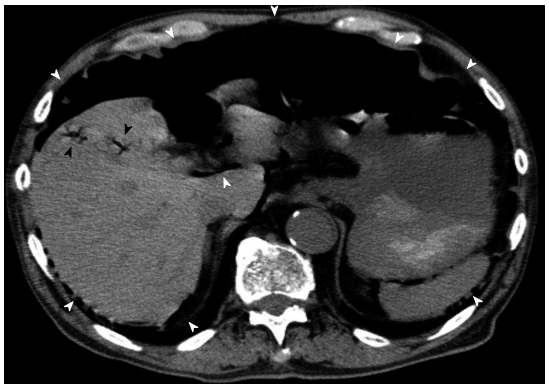

Figure 2 Computed tomography scans also showing widespread, massive pneumoperitoneum (white arrowheads). Moreover, portal venous gas was identified in close proximity to the liver, which was suggestive of portal venous gas in the peripheral portal system, and ascites was not observed (black arrowheads).

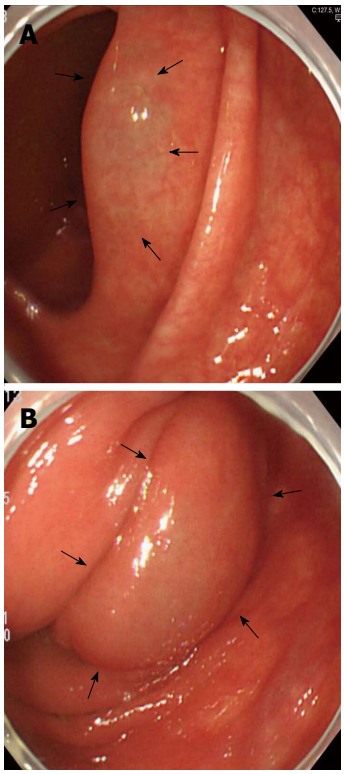

Figure 3 Total colonoscopy findings showing sporadic, round, and smooth elevated lesions, which are similar to submucosal tumors in the ascending colon (black arrows).

sical examinations did not show any peritoneal signs, although radiologic examinations showed marked findings. Consequently, he received conservative therapy with fasting, an intravenous infusion of antibiotics, and nasogastric intubation. The patient was relieved of the symptoms, and he was permitted to eat food orally 4 d after treatment. The patient has been doing well since hospital discharge.

Subsequently, the patient underwent upper endoscopy and total colonoscopy 1 mo after hospital discharge. Upper endoscopy findings showed no abnormality,

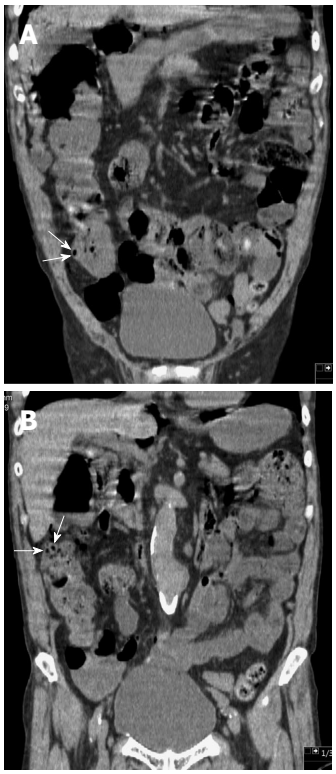

Figure 4 Computed tomography scans in the coronal plane showing sporadic intramural air-filled cysts in the ascending colon.

whereas total colonoscopy results demonstrated a sporadic, round, and smooth-surfaced elevated lesion mimicking a submucosal tumor in the ascending colon (Figure 3). On retrospective review, coronal sections on CT showed sporadic PCI in the ascending colon (Figure 4). The CT findings matched those of total colonoscopy. Thus, we diagnosed the patient's condition as PCI on follow-up examinations. PCI in the present case was retrospectively diagnosed as secondary PCI, because the patient had an underlying disease of chronic obstipation.

DISCUSSION

The most important point of the present case is whether surgical intervention is indicated when intra-abdominal free gas or PVG is observed. It is normal for clinicians to fear digestive tract perforation, as it can be fatal. PCI is one of the factors of pneumoperitoneum, but it has not been well recognized by clinicians such as gastroenterologists, surgeons, endoscopists, and radiologists. The term "pneumatosis intestinalis" was first described as primary PCI by Duo Vernoi while observing autopsy specimens in 1730[1]. Subsequently, Mayer used the term "pneumatosis cystoides intestinalis" to describe its occurrence in hogs in 1825, and the first well-documented case in humans was reported by Bang in 1876[10]. Recently, Koss reviewed cases with PCI and reported that approximately 85% of all cases of PCI were classified as secondary PCI,

Table 1 Causative factors of intraabdominal free gas

Pulmonary disease	Drugs	Infectious disease
Asthma	Alpha-glucosidase inhibitor	AIDS
Chronic bronchitis	Corticosteroids	Whipple' disease
Chronic obstructive pulmonary disease	Lactulose	Candida albicans
Positive end-expiratory pressure	Sorbitol	Clostridia
Pulmonary fibrosis	Chemotherapy agents	*Escherichia coli*
Gastrointestinal disorders	Gefitinib, Sorafenib, Cetuximab,	Mycobacterium tuberculosis
Pyloric stenosis	Sunitinib, imatinib, 5-FU, *etc.*	Intestinal parasites
Pyloric ulcer disease	Choral hydrate	Virus
Peptic ulcers	Caustic agents	Cytomegalovirus, Rotavirus,
Bowel obstruction	Mechanical causes	Adenovirus, Varicella zoster virus
Rupture of jejunal diverticula	Endoscopy	Autoimmune disease
Inflammatory bowel disease	Billiary stent perforation, Sclerotherapy	Scleroderma
Crohn's disease, ulcerative colitis	Barium enema	Lupus variants
Adynamic ileus	Operation	Polymyositis
Appendicitis	Jejunoileal bypass, Jejunostomy tubes,	Dermatomyositis
Toxic megacolon	Post-surgical anastomosis	Sarcoidosis
Volvulus	Organ transplantation	Polyarteritis nodosa
Carcinoma	Cardiac, Bone marrow, Kidney, Liver,	Vascular disease
Hirschsprung disease	Lung, Graft versus host disease	Mesenteric vascular disease
Celiac sprue	Trauma	Intestinal infarction and ischemia
Enteritis and colitis		Intestinal ischemia
Diverticulitis		Intestinal strangulation
Emphysematous gastritis		Collagen vascular disease
Bowel necrosis		

which results from other underlying diseases[11]. In the remaining 15% of cases, PCI was idiopathic or primary. Morris *et al*[12] reported that the incidence of PCI was 46% in the colon, followed by 27% in the small intestine, 7% in the colon and small intestine combined, 5% in the stomach, and 15% in other parts of the gastrointestinal tract. The most common localization of gas was in the submucosa (69.9%)[2]. On the basis of autopsy studies, its incidence in the general population has been estimated as three per 10000 individuals[13,14]. Its recognition has not been widespread in terms of frequency.

PVG is also a radiological sign of serious underlying gastrointestinal pathology. The mortality for PVG ranges between 75% and 90%. The most common cause of PVG is acute mesenteric ischemia. Although hepatic PVG helps distinguish between benign and life-threatening PCI, it may also occur with or without PCI due to nonischemic conditions. Mesenteric abscess formation, portomesenteric thrombophlebitis, sepsis, abdominal trauma, severe enteritis, cholangitis, chronic cholecystitis, pancreatitis, inflammatory bowel disease, diverticulitis, and gastrointestinal surgery or liver transplantation are considered causative factors of PVG[14,15]. The association between PVG and PCI seems to have been noted first in the article by Wolfe and Evans in 1955[15,16]. They reported gas in the portal veins of six living infants, three of whom had associated pneumatosis of the bowel. Khalil *et al*[13] reported that the combination of PCI and PVG is associated with bowel ischemia in about 70% of cases[13,17,18]. Sooby *et al*[15] classified 88 cases of PCI with PVG into three distinct subgroups: mechanical, ischemic, and benign. They also reported that of 88 patients with PCI, 19 were diagnosed as having benign PCI, including 6 patients with both PI and PVG[14]. Considering these cases, PCI may be one of the factors that induce PVG, which can also be fatal. Conversely, PCI and PVG are associated with each other, and they can occur together in various non-ischemic conditions that are not associated with an unfavorable outcome[13,14,18-20]. Even if life-threatening conditions of intraabdominal free gas, PCI, and PVG occur simultaneously, patients' conditions may not necessarily be serious. Thus, deepening the knowledge of these conditions is essential for clinicians (Table 1). In the present case, the mechanisms of these life-threatening conditions were connected, and the following hypotheses have been made. First, secondary PCI probably occurred due to mucosal disruption caused by chronic obstipation. Second, increased gas in the serosal or subserosal layer is partially transported via the mesenteric drainage veins to the hepatic portal veins. Sequentially, ruptures of serosal or subserosal cysts cause pneumoperitoneum. Therefore, physicians must determine whether it is best for patients to undergo surgical intervention. Considering these life-threatening conditions, the most important diagnostic information is patients' vital signs and complaints, and physical examination of the abdomen.

In general, surgical indications of PCI include cases suggestive of inconvertible intestinal obstruction or perforation, or patients with precancerous conditions[13,21]. Wu *et al*[2] mentioned that these complications associated with PCI occur in approximately 16.3% of cases. Hence, radiologic investigations are important for diagnosing PCI. Particularly, multidetector

CT is clearly beneficial, because it enables clinicians to make a more confident diagnosis of PCI by reformations in the coronal, sagittal, and axial planes[3,14]. However, radiologic or endoscopic examination does not necessarily show typical findings, which means that patients with PCI cannot be diagnosed definitively, as shown in the present case. We reached the definite diagnosis retrospectively by reformation of CT in the coronal plane.

We were able to avoid surgical intervention in the present case because of the following important factors: (1) the patient did not show any abdominal peritoneal signs; (2) arterial blood gas measurement did not show metabolic acidosis; (3) radiological examinations demonstrated that there was a considerable amount of free gas bilaterally in the subdiaphragmatic spaces together with PVG, but no ascites was observed; and (4) despite mild renal dysfunction, urine output was obtained, and the patient had stable vital signs. It was a bold decision to avoid surgical intervention, because the patient would have required prompt action if perforation or ischemia of the digestive tract perforation had occurred. Interestingly, nine of 27 patients with PCI died for an overall mortality rate of 33%. Eleven patients observed without surgery had a mortality rate of 18%, whereas those who underwent surgery had a mortality rate of 44%. The remaining nine patients who improved without surgery did not manifest any clinical signs that would have prompted surgery[22]. Therefore, it seems that some patients with PCI can be successfully managed with conservative treatment. However, 37% to 75% of patients with PVG have bowel infarction, and 10 of 12 patients with both PCI and PVG died within 48 h[8]. The long-term prognosis of PCI is unknown, and a long-term follow-up study is required to evaluate this further.

Thus, complementary evaluations such as blood gas analysis can be helpful. Knechtle *et al*[22] advocated the classification of clinical and laboratory values, including the assessment of arterial blood gas (pH, HCO_3^-), so that they could predict the occurrence of ischemic bowel and the patient's outcome. Khalil *et al*[13] propounded a decision-making algorithm after PCI is diagnosed. The proposal makes sense, because the checklists include a variety of essential criteria for diagnosing PCI, including physical conditions, physical examinations, and medical history intake. Obtaining the patient's medical history of comorbidities such as pulmonary disease, gastrointestinal disease, autoimmune disease, infectious disease, and vascular disease, as well as possible iatrogenic causes and the use of drugs is very important for cases of secondary PCI. The lack of knowledge may lead to misdiagnosis, which causes the patient to undergo unnecessary operations. As previously described, the fact that CT did not show ascites was favorable, because ascites often indicate ischemia or perforation of the gastrointestinal tract. Briefly, if CT demonstrates intraabdominal free gas with PVG in the absence of ascites, PCI is a possible diagnosis.

In conclusion, massive intraabdominal free gas with PVG does not always require surgical intervention. Therefore, the decision to perform surgery should be made on the basis of the knowledge of PCI. When clinicians encounter free gas with PVG in the abdomen, PCI should be considered when making the differential diagnosis.

ACKNOWLEDGMENTS

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COMMENTS

Case characteristics

An 81-year-old man presented with severe epigastralgia.

Clinical diagnosis

Physical examination showed severe epigastric tenderness and distension without any peritoneal signs.

Laboratory diagnosis

Regarding the hematological parameters, the white blood cell count and C-reactive protein level were mildly increased (10.500/μL and 0.91 mg/dL, respectively). Serum levels of blood urea nitrogen and creatinine were also increased (26.3 mg/dL and 1.29 mg/dL, respectively). Arterial blood gas analysis showed no abnormality (PH = 7.46).

Imaging diagnosis

Computed tomography scan showed that massive free gas was widespread bilaterally under the diaphragm. Moreover, portal venous gas was identified, but ascites was not observed.

Pathological diagnosis

There was no specimen to make a pathological diagnosis.

Treatment

The authors used a wait-and-see approach, because the physical examinations did not show any peritoneal signs, although radiologic examinations showed marked findings. Consequently, he received conservative therapy with fasting, an intravenous infusion of antibiotics, and nasogastric intubation. We were able to avoid surgical intervention.

Related reports

Pneumatosis cystoides intestinalis (PCI) is a rare entity. In addition, there are few reports of patients with PCI successfully treated without surgical intervention.

Term explanation

PCI is a rare clinical entity with an unknown etiology, and it is characterized by free gas in the submucosal or subserosal layer of the gastrointestinal tract.

Experience and lessons

The present case taught us the following lesson: the presence of intraabdominal free gas with portal venous gas (PVG) does not necessarily require surgical intervention. When gastroenterologists encounter free gas with PVG, PCI should be considered.

Peer-review

It is a case report of a patient with abdominal pain that was proved to be caused by PCI, and was managed conservatively. It is very interesting.

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