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FRONTIER

MicroRNA biomarkers predicting risk, initiation and progression of colorectal cancer

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Abstract

Colorectal cancer is a major global cause of morbidity and mortality. Current strategies employed to increase detection of early, curable stages of this disease are contributing to a reduction of the negative health impact from it. While there is a genetic component to the risk of disease, diet and environment are known to have major effects on the risk of an individual for developing the disease. However, there is the potential to reduce the impact of this disease further by preventing disease development. Biomarkers which can either predict the risk for or early stages of colorectal cancer could allow intervention at a time when prospects could be modified by environmental factors, including lifestyle and diet choices. Thus, such biomarkers could be used to identify high risk individuals who would benefit from lifestyle and dietary interventions to prevent this disease. This review will give an overview on one type of biomarker in the form of microRNAs, which have the potential to predict an individual's risk for colorectal cancer, as well as providing a highly sensitive and non-invasive warning of disease presence and/or progression. MicroRNA biomarkers which have been studied and whose levels look promising for this purpose include MiR-18a, MiR-21, MiR-92a, MiR-135b, MiR-760, MiR-601. Not only have several individual microRNAs appeared promising as biomarkers, but panels of these may be even more useful. Furthermore, understanding dietary sources and ways of dietary modulation of these microRNAs might be fruitful in reducing the incidence and slowing the progression of colorectal cancer.

Key words: Biomarkers; Epigenetics; Risk; Colorectal cancer; Predisposition; MicroRNA

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Core tip: The requirements for colonoscopic technologies in order to detect early stages of colorectal cancer are being superseded by highly sensitive microRNA technologies using various body fluids. As well as providing early warnings of the disease, these also potentially provide a highly sensitive marker of dietary efficacy in disease prevention or slowing of disease progression.

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INTRODUCTION

Colorectal cancer (CRC) is the second most common cancer in women and the third most common cancer in men[1]. In the earliest stages of CRC development, the lesion may not cause obvious symptoms, so individuals may not be prompted to go to a health practitioner until disease is advanced. Hence, many CRC patients present late, when their cancer is advanced and their prognosis for a permanently curative resection is less likely. As well as the asymptomatic nature of early CRC, other reasons why people may present with CRC at a late stage may be because of the insensitivity of screening tests to pick up precancerous lesions, or refusal to undergo a screening test for various reasons. In this review, we briefly consider current screening technologies[2], and then develop a rationale for a new highly sensitive screen using a novel biomarker.

The current CRC screening methods are faecal occult blood test, fecal immunochemical test, sigmoidoscopy and colonoscopy. Guaiac faecal occult blood tests are more effective when a repeat test is performed every 1 or 2 years[2]. Both guaiac faecal occult blood test and faecal immunochemical test have more of an important role in detecting early cancer rather than precancerous lesions as the tests only identifies a small proportion of patients with advanced adenomas[2]. Therefore, due to their low sensitivity for advanced adenomas, most advanced adenomas will go undetected and therefore these stool blood tests will not significantly contribute to prevention of cancer by detection and excision of adenomas. The limitation with sigmoidoscopy is that it is less likely to identify colon neoplasia in a proximal location[2]. In addition to this, sigmoidoscopy is a relatively inconvenient test as a bowel preparation and an office visit is needed[2]. Colonoscopy is comparatively a more attractive option than sigmoidoscopy as patients have their whole colon investigated and they can also have sedation[2]. However, colonoscopy is invasive, expensive and it carries a risk for complications[2]. Clearly, there is a need for a new biomarker or panel of biomarkers which are non-invasive, cost-effective and allows the identification of risk for CRC, for example by identifying patients with precancerous lesions. One such group of potentially highly sensitive biomarkers, microRNAs, will be examined in this manuscript.

RISK FACTORS FOR CRC

Certain non-modifiable and modifiable risk factors are known to be associated with CRC risk. Non-modifiable risk factors which increase an individual's risk of the disease include age, a personal history of adenomatous polyps or inflammatory bowel disease, a family history of adenomatous polyps or colorectal cancer and inherited genetic risk[3]. Modifiable risk factors include diet, physical activity, obesity, cigarette smoking and alcohol consumption[3].

There have been many studies relating various dietary factors to colorectal cancer risk. There is considerable evidence that certain types of dietary fibres reduce the risk of CRC[4]. However, there is also opposing evidence that indicates that other dietary fibres may increase the risk of CRC development[5]. Studies have shown an association between high intake of red and processed meats and increased risks of CRC[6,7]. For the relationship between folate intake and the risk of CRC, there have been inconsistent results[8]. The impact of protein intake on CRC risk is also unclear. Different studies have shown high protein intake to associate with both elevated and reduced risks of CRC[9,10]. These differences in results highlight the impact that the characteristics of the population study and the type of protein can have on the results[11]. There is evidence that obesity increases risk of colon cancer[12].

The human colonic microbiota is composed of bacteria, some of which can enhance while others can protect against colorectal carcinogenesis[13]. The burden of CRC may be alle*via*ted by using certain types of dietary fibre to manipulate the metabolic activity of the bowel microbiota or by changing the composition of the microbiota[13]. Low vitamin D status may also be associated with a higher risk of colorectal cancer[14]. High levels of low-density lipoprotein, triglycerides and total cholesterol may also be associated with increased risk[15]. However, further studies producing more convincing results are needed on this relationship. Reports have also investigated the relationship between minerals and risk of CRC. For example, very low or very high selenium concentrations seem to enhance the risk of cancer, while the optimal concentration and form of selenium may be protective[16].

EARLY STAGES IN CARCINOGENESIS

CRC arises following an accumulation of genetic and

epigenetic changes which transform normal colonic epithelial cells into cancerous cells[17-19]. Mutations in proto-oncogenes and tumour suppressor genes also contribute[17]. There are also many genetic polymorphisms which are being identified as increasing an individual's susceptibility to developing colorectal cancer. However, genetic variants such as mutations and polymorphisms are specific sequences of DNA and are therefore fixed and not modifiable. Hence, although genetic markers may be able to identify individuals at risk for colorectal cancer, they cannot be changed by lifestyle or dietary interventions.

Unlike genetic alterations, epigenetic changes are potentially modifiable. Epigenetic alterations are heritable changes in gene expression which do not alter the DNA sequence. Epigenetic mechanisms include DNA methylation, microRNA (miRNA) expression, histone modification and chromatin remodelling. Epigenetic alterations can increase, decrease or silence gene expression[19]. There has recently been a surge in the scientific literature of epigenetic biomarkers which are potentially associated with colorectal cancer[19-21]. In particular, there has been a focus on DNA methylation and miRNA expression patterns, the latter of which will be the subject of this review.

The majority of CRCs arise from localised precursor lesions called adenomas. Thus, individuals with colorectal adenomas are at increased risk of CRC[17]. Removal of this adenoma can prevent it from developing into cancer. An epigenetic biomarker which is differentially expressed in adenoma patients compared to healthy controls would identify those people who have an increased risk for developing CRC. As the presence of adenomas is known to increase risk for CRC, epigenetic biomarkers which detected adenomas have been included in this review.

BIOMARKERS

A biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention"[22].

Environmental factors have been linked to aberrant epigenetic modifications which contribute to cancer formation. In colorectal cancer, there is the potential to link lifestyle and diet risk factors to aberrant epigenetic alterations in the very early stages of colorectal cancer development. As both epigenetic changes and lifestyle choices are modifiable, there is the possibility that positive lifestyle interventions can alter epigenetic mechanisms to change gene expression and reduce the risk for colorectal cancer. This modified level or expression of the epigenetic markers could be measured as a biomarker to reflect the risk status of the individual.

Many biomarkers in the literature are either tissue, stool or blood-based[22]. Tissue based biomarkers require a sample of tissue in which to measure the expression of the epigenetic biomarker. This is usually taken through a biopsy during colonoscopy and is thus an invasive technique which requires colonoscopy and excision of tissue. An ideal biomarker is non- invasive so this review only included biomarkers which were measured from a stool or blood sample. The details of the studies are provided in Table 1[23-36], and discussed subsequently.

MICRORNA IN CRC RISK

While some studies had considered peripheral blood mononuclear cells which are important for studying DNA or gene expression changes, the small size of miRNAs means that they are more readily detected in plasma samples, or in faecal samples. Thus, the following discussion mostly considers such samples.

MiR-21 has been investigated in several different studies. Link *et al*[23] showed that miR-21 was increased in stool samples from adenoma subjects compared to normal colonoscopy subjects. However, in another stool-based study, no difference was found in miR-21 levels between patients with polyps and controls[26]. Two studies have demonstrated that miR-21 is overexpressed in the serum of patients with advanced adenomas (AA), as compared to controls[24,25]. Two findings from one of the serum-based studies indicated that miR-21 in the serum of CRC patients is secreted from CRC tissue, which is significant as it increases the diagnostic specificity of blood-based levels of miR-21[25]. Initially, Toiyama *et al*[25] observed a statistically significant correlation between tissue miR-21 level and matching serum samples from a small number of patients with CRC. Surgical removal of CRC led to a statistically significant reduction of miR-21 in the serum of these same patients. However, they identified two possible limitations of miR-21 as a colorectal neoplasia biomarker. Firstly, it is presently difficult to be certain that changed expression levels of circulating miR-21 are specifically caused by colorectal neoplasia, because circulating miR-21 has been reported to be associated with other cancers, such as breast cancer, glioblastoma and pancreatic cancers[25]. Secondly, all their study samples were from subjects of Japanese origin[25]. Hence, the lack of ethnic diversity may hamper this study's applicability to other populations. Luo *et al*[27] did not find any differences in plasma miR-21 levels of AA patients, compared to controls. However, these authors acknowledged that their observed lack of differences in miRNA expression levels may have been because of inadequate power to distinguish modest differences due to small sample sizes.

MiR-92a has also been examined in a number of studies. It was shown to be overexpressed in the serum[24], plasma[31] and stool[26] of AA patients,

Table 1 Summary of experiments relevant to microRNA detection of colorectal cancer

miRNA	Sample size		Findings	Specimen type	Ref.
	Cases (*n*)	Controls (*n*)			
miR-21 miR-106a miR-17 miR-143 miR-622 miR-654-3p	9 non-advanced adenomas and AA 10 CRC	10 controls (normal colonoscopy)	miR-21, miR-106a: Colorectal neoplasia (adenoma, CRC) patients had higher stool expression of these two miRNA compared to normal colonoscopy subjects ($P < 0.05$). Adenoma patients had higher stool miR-21 and miR-106a expression compared to CRC patients miR-17, miR-143, miR-622, miR-654-3p: No differences between groups	Stool	Link *et al*[23]
miR-21 miR-92a miR-31 miR-18a miR-106a	50 AA 200 CRC	80 controls (do not have a current or previous malignancy or inflammatory condition)	miR-21, miR-92a: miR-21 and miR-92a levels in CRC patients and AA patients were significantly higher compared to controls (all $P < 0.05$). miR-21 yielded an AUC of 0.709 in differentiating AA from controls. miR-92a yielded an AUC of 0.701 in differentiating AA from controls. Both miRNA together yielded an AUC of 0.722 in differentiating AA from controls miR-18a, miR-31, and miR-106a: No significant differences between groups	Serum	Liu *et al*[24]
miR-21 miR-31	43 AA 60 postoperative patients 186 CRC	53 controls (negative colonoscopic examination, no prior diagnosis of any other malignancy)	miR-21: Serum levels were increased in adenomatous polyp patients compared with controls ($P < 0.001$). Serum miR-21 levels yielded an AUC of 0.803 (95%CI: 0.669-0.869) in differentiating AA from controls. The sensitivity, specificity, positive predictive value and negative predictive values were 76.8 % and 81.1%, 76.7%, and 81.1%, respectively, at a cut-off value of 0.0013	Serum	Toiyama *et al*[25]
miR-92a miR-21	44 patients with minor polyp (defined as hyperplastic polyp or adenoma less than 1 cm in diameter) 13 AA 88 CRC	101 controls (asymptomatic individuals)	miR-92a: Stool miR-92a was significantly increased in polyp patients compared with controls ($P < 0.0001$). Sensitivity of 56.1% for polyp, specificity of 73.3%. Higher sensitivity for AA than minor polyps ($P < 0.05$). The removal of AA led to a decrease in stool miR-92a level ($P < 0.05$). miR-21: No difference between polyps and controls	Stool	Wu *et al*[26]
miR-29a, miR -106b, miR -133a, miR -342-3p, miR -532-3p miR-18a, miR -20a, miR -21, miR -92a, miR -143, miR -145, miR -181b	Marker validation phase 50 AA	Marker validation phase 50 controls (free of colorectal neoplasms)	No statistically significant differences between AA patients and controls for any of the investigated miRNA	Plasma	Luo *et al*[27]
miR-10a, miR-29a, miR-31, miR-92a, miR-100, miR-125b, miR-184, miR-187, miR-196a, miR-200b, miR-203, miR-17-3p	73 non-advanced adenoma 43 AA 8 CRC	48 controls (polyp-free)	No statistically significant associations with non-advanced adenoma or AA for any of the investigated miRNA	Plasma	Adams *et al*[28]
miR-34a miR-150 miR-923	Discovery set 8 polyp 16 adenoma 8 CRC (stage I/Ⅱ) 8 CRC (stage Ⅲ/Ⅳ) Validation set 20 polyp 20 adenoma 23 CRC (stage I/Ⅱ) 14 CRC (stage Ⅲ/Ⅳ)	Discovery set 8 controls Validation set 20 controls	miR-34a: Validation cohort: Significantly higher in adenoma group compared to controls (FC 2.09, $P = 0.028$). Significantly higher in adenoma group compared to the polyp group (FC 2.71, $P = 0.002$). miR-923: Validation cohort: No significantly different levels	Plasma	Aherne *et al*[29]

miR-18a miR-15b miR-19a miR-19b miR-29a miR-335	Set 1 20 AA 21 CRC Set 2 40 AA 42 CRC	Set 1 20 controls Set 2 53 controls	miR-18a: Set 1 and Set 2: Significantly overexpressed in AA patients compared to controls in both sets. Set 1: Good discriminative capacity in AA patients (AUROC, 0.84; 95%CI: 0.72-0.96; sensitivity [S], 80%; specificity [Sp], 80%). Set 2: Lower discriminative capacity in AA patients (AUROC, 0.64; 95%CI: 0.52- 0.75; S, 72%; Sp, 57%)	Plasma	Giráldez *et al*[30]
miR-29a, miR-92a,	Large-scale validation 37 AA 100 CRC	Large-scale validation 59 controls (negative results of health examination including blood test, chest X-ray, abdominal ultrasound examination, fecal occult-blood testing, rectal touch, CT scan and colonoscopy. None of these controls had previously been diagnosed with any types of malignancy previously)	miR-29a and miR-92a: Significantly higher in AA compared to controls (P < 0.0001 for miR-29a, P < 0.0001 for miR-92a). Both miRNAs together yielded an AUC of 0.773 (95%CI: 0.669-0.877), sensitivity 73.0% and specificity 79.7%, in discriminating AA. miR-29a: Yielded an AUC of 0.769 (95%CI: 0.669-0.869) for differentiating AA from controls. The sensitivity was 62.2% and specificity 84.7%, at a cut-off value of 1.210 for miR-29a. The odds ratio for cases with miR-29a > 1.210 being associated with AA was 12.20 (95%CI: 4.350-34.237). miR-92a: Yielded an AUC of 0.749 (95%CI: 0.642-0.856) for differentiating AA from controls. Sensitivity 64.9% and specificity 81.4%, at a cut-off value of 1.682 for miR-92a. The odds ratio for cases with miR-92a > 1.682 being associated with AA was 4.56 (95%CI: 1.893-10.988)	Plasma	Huang *et al*[31]
A panel of 8 miRNAs miR-532-3p + miR-331 + miR-195 + miR-17 + miR-142-3p + miR-15b + miR-532 + miR-652	Initial Screening 9 adenoma 20 CRC (stage Ⅲ/Ⅳ) Validation 16 adenoma 15 CRC (stage Ⅰ/Ⅱ) 15 CRC (stage Ⅲ) 15 CRC (stage Ⅳ)	Initial Screening 12 controls (without CR neoplasia) Validation 26 controls (without CR neoplasia)	Initial Screening 15 out of 380 screened miRNAs most dys-regulated in plasma of adenoma patients compared to controls (P < 0.05, FDR: 5%). Validation A panel of 8 plasma miRNAs yielded an AUC of 0.868 (95%CI: 0.76-0.98), sensitivity 88% and specificity 64% in differentiating adenoma from controls	Plasma	Kanaan *et al*[32]
miR-601 miR-760	Large scale validation 43 AA 90 CRC	Large scale validation 58 controls	miR-601: AUC of 0.638, sensitivity of 72.1% and specificity of 51.7% in differentiating AA from controls miR-760: AUC of 0.682, sensitivity of 69.8% and specificity of 62.1% in differentiating AA from controls miR-601 + miR-760: Significantly decreased in colorectal neoplasia (AA and CRC) compared to controls. Both miRNAs together yielded AUC of 0.683, sensitivity 72.1% and specificity 62.1% in differentiating AA from controls	Plasma	Wang *et al*[33]
miR-135b miR-31	110 adenomas < 1 cm in size 59 AA 42 IBD 104 CRC	109 controls (normal colonoscopy)	miR-135b: Significantly increased in adenoma subjects (median, 28.4; IQR, 0.2-79.7; P < 0.0001) compared to controls (median, 0; IQR, 0-30.8). No significant difference in IBD subjects compared to controls. AUC of 0.71 for detection of adenoma. Sensitivity of 73% for AA, 61% for adenoma < 1 cm in diameter, 65% for any adenoma and specificity of 68%, at a cut-off of 14 copies/ng of stool RNA. Sensitivity of 44% for adenoma < 1 cm, 46% for AA, and specificity of 80%, at a cut-off of 38 copies/ng of stool RNA. Removal of AA or CRC resulted in a significant reduction of stool miR-135b. miR-31: No significant differences between groups	Stool	Wu *et al*[34]
miR-18a miR-221	151 adenoma 48 AA 198 CRC	198 controls (normal colonoscopy)	miR-18a, miR-221: No significant up-regulation in adenoma or AA	Stool	Yau *et al*[35]
A panel of 4 miRNAs miR-19a-3p + miR-223-3p + miR-92a-3p + miR-422a	Validation of the diagnostic performance of the miRNA panel: 73 adenoma 117 CRC	Validation of the diagnostic performance of the miRNA panel: 102 controls (healthy individuals seeking a routine health check- up)	Validation of the miRNA panel The miRNA panel yielded an AUC of 0.765 (95%CI: 0.669-0.845) in differentiating adenoma from controls	Serum	Zheng *et al*[36]

CRC: Colorectal cancer; AA: Advanced adenomas; IBD: Inflammatory bowel disease.

compared to controls. However, Luo *et al*[27] did not show a difference of miR-92a levels in the plasma of AA patients compared to neoplasm-free controls. Furthermore, Adams *et al*[28] did not find plasma miR-92a levels to be associated with non-advanced adenomas or AA. The cause of these discrepancies is unclear, but may have been due to differing samples sizes and patient characteristics including ethnicity. A notable finding that supported the involvement of miR-92a in CRC came from the stool based study of miR-92a from Wu *et al*[26], as they found that following the removal of AA or cancer, there was a significant reduction in stool miR-92a.

Giráldez *et al*[30] found that miR-18a was over-expressed in the plasma of AA, compared to controls. However, these findings were not concordant with another study which found no differences in plasma miR-18a levels in AA samples, compared to neoplasm-free controls[27]. Another study did not find differences in miR-18a levels between serum samples from AA individuals and controls[24]. Furthermore, no upregulation of miR-18a was seen in stool samples from adenoma subjects[35]. Thus, the present balance of evidence does not support miR-18a being a useful biomarker.

Similarly, miR-31 also does not seem like a useful biomarker at present. A number of studies have not been able to find a difference in miR-31 expression levels between adenoma patients and controls in serum[24,25], plasma[28] or stool[34] samples. A large case-control study revealed the potential utility of miR-135b for detecting adenoma[34]. The group's findings suggested that overexpression of this miRNA is specific for colorectal neoplasia, as removal of AA and CRC led to a significant reduction in the expression of stool miR-135b. Furthermore, this study included controls with inflammatory bowel disease and a lower level of stool miR-135b was found in these controls, giving further evidence that miR-135b upregulation is specific for colorectal neoplasia. The initial findings for miR-135b appear promising and some strengths of this study include larger cohort numbers and inclusion of IBD controls. However, some limitations of this study were identified by the authors; the findings of this study may not be representative of the community screening setting due to recruitment from limited locations and the inclusion of some symptomatic patients[34]. Also, the impact of attaining a stool sample before compared to after colonoscopy on miRNA levels was not analysed and in this study, stool was sampled one week before colonoscopy in all AA patients.

Researchers have investigated the discriminative capability of both single miRNAs and panels of miRNAs in differentiating adenoma patients from controls. It may be more useful in clinical practice to use panels of miRNA rather than a single miRNA to discriminate individuals with colorectal adenomas from those without, as there is doubt that a single miRNA has high enough specificity for it to be used alone as a biomarker of colorectal neoplasia[32]. The reports of specific miRNA being differentially expressed in multiple cancers lend support to this idea[27]. Panels of plasma and serum miRNA have been tested for their ability to discriminate adenomas from controls. A panel of 8 plasma miRNAs (miR-532-3p, miR-331, miR-195, miR-17, miR-142-3p, miR-15b, miR-532, miR-652) yielded an AUC of 0.868 (95%CI: 0.76-0.98), sensitivity 88% and specificity 64% in differentiating adenoma from controls[32]. However, the authors pointed out that to investigate specificity more thoroughly, individuals with inflammatory diseases and other tumours should have been included among their controls. A serum miRNA panel composed of 4 miRNA (miR-19a-3p, miR-223-3p, miR-92a-3p and miR-422a) yielded an AUC of 0.765 (95%CI: 0.669-0.845) in differentiating adenoma from controls[36].

MiR-760 had an AUC value of 0.682, sensitivity of 69.8% and specificity of 62.1% in differentiating AA from controls[33]. MiR-601 had an AUC of 0.638, sensitivity of 72.1% and specificity of 51.7% in this same study. When both miRNA were combined, an AUC of 0.683, sensitivity of 72.1% and specificity of 62.1% was achieved in differentiating AA from controls. The 2 miRNA together demonstrated an AUC, sensitivity and specificity similar to miR-760 alone and therefore, the addition of miR-601 to miR-760 was not considered to be useful[33]. This highlights the importance in analysing the individual contribution of panel components to the discriminatory power of the panel overall to identify those markers which should or should not be included.

As noted in sections on specific miRNAs above, there were inconsistences in findings between studies for the same miRNA. Firstly, this may have been caused because of the different type of sample used (stool, plasma or serum). Methodologic differences in collection procedures, specimen preparation/processing, miRNA extraction, miRNA detection and measurement, data acquisition, data normalisation, quantification methods may have also contributed to different results for studies. For example, the collection of blood samples before or after colonoscopy may have affected results. MiRNA levels normalised to different internal controls could also contribute to disparities in results. The differences in results may also be due to differences in subject characteristics, including age, gender and ethnicity.

One group has reported that most of the reported circulating miRNA biomarkers for cancer are highly expressed in blood cells and therefore, haemolysis and variations in blood cell counts can significantly alter miRNA levels in plasma[37]. Thus, altered circulating miRNA levels discovered in cancer studies reported to have been associated with cancer may in

fact reflect blood cell effects, instead of cancer tissue specific origin[37]. This may suggest that miRNAs that are not expressed in blood cells should be investigated if attempting to identify circulating miRNAs biomarkers that are very specific for cancer[37].

PROSPECTS FOR MODULATING THE MICRORNAS IMPLICATED IN THESE STUDIES

While early detection of CRC is desirable for enabling early surgical or pharmacologic intervention, there is also considerable interest in the question as to whether diet or other lifestyle changes could affect the relevant microRNAs. A summary of some relevant dietary factors is provided in Table 2, and discussed below.

Tarallo *et al*[38] studied a panel of seven human microRNAs in plasma and stool samples from 24 healthy individuals with differing dietary habits. Eight of these were vegans, eight vegetarians and eight on an omnivorous diet, and the groups had similar age and sex distributions. They found that miR-92a was differentially expressed in both plasma and stool samples, and was very significantly affected by diet. This MicroRNA was also associated with low body mass index. Although miR-16, miR-21, mir-34a and miR-222 showed associations with diet and lifestyle factors, the data were not consistent between stool and plasma.

Davidson *et al*[39] used a hypothesis-drive approach to study dietary modulation of microRNA expression. Specifically, they considered the effects of a long chain omega-3 polyunsaturated fatty acid (lc n-3 PUFA)-enhanced diet on the development of carcinogen- induced CRC in a Sprague-Dawley rat model. The animals were fed diets containing corn oil or fish oil, and injected with the colon-specific carcinogen, azoxymethane, or saline as negative control. They quantified the effects of the diets on expression of 368 different microRNAs in the colonic mucosa. It appeared that let-7d, miR-15b, miR-107, miR-191 and miR-324-5p were the most strongly significantly affected by diet X carcinogen interactions.

The fish oil-fed animals showed the smallest number of differentially expressed miRNAs between carcinogen and control treatments, probably because the fish oil was protecting against carcinogen-induced inflammation. Using a mouse transplantable tumour model, Tsoukas *et al*[40] also related protection against tumour growth and progression associated with microRNA dysregulation, to levels of lc n-3 PUFAs. These nutrients have also been shown to have a beneficial effect on the modulation of MiR-21 expression in breast cancer cells[41]. Dietary lipid intake will also modulate serum lipoproteins, and there is evidence that miRNAs are transported to target cells *via* binding to lipoproteins such as LDL and HDL cholesterol[42].

MiR-155 is carried mainly by HDL. Although not one of the main miRNAs specifically identified in CRC, it is well characterized as an inflammatory regulator[43]. Kim *et al*[43] studied the levels of miR-155 among HDL isolated from smokers and non-smokers, following eight weeks daily consumption of high dose Vitamin C. They reported several potentially beneficial effects in MiR-155 expression and consequent inflammation through an improvement of lipoprotein parameters.

Singh *et al*[44] studied Vitamin D regulation of miRNA across different cancer cell models, representing non-malignant and malignant cells. They exposed the cells to 30 min of treatment with vitamin D3 (1,25-dihydroxyvitamin D3) or 1,25(OH)2D3. Across all cells, 111 miRNAs were significantly modulated by the vitamin treatment, but only 5 miRNAs were modulated in more than one cell model, and of these only 3 miRNAs were modulated in the same direction. An integrative network-based analysis used a publicly available data set to study the role of 1,25(OH)2D3 in cancer cells on levels of microRNAs[45]. Pathway analysis revealed 15 significantly altered pathways, including eight somewhat general, mostly cell cycle-related pathways, and seven cancer-specific pathways. The authors identified a new vitamin D-microRNA network, including six differentially expressed microRNAs (MiR-29a, MiR-371-5p, MiR-1915, MiR-663, MiR-134 and MiR-542-5p). All six were up-regulated in 1,25(OH)2D3-treated cancer cells in the studies by Kutmon *et al*[45].

MiR-155 is a key regulator of Toll-like receptor (TLR) signaling that plays a pivotal role in immune response and through this, may also play a role in CRC. Li *et al*[46] used a rodent model to show that 1,25(OH)2D3 modulates innate immune response by targeting the miR-155-SOCS1 axis. Jorde *et al*[47] supplemented human volunteers with high doses of 1,25(OH)2D3 for 12 mo. They found significant changes with miR-532-3p and miR-221 from baseline to 12 mo that was significantly different between the vitamin D and placebo group. In colon human cancer cell models, MiR-22 and several other miRNA species have been identified as 1,25(OH)2D3 targets[48].

Vitamin E has also been shown to play a role in miRNA regulation, at least in rat liver[49]. These authors fed rats for 6 mo with vitamin E-sufficient or deficient diets, after which they estimated the hepatic concentrations of miRNAs that had been previously associated with this vitamin (miRNA-122a in lipid metabolism and miRNA-125b in cancer and inflammation). Concentrations of both these miRNAs were decreased in conditions of vitamin E deficiency.

Minerals may also affect miRNA expression and activity. In particular, Selenium (Se) showed effects on the expression of a number of genes, especially

Table 2 Summary of dietary regulation of microRNAs, potentially relevant to colorectal cancer

MicroRNA	Study population	Diet or nutrient	Analysis method	Findings	Specimen type	Ref.
miR-16 miR-21 miR-34a miR-92a miR-106a miR-146 miR-222	Italian-based 8 vegans 8 vegetarians 8 omnivores	Meat, processed meat, fish, cheese	Food frequency and lifestyle questionnaire	miR-92a was significantly decreased by meat and dairy products, and associated with low body mass index. Weaker associations found between miR-21 levels and vegetable intake	Plasma and stool	Tarallo *et al*[38]
Let-7d miR-15b miR-107 miR-191 miR-324-5	Sprague-Dawley rats, treated with saline or the carcinogen, azoxymethane	Corn oil *vs* fish oil in the diet	Effects of diets on the expression of 368 miRNAs in the colonic mucosa	The five identified miRNAs were the most strongly affected by diet X carcinogen actions. The fish fed animals showed the smallest number of differentially expressed miRNAs - interpreted as due to a reduction in inflammation	Colonic mucosa	Davidson *et al*[39]
miR-1903 miR-467c miR-368 miR-927c	Female athymic nude mice, injected with HT-29 colon cancer cells	Corn oil *vs* ground walnuts in the diet	Effects of the diets on the expression of four microRNAs in the colon tumours	The first three of these microRNAs were down-regulated and the latter up-regulated in expression. These data were related to significant increases in α-linolenic, eicosapentaenoic, docosahexaenoic and total omega-3 acids, and a decrease in arachidonic acid in the walnut fed mice	Colorectal tumour tissue	Tsoukas *et al*[40]
miR-155	Young subjects (22 + 2 yr), smokers and non-smokers	High dose vitamin C daily for 8 wk	Expression level of miR-155 in HDL3	miR-155 reduced in HDL fraction by 49% in non-smokers and 75% in smokers after 8 wk supplementation. This effect was related to a reduction in reactive oxygen species	Serum lipoprotein levels	Kim *et al*[43]
miR-98 miR-92a miR-30e miR-140-5p miR-138	7 different prostate cell models including malignant and non-malignant	30 min treatment with 1a,25(OH)2D3	MiRNA microarray analyses	111 miRNAs showed changed expression levels, but only 5 were seen affected in more than one cell line and only 3 were changed in the same direction	Total mRNA and miRNA from each cell line.	Singh *et al*[44]
miR-22 miR-29ab miR-134 miR-1207-5p miR-371-5p miR-17 miR-20a	LNCaP human prostate cancer cells	48 h treatment with 100 nmol/L 1,25(OH)2D3 compared with non-treated control, cells	Agilent human microRNA v3 microarrays to measure microRNA expression	Four hundred and twenty genes were up-regulated and 413 genes down-regulated in the 1,25(OH)2D3-treated cells. The most strongly affected are those identified in column 1 (the last two of these miRNAs is downregulated)	Integrative network-based analysis using a publicly available data set	Kutmon *et al*[45]
miR-155	RAW264.7 macrophage cells stimulated with lipopolysaccharide (LPS)	24 h in the presence of EtOH or 20 nmol/L 1,25(OH)2D3	miRNA profiling by microarrays	Several miRNAs were induced by LPS and suppressed by 1,25(OH)2D3, of which miR-155 was on the top of the list, suppressing about 50% of the LPS induction	Total mRNA and miRNA from each cell line	Li *et al*[46]
miR-22	SW480-ADH and HCT116 colon cancer cells	10^{-7} mol/L 1,25(OH)2D3 for 24, 48 or 96 h	miRNA profiling by microarrays	Although there were 12 microRNAs that showed differential expression with and without vitamin D, miR-22 showed the most consistent differences	Total miRNA from each cell line	Alvarez-Díaz *et al*[48]
Let-7f Let-7a miR-151-5p miR-22 miR-221 miR-28-5p miR-552-3p miR-766 miR-99b	Males, generally in good health, with no diabetes or other concomitant diseases	High dose vitamin D3 (20000-40000 IU per week)	Quantitative real-time PCR	In 10 pilot subjects, 136 miRNAs were changed in expression in one or more plasma samples drawn at baseline and after 12 mo of vitamin D supplementation. The twelve miRNAs that showed the greatest change in expression in the pilots were further measured in RNA from baseline and 12 mo plasma samples in 40 subjects given vitamin D and 37 subjects given placebo	Plasma	Jorde *et al*[47]
miR-122a miR-125b	Fischer 344 rats	0, 12 or 24 mg/kg	Quantitative real-time PCR	Vitamin E sufficiency resulted in increased concentrations of miRNA-122a and miRNA-125b	Liver tissue	Gaedicke *et al*[49]

miR-625 miR-492 miR-373 miR-22, miR-532-5p miR-106b miR-30b miR-185 miR-203 miR1308 miR-28-5p miR-10b	CaCO2 human colon cancer cells	Selenium-deficient or sufficient medium	Microarray validated with quantitative real-time PCR	Selenium deficiency resulted in altered expression of 12 genes	Total miRNA from combined cells of each treatment	Maciel-Dominguez *et al*[50]
miR-21	U251 human glioblastoma cells	10, 50 or 100 umol/L Resveratrol for 72 h	Quantitative real-time PCR	Resveratrol inhibited miR-21 expression which in turn suppressed NF-κB activity. However, over-expression of miR-21 could reverse the effect of resveratrol on NF-κB activity and apoptosis	Cell extracts	Li *et al*[54]
miR-21	Estrogen-dependent MCF-7 and estrogen receptor-negative p53 mutant MDA-MB-468 human breast cancer cells	0, 30 or 60 umol/L	Quantitative real-time PCR	Cells were studied either in tissue culture or as a xenograft in BALB/C female athymic mice miR-21 was up-regulated in DIM-treated MCF-7 cells, but not in the ER negative, p53 mutant MDA-MB-468 cells	Cell extracts	Jin[55]
miR-30b miR-1224-3p miR-197 miR-523-3	HepG2 human hepatocarcinoma cells	3-3′-Diindoyl-methane for 24-96 h 50 mg/L of epigallocatechin gallate (EGCG), 100 mg/L of grape seed extract (GSPE) or 100 mg/L of cocoa proanthocyanidin extract (CPE)	Microarray analysis validated by quantitative real-time PCR	MiR-30b was downregulated by all three treatments, while treatment with GSPE or CPE upregulated miR-1224-3p, miR-197 and miR-532-3p	Cell extracts	Arola-Arnal *et al*[57]
miR-210 (plus 13 other miRNAs upregulated and 7 down-regulated)	Tobacco carcinogen-induced lung cancer in A/J mice	Purified mouse chow containing 0.4% EGCG	Microarray analysis validated by quantitative real-time PCR	MiR-210 had been previously found upregulated by EGCG in *in vitro* experiments, but this ranked behind the 13 most strongly upregulated miRNAs (miR-2137, miR-449a, miR-144, miR-486, miR-3107, miR-193, miR-5130, miR-2861, miR-511-3p, miR-763, miR-3473, miR-211, miR-210) or seven most down regulated in this *in vivo* study	Tumour tissue, all tumours from a single mouse combined to a single sample	Zhou *et al*[58]

glutathione peroxidase 2 and selenophosphate synthetase 2, through altering the profile of miRNAs in an intestinal cell line[50]. Following exposure of CaCO2 cells to Se-deficient medium for 72 h, there were alterations in the levels of twelve miRNA: miR-10b, miR-22, miR-28-5p, miR-30b, miR-106b, miR-185, miR-203, miR-373, miR-492, miR-532-5p, miR-625 and miR-1308. In particular, silencing of miR-185 increased GPX2 and SEPSH2 expression.

A range of polyphenols have been found to interact with and be affected by miRNAs. Of particular interest to CRC may be the regulatory effects of curcumin on miR-21[51]. MiR-21 has been found to mediate a range of effects of curcumin on cancer cells, including cell proliferation, cellular senescence or apoptosis, metastasis and anti-cancer drug resistance. MiR-21 was found to suppress the anticancer activities of curcumin by targeting the PTEN gene in human non-small cell lung cancer A549 cells[52]. In turn, curcumin has been shown to decrease the levels of miR-21 through increasing miR-21 exosome exclusion from cancer cells, and also through inhibiting the transcription of the miR-21 gene by binding to its promoter. At a 20-40 mmol dose, curcumin treatment led to a significant reduction of microRNA-21 expression, as compared to that in untreated cells.

Resveratrol (trans-3,4′,5-trihydroxystilbene) has been shown to induce the expression of miR-663, a tumor-suppressor and anti-inflammatory microRNA, while downregulating miR-155 and miR-21[53]. These authors suggest that the use of resveratrol in therapeutics may be optimised by considering the effects of the selected dose on the expression of miR-155 or miR-21. The authors also suggested that the activity of resveratrol might be enhanced by finding ways to manipulate the levels of its key target microRNAs, such as miR-663. More generally, resveratrol is known to play an important role in inhibiting proliferation and inducing apoptosis of cancer cells[54]. These authors found a dose-dependent decrease in cancer cell viability following resveratrol

treatment. As well as effects on cellular signalling pathways, resveratrol inhibited miR-21 expression, which in turn could suppress nuclear factor-kappaB activity. Conversely, over-expression of miR-21 was found to inhibit the beneficial antitumour effects of resveratrol.

3,3'-Diindolylmethane (DIM) is a cancer-preventive phytochemical that is found in Brassica vegetables. At least in a human breast cancer cell line, DIM was shown to inhibit cell growth through a miR-21-mediated mechanism[55]. These effects were related to differential modulation of cellular signalling pathways that led to arrested cell-cycle progression of the human cancer cells.

Proanthocyanidins are highly abundant and found in a range of food plants including cinnamon, cocoa beans, grape seed, grape skin and various berries. They have been found to have positive health effects on a variety of metabolic disorders associated with inflammation, largely through their effects on genomic stability[56]. Arola-Arnal and Blade studied the effects of proanthocyanidin-rich natural extracts in modulating miRNA expression[57]. They used microarray analysis and Q- PCR, to study miRNA expression in colonic HepG2 cells treated with a grape seed proanthocyanidin extract (GSPE), cocoa proanthocyanidin extract (CPE) or the green tea polyphenol epigallocatechin gallate (EGCG). They found that miR-30b was downregulated by all three treatments, while treatment with GSPE or CPE upregulated miR-1224-3p, miR-197 and miR-532-3p.

In other models, Zhou *et al*[58] found that EGCG upregulated the expression of miRNAs such as miR-210, thereby reducing cell proliferation. They used functional genomic approaches to study the role of miRNA in EGCG inhibition of carcinogen-induced mouse tumors. They identified changes in the expression levels of 21 microRNAs for which they found 26 potential targeted genes relating to cancer inhibition.

CONCLUSION

The available studies summarised herewith begin in 2010, at a time when miRNA methodologies were becoming increasingly more sensitive. While not all hypothesised miRNAs have proved as useful as initially hoped, it would seem that either individual miRNAs or a panel of these would have very significant prognostic value and enable an intensive lifestyle intervention to prevent what would otherwise be the natural course of disease. The identification of potential biomarkers that reliably detect or diagnose early stages of CRC or evidence of CRC progression is urgently needed.

An increasing body of evidence suggests that epigenetic changes contribute to carcinogenesis, and miRNAs are prominent among these. There is no question but that deregulation of miRNAs plays an important role in human carcinogenesis. Overall, miRNAs appear to be a promising class of biomarkers for CRC. However, further research is needed to validate previous findings and increase our current understanding of the identified miRNAs. The applicability of some studies in other populations is uncertain as many subjects were recruited from a single ethnic group. Thus, future studies could confirm the utility of miRNA for other ethnic groups by recruiting subjects from multiple centres and various populations.

Many of the studies had small sample sizes and this may have contributed to the contradictory findings of some of the reports. Hence, stronger studies in the future would do well to increase their sample sizes to determine whether certain miRNA levels are consistently modified. As well as determining that specific miRNA levels are modified in patients with AA, it would also be useful to determine how specific these changes are for colorectal neoplasia. Future studies may also consider whether the asymptomatic or symptomatic status of subjects causes differences in miRNA expression levels. Analyses of miRNA expression should be separated for asymptomatic and symptomatic individuals to examine any differences which may exist. For most of the miRNA studies so far, controls consist of individuals who were determined to be free from colorectal neoplasia following a colonoscopy. However, it is known that miRNA are dysregulated in different cancers and other diseases. Hence, to investigate the specificity of miRNA for colorectal neoplasia, groups should include control individuals with different diseases including conditions affecting various organs to confirm the specificity of individual miRNA transcripts. On another note on controls, it is important to obtain comprehensive information on the health of potential controls to confirm those in the "healthy" control groups are indeed healthy to the best available knowledge. Another way to confirm the specificity of miRNA for colorectal neoplasia is to analyse the relationship between miRNA expression in neoplastic tissue and miRNA expression from blood and stool samples. If there is concordant expression between the two, it makes it more likely that the miRNA was secreted from the colorectal neoplastic lesion. This would be an important finding as this would increase the specificity of this miRNA for colorectal neoplasia[20]. Another issue for circulating miRNA research is the lack of consensus on precise and robust internal controls[27,31]. Luo *et al*[27] chose miR-16 as an internal control in their plasma-based miRNA tests. There is also room for improvement in the assay technologies used to measure miRNAs. qRT-PCR cannot precisely quantify many miRNAs

in plasma which are present in too low levels so some miRNAs cannot be included in studies or were included but their performance may not be accurately reflected in results[27,28]. Other methods to detect and quantify low-level miRNAs with higher sensitivity would be desirable in future investigations. MiRNA with initial promising results should be further studied in larger study populations to verify reproducibility. To validate miRNA further, the performance of selected miRNA should be compared to the performance of current accepted screening tools, such as fecal occult blood tests. All promising miRNA should undergo testing in large prospective trials if they are to be accepted as a screening tool in routine clinical practice.

A biomarker will be useful for screening or the early detection of cancer only if it can be detected in a non-invasive or minimally invasive fashion without tissue biopsy. Increasing evidence has indicated that miRNAs in serum, stools or other body fluids may become important biomarkers for the detection of early CRC. It is hoped that such miRNA markers will be translated into clinical use in the near future, enabling early diagnosis of CRC development and an accurate assessment of disease progression. Such advances would allow patients to receive early treatment and ultimately improve survival.

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REVIEW

Polyphosphate and associated enzymes as global regulators of stress response and virulence in *Campylobacter jejuni*

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Abstract

Campylobacter jejuni (*C. jejuni*), a Gram-negative microaerophilic bacterium, is a predominant cause of bacterial foodborne gastroenteritis in humans worldwide. Despite its importance as a major foodborne pathogen, our understanding of the molecular mechanisms underlying *C. jejuni* stress survival and pathogenesis is limited. Inorganic polyphosphate (poly P) has been shown to play significant roles in bacterial resistance to stress and virulence in many pathogenic bacteria. *C. jejuni* contains the complete repertoire of enzymes required for poly P metabolism. Recent work in our laboratory and others have demonstrated that poly P controls a plethora of *C. jejuni* properties that impact its ability to survive in the environment as well as to colonize/infect mammalian hosts. This review article summarizes the current literature on the role of poly P in *C. jejuni* stress survival and virulence and discusses on how poly P-related enzymes can be exploited for therapeutic/prevention purposes. Additionally, the review article identifies potential areas for future investigation that would enhance our understanding of the role of poly P in *C. jejuni* and other bacteria, which ultimately would facilitate design of effective therapeutic/preventive strategies to reduce not only the burden of *C. jejuni*-caused foodborne infections but also of other bacterial infections in humans.

Key words: *Campylobacter jejuni*; Stress tolerance; Inorganic polyphosphate; Virulence; Colonization/infection

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Core tip: Recent studies show that inorganic polyphosphate (poly P) plays several important roles in the biology of *Campylobacter jejuni* (*C. jejuni*), a major cause of bacterial foodborne gastroenteritis in humans. This review summarizes the latest findings on the role of poly P in *C. jejuni* stress survival and virulence, provides directions for future investigation, and discusses the potential of polyphosphate kinase enzymes as drug/vaccine targets to control *C. jejuni* infections in humans.

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INTRODUCTION

Campylobacter (Greek word "*Kampylos*" means curved) species are curved to spiral-shaped, non-spore forming, Gram-negative bacteria that contain a single flagellum at one or both ends. Most of the *Campylobacter* species are microaerophilic and use a respiratory type of metabolism; however, some species prefer to grow anaerobically. *Campylobacter* species are chemoorganotrophs - they primarily depend on amino acids or Kreb's cycle intermediates for energy. Based on their optimal growth temperature, *Campylobacter* species are classified into thermophilic and non-thermophilic species. The thermophilic *Campylobacter* species grow optimally at 42 ℃ [*Campylobacter jejuni* (*C. jejuni*) and *Campylobacter coli* (*C. coli*)], while the non-thermophilic species grow optimally at the range between 30-37 ℃ (*Campylobacter fetus*). The genus *Campylobacter* consists of 34 species and 14 subspecies, which are human and animal pathogens (http://www.bacterio.net/campylobacter.html, last accessed 17 March 2016). Several of these species cause a plethora of clinical manifestations in humans (Table 1); however, *C. jejuni* and *C. coli* are the predominant species associated with human disease[1].

C. jejuni is one of the major causes of bacterial foodborne diarrheal disease in the United States and worldwide[2]. According to Foodborne Diseases Active Surveillance Network, *Campylobacter* spp. account for 9% of total foodborne illnesses, 15% of hospitalizations and 0.1% of deaths from foodborne illnesses each year[3]. In immunocompetent patients, *C. jejuni* infection typically manifests as a self-limiting acute gastroenteritis characterized by severe watery and sometimes bloody diarrhea, fever, nausea, and vomiting. However, in immunocompromised patients, *C. jejuni* can cause a severe, life-threatening disease, often requiring hospitalization and antibiotic treatment. In addition to causing gastroenteritis, *C. jejuni* is also associated with post-infection complications such as Guillain-Barre syndrome, which is a rare neuromuscular disease that is thought to occur in 1 in 1000 individuals infected with *C. jejuni*[4]. Other sequelae such as Reiter's syndrome, inflammatory bowel syndrome, and immunoproliferative small intestinal disease also significantly add to the burden of *C. jejuni* infection[5-7].

Unlike in humans, *C. jejuni* lives as a commensal in the gut of a variety of domestic and wild animals and birds[4]. Epidemiological studies show that the majority (50%-80%) of human infections are acquired by consumption of contaminated poultry and poultry products and that nearly 90% of the poultry flocks in the United States are colonized with *C. jejuni*, suggesting that controlling *C. jejuni* colonization in poultry is an effective strategy to control human infections[4]. Rarely, contaminated raw milk, water, and vegetables also serve as sources of human infection[1,4]. Although transmission through ingestion of contaminated food and water is the major route, person-to-person contact, as well as contact with pets or their feces is not uncommon[1,4]. Although *C. jejuni* is a major public health concern worldwide, the genetic determinants that contribute to its ability to survive in different host and non-host environments, to colonize poultry and other domestic animals, and to cause disease in humans are relatively poorly understood.

C. jejuni is unique among the enteric bacterial pathogens in that it lacks many classical stress response and virulence mechanisms. More specifically, *C. jejuni* lacks the stationary phase sigma factor RpoS, the heat shock sigma factor RpoH, the cold shock protein CspA, the oxidative stress response genes SoxRS, OxyR, SodA and KatG, the osmoprotectants ProU, OtsAB and BetAB, and the leucine-responsive global regulator Lrp, which are all essential for stress tolerance in other enteric pathogens[8]. These findings are consistent with the unusual sensitivity of *C. jejuni* to various environmental stresses *i.e., C. jejuni* is unable to grow in the presence of oxygen, has a narrow growth temperature range, is normally incapable of multiplication outside the host, does not survive well on dry surfaces, cannot withstand high temperaturature, and is more sensitive to osmotic and low pH stresses[8]. *C. jejuni* also lacks many classical virulence mechanisms, including a type Ⅲ secretion system and exotoxins, which play important roles in the pathogenesis of other enteric bacteria[9]. Several *C. jejuni* strains contain type Ⅳ and type Ⅵ secretion systems but the absence of these systems in many pathogenic *C. jejuni* strains questions

Table 1 Comprehensive summary of *Campylobacter* infections in humans

Type	Clinical form/presentation	Risk factors	Target site	Symptoms	Species associated
(A) Intestinal	Periodontal diseases	Oral bleeding, increased vascular permeability[70], and pregnancy[71]?	Oral cavity	Bleeding, tenderness, and tooth loss	*C. rectus*[70], *C. gracilis*, *C. showae*[72] and *C. concisus*[73]
	Esophageal diseases (gastroesophageal reflux disease, Barrett's esophagus, and esophageal adenocarcinoma)	Mucosal damage due to stomach contents regurgitation[1]	Esophagus	Heart burn, regurgitation, bloating, bad breath, nausea, and abdominal pain	*C. concisus*[74]
	Self-limited gastroenteritis, most common form	All factors as described in review	Jejunum and ileum	Diarrhea, fever, and abdominal pain	*C. jejuni* and *C. coli*[1,75]
	Post-infectious functional gastrointestinal disorder, irritable bowel syndrome, and functional dyspepsia	Infection with *C. jejuni* and other species[1]		Diarrhea, constipation or both, and abdominal pain	*C. jejuni*, *C. coli* and *C. consisus*[76]
	Inflammatory bowel disease, Crohn's diseases (CD), and ulcerative colitis (UC)	Gut dysbiosis[77]	CD-any part of intestine UC- colon	Diarrhea, fever and fatigue, abdominal pain, weight loss, and reduced appetite	*C. concisus*, *C. showae*, *C. hominis*, *C. rectus*, and *C. ureolyticus*[78]
	Colorectal cancer	Gut dysbiosis[79]	Colon	Diarrhea, constipation, abdominal pain, weight loss, and rectal bleeding	*C. showae*[80]
	Cholecystitis	Gall stones[1]	Gall bladder	Pain and tenderness in right abdomen, nausea, vomiting, and fever	*C. jejuni*[81]
(B) Extra-intestinal	Guillain-Barre syndrome	*C. jejuni* infection[82]	Nervous system	Progressive symmetric weakness in limbs, below or lack of reflex (hyporeflexia)	*C. jejuni*[1,82]
	Miller Fisher syndrome	*C. jejuni*[83]	Nervous system	Oculo-motor weakness	*C. jejuni*[83]
	Reactive arthritis	Infection with enteric bacterial pathogens, including *Campylobacter*[84]	Joints, eyes, and genitourinary tract	Pain and stiffness of joints, swollen toes, eye inflammation, and urinary problems	*C. jejuni* and *C. coli*[1,84]
	Cardiovascular complications	Immuno-compromised condition, and bacteremia[1]	Cardiovascular system	Chest pain leading to arrhythmia, dilated cardiomyopathy, and sudden death due to congestive heart failure	*C. jejuni* and *C. fetus*[85,86]
	Meningitis	Immuno-compromised condition[1,87]	Meninges of brain and spinal cord	Headache with nausea or vomiting, seizures, sensitive to light, and loss of appetite	*C. jejuni* and *C. fetus*[88,89]
	Abscesses of breast, brain, vertebra, and liver	Secondary bacterial infection	Breast, brain, vertebra, and liver	Varies with target organ	*C. rectus*[90], *C. curvus*, *C. gracilis* and *C. showae*[91]
	Reproductive complication	Bowel infection, and periodontal disease[92]	Uterus, and placenta	Preterm birth, low birth weight, and intra-uterine growth restriction	*C. jejuni*, *C. coli*, *C. fetus* and *C. upsaliensis*[92]

Campylobacter infection types, clinical presentations, risk factors, target organ or tissues involved, symptoms and associated *Campylobacter* species are described.

their requirement for virulence[10,11]. Nevertheless, the increasing incidence of *C. jejuni* infections in humans suggests that this organism may have evolved alternative mechanisms for stress survival and virulence. One such mechanism that has been relatively well characterized by several recent studies and plays important roles in *C. jejuni* survival and virulence involves inorganic polyphosphate (poly P). In this review, we summarize recent data on the role of poly P and related enzymes in *C. jejuni* biology, with particular focus on stress survival and virulence.

INORGANIC POLYPHOSPHATE

Even after the discovery of the subatomic particle (the God particle), the origin of the universe is still a debated topic. However, there is almost a universal agreement that phosphate has played a key role in the origin of life on earth. Poly P is a linear polymer of ten to hundreds of phosphate residues linked by high-energy phosphoanhydride bonds. Poly P granules were first seen in bacteria as "Volutin granules" or "Metachromatic granules", named after their tendency to stain pink with basic blue dyes[12]. Indeed, the presence of metachromatic granules was used as a diagnostic feature for pathogenic bacteria such as *Corynebacterium diptheriae*. Later with the advent of electron microscopy, these granules were identified in nonpathogenic bacteria, which refuted the idea of using poly P granules as a marker for pathogenic bacteria[12]. Recent studies have shown that poly P is essential for numerous cellular functions in bacteria,

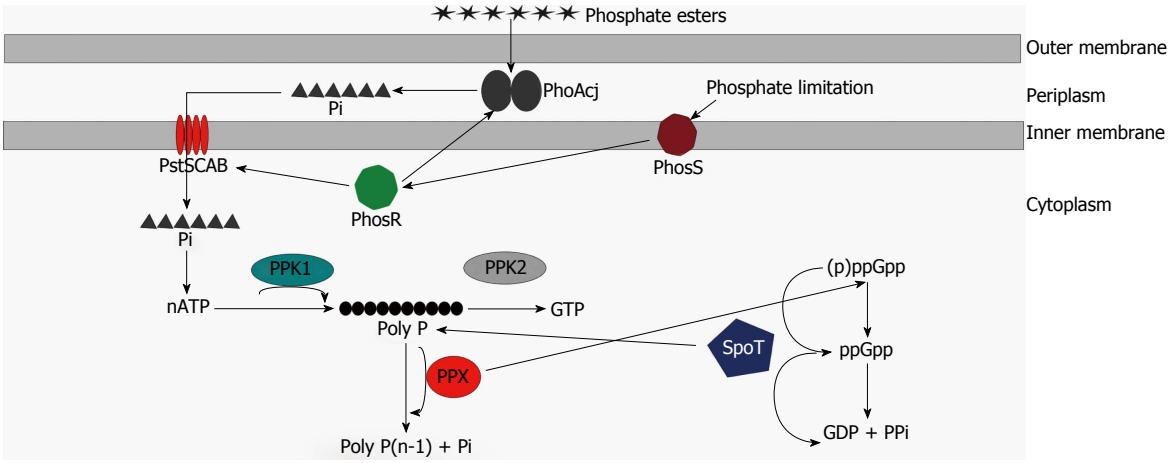

Figure 1 Model of poly P metabolism in *Campylobacter jejuni*. Phosphate esters are hydrolyzed to inorganic phosphate (Pi) by alkaline phosphatase (PhoAcj) in the periplasm. Phosphate uptake proteins and alkaline phosphatase are directly regulated by the PhosS/PhosR two-component system. Pi is transported across the inner membrane *via* the high affinity phosphate transport system PstSCAB. ATP generated from Pi is utilized for poly P synthesis by PPK1. PPK2 utilizes poly P to generate GTP, while PPX hydrolyzes poly P back to Pi. PPX also affects conversion of (p)ppGpp to ppGpp. SpoT is a bifunctional enzyme involved in both ppGpp synthesis as well as ppGpp hydrolysis. SpoT is also linked to poly P metabolism and a *spoT* mutant has reduced ability to accumulate poly P.

such as energy source, phosphate reservoir, cation sequestration, buffering role against alkali, participation in membrane transport, cell envelope formation and function, regulator of enzyme activities, gene activity control and development, chromatin destabilization, DNA replication and phage production, sporulation and germination, bacterial virulence/pathogenesis, and regulator of stress and survival[13]. Additionally, bacteria capable of storing large amounts of intracellular poly P are used in the biological treatment of wastewater[14].

GENERAL ASPECTS OF POLY P METABOLISM IN BACTERIA

Polyphosphate kinase 1 (PPK1) is the principal enzyme responsible for poly P synthesis in many bacteria[15,16]. PPK1 is highly conserved; homologs of this enzyme have been found in over 100 bacterial species, including 20 major human and animal pathogens. PPK1 is perhaps the most known of all poly P-related enzymes for its role in bacterial survival under conditions of stress, virulence, and host colonization[13]. Many bacterial species contain another enzyme, PPK2, which preferentially mediates poly P-driven generation of guanosine triphosphate (GTP)[17,18], a molecule known to have important roles in cell signaling and DNA, RNA, protein, and polysaccharide synthesis[19,20]. Similar to PPK1, PPK2 is also widely conserved in bacteria, including major human pathogens such as *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, and *Vibrio cholerae* among others[17]. Although relatively less studied compared to PPK1, few recent studies have shown a role for PPK2 in bacterial survival and virulence[18]. The third family of enzymes involved in poly P metabolism includes exopolyphosphatases, which degrade poly P to inorganic phosphate[13,21]. Many bacteria contain 2 types of exopolyphosphatases[22], one that only hydrolyzes poly P (hereafter referred to as PPX) and the other that, in addition to hydrolyzing poly P, also hydrolyzes guanosine pentaphosphate (pppGpp) to guanosine tetraphosphate (ppGpp) (hereafter referred to as PPX/GPPA)[13,23]. ppGpp is a signaling molecule that plays an important role in bacterial stringent response induced by starvation[13,23]. Of all the poly P-metabolizing enzymes, PPX enzymes are the least understood with regards to their role in bacterial survival and virulence.

POLY P METABOLISM IN *C. JEJUNI*

C. jejuni contains all the enzymes involved in poly P metabolism- PPK1, PPK2, and 2 PPX/GPPA enzymes (Figure 1). Two independent studies have evaluated the contribution of PPK1 to poly P metabolism in *C. jejuni*[24,25]. Using a toluidine blue O assay, both studies demonstrated that the *C. jejuni* wildtype strain accumulated more poly P in stationary phase than in mid-log and transition phases and that deletion of *ppk1* significantly reduced poly P accumulation (Figure 1)[24,25]. The *C. jejuni ppk1* mutant retained a modest ability to synthesize poly P[24,25]; thus, the alternative enzyme that contributes to residual poly P levels in the *ppk1* mutant remains to be identified. Using electron microscopy, the authors further demonstrated that the *C. jejuni* wildtype strain contained several poly P-like granules and that the *ppk1* mutant contained fewer of these granules[25]. More recently, a detailed ultrastructure analysis of *C. jejuni* at 5 nm resolutions also showed the presence of orange, poly P storage granules in *C. jejuni*[26]. Although the two studies suggested that the observed granules are poly P granules, additional work is required to confirm that

Table 2 Phenotypes associated with enzymes of poly P and ppGpp metabolism

Phenotype	PPK1	PPK2	PPX1	PPX2	PPX1-PPX2	PhoAcj	SpoT
Poly P metabolism							
Poly P synthesis/accumulation	↑	-	-	-	-	↑	↑
Poly P-dependent GTP synthesis	-	↑	NT	NT	NT	NT	NT
Poly P degradation	-	-	↑	↑	↑	NT	NT
Maintenance of ATP:GTP ratio	NT	↑	NT	NT	NT	NT	NT
ppGpp metabolism							
ppGpp synthesis	NT	-	↑	-	↑	NT	↑
Stress survival							
Stationary phase survival and growth	-	-	-	-	-	-	↑
Survival under low CO_2	NT	NT	NT	NT	NT	NT	↑
Osmotic shock survival	↑	↑	↑	↑	↑	↑	NT
Low nutrient stress survival	↑	↑	↑	↑	↑	↑	NT
Aerobic stress survival	-	↑	NT	NT	NT	NT	↑
VBNC formation	↑	↑	NT	NT	NT	NT	NT
Natural transformation	↑	NT	NT	NT	NT	NT	NT
Antimicrobial resistance	↑	↑	NT	NT	NT	↓	R
Virulence-related							
Motility	-	-	↑	↑	↑	NT	NT
Biofilm formation	↓	↓	↑	↑	↑	↓	NT
Resistance to complement-mediated killing	NT	NT	↑	↑	↑	NT	NT
Adherence	NT	-	NT	NT	NT	NT	↑
Invasion	-	↑	↑	↑	↑	↑	↑
Intraepithelial survival	↑	↑	↑	↑	↑	↑	↑
Chicken colonization	↑	↑	NT	NT	NT	↑	NT

NT: Not tested; R: Tested only for Rifampicin resistance; -: Phenotype absent; ↑: Phenotype is positively regulated *i.e.*, deletion of the gene reduces the phenotype when compared to wildtype; ↓: Phenotype is negatively regulated *i.e.*, deletion of the gene enhances the phenotype when compared to wildtype.

these granules are indeed poly P granules.

In another study, the role of PPK2 in poly P metabolism was investigated[27]. The authors of this study demonstrated that a *ppk2* mutant was deficient in poly P-dependent GTP synthesis. The authors also showed that the ratio of ATP:GTP was altered in the *ppk2* mutant, thus suggesting that PPK2 is required for poly P-dependent GTP synthesis as well as for maintenance of nucleotide balance in the cell (Figure 1). GTP is a signaling molecule that modulates many physiological functions in bacteria[19,20]. Unlike studies in other bacteria, which showed that PPK2 possesses poly P-synthetic activity[28], the authors demonstrated that poly P levels were unaltered in the *C. jejuni ppk2* mutant when compared to wildtype, suggesting that PPK2 does not appear to contribute to poly P synthesis in *C. jejuni*[27].

Malde *et al*[29] evaluated the contributions of PPX/GPPA enzymes to poly P metabolism in *C. jejuni*. They demonstrated that both *ppx1/gppa* and *ppx2/gppa* mutants accumulated more poly P than the wildtype strain and that the *ppx1/gppa* mutant accumulated relatively more poly P than the *ppx2/gppa* mutant when compared to wildtype. Based on these data, the authors suggested that PPX1/GPPA and PPX2/GPPA are involved in poly P degradation and that PPX1/GPPA is probably the primary enzyme that contributes to poly P degradation in *C. jejuni* (Figure 1)[29]. However, whether PPX/GPPA enzymes possess PPX activity needs to be further experimentally confirmed.

PPKS: ROLE IN *C. JEJUNI* SURVIVAL AND VIRULENCE

Mutations in *ppk1* and *ppk2* genes have resulted in a variety of phenotypic changes in *C. jejuni*. Although the mechanisms underlying these phenotypes are poorly understood, the phenotypes as such are significant and relevant to *C. jejuni* pathogenesis. In the following sections, we will review our latest understanding of the role of PPKs in these phenotypes, which are summarized in Table 2.

PPKs: Role in growth and stationary phase survival

Several studies evaluated the effect of *ppk1* and *ppk2* mutations on *C. jejuni* growth and demonstrated that these mutations do not affect *C. jejuni* growth[24,25,27]. In other organisms, *ppk1* mutants are defective in stationary phase growth and survival[30,31]. The *C. jejuni* wildtype strain accumulated more poly P in stationary phase than in mid-log and transition phases and more genes were differentially expressed in the *ppk1* mutant in stationary phase when compared to mid-log phase[24,25,32]. However, the *C. jejuni ppk1* mutant grew and survived similar to wildtype in stationary phase. A possible explanation for this difference is that *C. jejuni* lacks RNA polymerase, sigma S (RpoS), which regulates stationary phase survival and itself is modulated by poly P in other organisms[8,33].

PPKs: Role in stress survival and adaptation

C. jejuni encounters a number of unfavorable

environments both in the external environment during transmission as well as in the host during colonization/infection[8]. Poly P is known for its role in modulating stress tolerance in bacteria[13]. Two independent studies by Candon *et al*[24] and Gangaiah *et al*[25] demonstrated that the *ppk1* mutant had a reduced ability to survive under low nutrient stress and osmotic shock. Similarly, in another study, Gangaiah *et al*[27] showed that the *ppk2* mutant, in addition to displaying a reduced ability to survive under low nutrient stress and osmotic shock, also had reduced ability to survive under aerobic stress. Bacteria generally respond to nutrient stress by eliciting a stringent response regulated by ppGpp and/or a general stress response regulated by RpoS. A *C. jejuni* bifunctional (p)ppGpp synthase/hydrolase (*spoT*) mutant, which is defective in eliciting a stringent response, was compromised in its ability to survive under low nutrient stress[34]. Thus, to rule out the possible role of ppGpp in PPK1-mediated nutrient stress survival phenotype, it would be interesting to determine if ppGpp levels are altered in the *ppk1* mutant. Transcriptome analysis revealed that, despite *C. jejuni* lacking RpoS, homologs of several genes involved in general stress response were downregulated in *ppk1* and *ppk2* mutants; this might partly explain the stress survival deficiencies in the *ppk* mutants[32]. In *E. coli*, poly P also affects stress tolerance by several other mechanisms- poly P is thought to be involved in sensing minor changes at the cell surface, serves as a source of energy during times of stress, and regulates Lon protease, which degrades nonessential proteins providing free amino acids for survival during stress[13,35-38]. Whether such mechanisms also play a role in poly P-mediated stress resistance in *C. jejuni* is worth investigating.

PPKs: Role in viable but nonculturable cell formation

C. jejuni is also capable of forming viable but nonculturable cell (VBNC) under stressful conditions; this phenotype is thought to provide a survival advantage to *C. jejuni* during times of stress[39,40]. Two recent studies showed that both *ppk1* and *ppk2* mutants were compromised in their ability to form VBNCs after formic acid treatment[25,27]. The reduced ability of the *ppk* mutants to form VBNCs could be due to the fact that these mutants were more sensitive to other stresses such as low nutrient stress and osmotic shock. Poly P can serve as a source of energy during times of stress[13]; this could also contribute to the reduced ability of the *ppk1* mutant to form VBNCs. A *ppk2* mutant had similar poly P levels but was compromised in its ability to regulate nucleotide balance; thus, whether altered nucleotide balance is the cause of the *ppk2* mutant's reduced ability to form VBNCs needs to be further studied. One might also speculate that PPK enzymes, as global regulators, might modulate VBNC formation by affecting the expression of other genes involved in VBNC formation; however, transcriptome analysis of *ppk* mutants did not appear to provide any additional insights into this mechanism. For more information on the role of poly P in VBNC formation in *C. jejuni*, refer to the review by Kassem *et al*[39].

PPKs: Role in antimicrobial resistance and natural transformation

C. jejuni is increasingly becoming resistant to several clinically-relevant antimicrobials[41-43]. Two recent studies demonstrated that both *ppk1* and *ppk2* mutants were more susceptible to several antimicrobials (erythromycin, cefotaxime, ciprofloxacin, rifampin, polymyxin, and tetracycline) compared to their respective wildtypes[25,27]. The stringent-response mediator ppGpp affects bacterial resistance to antimicrobials[44]; studies looking at ppGpp levels in the *ppk1* mutant would provide some insights on this mechanism. Both *ppk1* and *ppk2* mutants had altered outer membrane profiles, suggesting that membrane permeability of these mutants may be compromised[45]; where altered membrane permeability could be the cause of increased susceptibility of *ppk* mutants to antimicrobials. Stress resistance is an emerging mechanism of antimicrobial resistance in bacteria[44]; thus, the susceptibility of *ppk* mutants to antimicrobials may also be explained by their general sensitivity to other stresses. In *E. coli*, poly P is known to regulate adaptive evolution and ribosome fidelity, both of which modulate resistance to antimicrobials[46]. Whether these mechanisms play a role in PPK1-mediated resistance to antimicrobials in *C. jejuni* also need to be investigated.

C. jejuni is naturally competent (*i.e,* ability to take up DNA from its surroundings) and this feature impacts the organism's ability to adapt to different environments as well as to acquire antimicrobial resistance genes. Gangaiah *et al*[25] showed that a *ppk1* mutant was compromised in its ability to acquire foreign DNA for natural transformation. Poly P is a component of the membrane channels that mediate DNA uptake[47,48]; the potential of such a mechanism for PPK1 to mediate natural competence in *C. jejuni* should be explored. Evidence suggests that outer membrane composition of the *ppk1* mutant is altered[45]; whether this influences the ability of *C. jejuni* to take up DNA is also unknown.

PPKs: Role in motility and biofilm formation

Several recent studies have evaluated the role of PPKs in virulence-associated phenotypes such as motility, biofilm formation, adherence, invasion, and intracellular survival[24,25,27,45]. Motility is an essential *C. jejuni* virulence mechanism[49]. Three independent studies demonstrated that both PPK1 and PPK2 were not required for *C. jejuni* motility[24,25,27]. This is in contrast to other organisms, where PPK1 is required for motility. Surprisingly, Chandrashekhar *et al*[32] demonstrated that several flagella-associated genes

Table 3 Summary of chicken colonization phenotypes of *ppk1* and *ppk2* mutants

Study	Organ/ feces	Inoculation dose (CFU/chick)	Wildtype (CFU/g)	*ppk1* mutant (No. of chicks colonized)	*ppk2* mutant (No. of chicks colonized)
Candon *et al*[24] 2007	Ceca	1.5×10^5	1.8×10^8	0/10 chicks	NS
		1.5×10^6	1.8×10^8	8/10 chicks	NS
		1.5×10^7	1.8×10^8	10/10 chicks at an average of 1.8×10^8 CFU/g	NS
Gangaiah *et al*[25] 2009 and 2010	Ceca	1.0×10^3	1.0×10^6	0/5 chicks	0/5 chicks
		1.0×10^4	1.0×10^8	0/5 chicks	0/5 chicks
		1.0×10^5	8.0×10^8	5/5 chicks at an average of 2.0×10^4 CFU/g	5/5 chicks at an average of 9.0×10^3 CFU/g
	Bursa	1.0×10^3	1.0×10^4	0/5 chicks	0/5 chicks
		1.0×10^4	1.0×10^5	0/5 chicks	0/5 chicks
		1.0×10^5	1.5×10^5	5/5 chicks at an average of 1.0×10^2 CFU/g	5/5 chicks at an average of 8.0×10^2 CFU/g
	Feces	1.0×10^3	1.0×10^5	0/5 chicks	0/5 chicks
		1.0×10^4	1.0×10^7	0/5 chicks	0/5 chicks
		1.0×10^5	3.0×10^7	5/5 chicks at an average of 1.5×10^3 CFU/g	5/5 chicks at an average of 8.0×10^3 CFU/g

NS: Not studied.

and flagellar glycosylation genes were downregulated in the *ppk* mutants; the authors hypothesized that the degree of downregulation in the *ppk* mutants may not have been enough to impair motility. Although motility was not affected by PPKs in *C. jejuni*, the potential role of PPKs on other phenotypes associated with flagella such as autoagglutination, secretion, and/or invasion needs further investigation.

Biofilms play an important role in *C. jejuni* tolerance to environmental stresses, disinfectants, and antimicrobials, as well as facilitating colonization of animal and human hosts[50-52]. Two independent groups demonstrated that the *ppk1* mutant formed higher amount of biofilms than the wildtype[24,25]. This finding is in contrast to other organisms, where PPK1 is required for biofilm formation[13]. In another study, Gangaiah *et al*[27] demonstrated that *ppk2* mutant also formed higher amount of biofilms than its parent. Drozd *et al*[53] went onto characterize how PPK enzymes modulate biofilm formation in *C. jejuni* and demonstrated that *ppk1* and *ppk2* mutants formed higher adherent colonies on day 1 and 2 compared to wildtype. This study suggested that, compared to mutant strains, the wildtype might spend more time in planktonic phase, delaying biofilm formation[53]. The authors further demonstrated that *ppk1* and *ppk2* mutants had reduced calcofluor white reactivity, suggesting that polysaccharide structures might be altered in the mutant strains[53]. The quorum sensing molecule Autoinducer-2, which is required for biofilm formation, was increased in both mutants[53]. Finally, the authors evaluated the mutants for altered expression of genes involved in biofilm formation and found that several genes (*pglH, kpsM, cj0688, neuB1, neuB1, fliS,* and *maf5*) involved in biofilm formation were altered in *ppk1* and *ppk2* mutants[53].

PPKs: Role in adherence, invasion and intracellular survival

Adherence, invasion, and intracellular survival are also essential virulence mechanisms for *C. jejuni*[54]. Recent studies demonstrated that both PPK1 and PPK2 are important for invasion and intracellular survival within INT-407 human intestinal epithelial cells[24,25,27]. Pina-Mimbela *et al*[45] showed that *ppk1* and *ppk2* mutants had qualitative and quantitative differences in outer membrane composition compared to wildtype. They also demonstrated that the differences in outer membrane composition in the mutants were directly related to the ability of *C. jejuni* to invade and survive within INT-407 cells[45]. The authors of this study went one step further to evaluate which fractions were associated with invasion and intracellular survival[45]. They found that outer membrane proteins were uniquely associated with invasion, whereas outer membrane proteins, lipids, and lipoglycans all were associated with intracellular survival[45].

PPKs: Role in chicken colonization

Two independent studies evaluated the role of PPK1 in *C. jejuni* colonization in day-old chicks (Table 3)[24,25]. Candon *et al*[24] demonstrated that, after 7 days of oral inoculation, the wildtype colonized the ceca of chicks at a minimum average of 1.79×10^8 CFU/g of cecal contents at all inoculation doses examined. The *ppk1* mutant colonized the ceca of all chicks at a rate similar to that of wildtype at a dosage of 1.5×10^7 CFU/chick, of 8 out of 10 chicks at a dosage of 1.5×10^6 CFU/chick, and of 0 out of 10 chicks at a dosage of 1.5×10^5 CFU/chick[24]. In another study, Gangaiah *et al*[25] evaluated the role of PPK1 in colonization of ceca and bursa as well as *C. jejuni* load in feces using low inoculation doses *i.e.,* 10^3, 10^4, and 10^5 CFU/chick. They demonstrated that, after 8 d of oral inoculation, the *ppk1* mutant colonized the ceca and bursa and showed a *C. jejuni* load in feces of all chicks but at a significantly lower rate than the wildtype at a dosage of 10^5 CFU/chick[25]. While the wildtype colonized to an average of about 1.0×10^8 CFU/g of ceca and about 1.0×10^5 CFU/g of bursa, and showed a *C. jejuni* load of about 1.0×10^7 CFU/g of feces, none of the chicks were colonized with the mutant strain at a dosage of 10^4 CFU/chick[25]. Similarly, while the wildtype colonized to an average of about 1.0×10^6 CFU/g of ceca and about $1.0 \times$

10^4 CFU/g of bursa, and showed a *C. jejuni* load of about 1.0 × 10^5 CFU/g of feces, none of the chicks were colonized with the mutant strain at a dosage of 10^3 CFU/chick[25]. Except for 10^5 CFU/chick, the two studies used different dosages for inoculation. Both studies were done in the same strain background; thus, the reason for the difference in the results between the two studies is unclear.

Furthermore, Gangaiah *et al*[27] evaluated the role of PPK2 in colonization of ceca and bursa as well in *C. jejuni* load in feces using 10^3, 10^4, and 10^5 CFU/chick inoculation doses (Table 3). They demonstrated that, at 10^5 CFU/chick inoculation dose, the *ppk2* mutant colonized the ceca and bursa as well as showed a *C. jejuni* load in feces of all chicks but at a significantly lower rate than the wildtype[27]. While the wildtype colonized to an average of about 1.0 × 10^8 CFU/g of ceca and about 1.0 × 10^5 CFU/g of bursa, and showed a *C. jejuni* load of about 1.0 × 10^7 CFU/g of feces, none of the chicks were colonized with the mutant strain at a dosage of 10^4 CFU/chick, except for 1 chick[27]. Similarly, while the wildtype colonized to an average of about 4.0 × 10^6 CFU/g of ceca and about 1.0 × 10^4 CFU/g of bursa, and showed a *C. jejuni* load of about 1.0 × 10^5 CFU/g of feces, none of the chicks were colonized with the mutant strain at a dosage of 10^3 CFU/chick[27].

It is intriguing that both *ppk1* and *ppk2* mutants behave similar with respect to chicken colonization. Several *C. jejuni* genes have been identified to be important for chicken colonization[55]; transcriptome and outer membrane proteome analyses showed that these genes did not appear to be regulated by PPK1 and PPK2[32,45]. Thus, the mechanisms underlying the contribution of poly P to chicken colonization are unclear. Both *ppk1* and *ppk2* mutants showed dose-dependent colonization defects in day-old chicks[24,25,27]. The authors hypothesized that the dose-dependency may be related to the hyperbiofilm phenotype of *ppk1* and *ppk2* mutants[24,25,27]. According to this hypothesis, at lower inoculation doses, the *ppk* mutants are more sensitive to *in vivo* stresses and thus, display no or reduced colonization. At higher doses, the *ppk* mutants form hyperbiofilms, conferring resistance to *in vivo* stresses and thus, similar colonization of the mutants as that of wildtype. Additional *in vivo* studies complementing the mutants to rule out the effect of secondary mutations on colonization are warranted.

PPX/GPPA ENZYMES: ROLE IN *C. JEJUNI* SURVIVAL AND VIRULENCE

Malde *et al*[29] demonstrated that mutations in *ppx/gppa* genes were associated with a variety of phenotypes in *C. jejuni*. In this study, it was shown that *ppx/gppa* mutants were deficient in survival under nutrient limitation and osmotic stress. The authors further showed that the nutrient survival defect in the mutants could be complemented by amino acid supplementation. Based on these findings, it was hypothesized that the nutrient survival phenotype in the mutants is likely due to reduced ppGpp or increased poly P levels. Both *ppx/gppa* mutants had increased poly P levels; however, only *ppx1/gppa* mutant had reduced ppGpp levels when compared to wildtype but not *ppx2/gppa* mutant, which indeed accumulated more ppGpp than the wildtype[29]. This suggests that the nutrient survival defect in the mutants is more likely due to increased poly P levels rather than due to reduced ppGpp levels.

Further, Malde *et al*[29] also demonstrated that *ppx/gppa* mutants were compromised in several virulence-associated phenotypes such as motility, biofilm formation, and invasion and intracellular survival within human intestinal epithelial cells. Unlike *ppk1* and *ppk2* mutants, which had similar motility as the wildtype, the *ppx/gppa* mutants were defective in motility compared to wildtype. The *ppx/gppa* mutants also had decreased ability to form biofilms. Poly P is essential for chelation of cations[13], which are required for biofilm formation[56]; whether such a mechanism is the cause of reduced biofilms in *ppx/gppa* mutants is an interesting question. The group further went on to test the contributions of PPX/GPPA enzymes to serum resistance and demonstrated that *ppx/gppa* mutants were resistant to human complement but not to chicken complement.

Although the contributions of PPX/GPPA enzymes to *C. jejuni* biology is well characterized at the phenotypic level using *in vitro* assays, it remains to be understood if these contributions impact colonization of *C. jejuni* in day-old chicks and infection in humans. The mechanisms underlying the PPX/GPPA-associated phenotypes are also largely unknown; a transcriptome analysis of the *ppx* mutants would provide some insights on this aspect. In other bacteria, it has been shown that excess poly P in the *ppx* mutants restricts growth and downregulates metabolism, which is thought to have caused the underlying phenotypes[57]. The *C. jejuni ppx/gppa* mutants grew very similar to the wildtype strain, yet were compromised in several phenotypes (*i.e.,* motility, biofilm formation, nutrient stress survival, invasion and intracellular survival, and resistance to human complement-mediated killing), suggesting that growth restriction and metabolic downshift are less likely the reasons for PPX-dependent phenotypes in *C. jejuni*. Phenotypes associated with excess of poly P were modest compared to those associated with poly P deficiency, suggesting that lack of poly P impacts *C. jejuni* biology more than excess of poly P. It is also intriguing to note that the phenotypes of the double mutant lacking both *ppx1/gppa* and *ppx2/gppa* genes were more severe compared to those of the individual mutants, suggesting some degree of functional redundancy in these enzymes; the reason behind this functional redundancy is unclear. Overall, findings from the analysis of *ppx/*

gppa mutants suggest that poly P levels are tightly regulated and that dysregulation of poly P levels as seen in the *ppx/gppa* mutants compromises *C. jejuni*'s survival and virulence properties.

LINK BETWEEN POLY P AND (P)PPGPP METABOLISM IN *C. JEJUNI*

Malde *et al*[29] assessed the role of PPX/GPPA enzymes in ppGpp synthesis and demonstrated that PPX1/GPPA but not PPX2/GPPA is important for ppGpp synthesis. In another study, Candon *et al*[24] demonstrated that a *spoT* mutant, which is deficient in ppGpp synthesis, had significantly reduced poly P levels than its parent. These findings suggest that (p)ppGpp and poly P metabolism are linked in *C. jejuni* (Figure 1). Both, (p)ppGpp and poly P metabolism are known to be linked in *E. coli*; in this organism, ppGpp is known to modulate poly P levels by inhibiting PPX enzymes[58,59]. Analogous to *E. coli*, the authors speculated that (p)ppGpp might modulate poly P levels in *C. jejuni* by inhibiting PPX enzymes, which would not only affect the dynamic balance between poly P degradation by PPX enzymes but also affects poly P synthesis by PPK1. However, this hypothesis remains to be experimentally confirmed. In *Pseudomonas aeruginosa*, PPK2 affects (p)ppGpp accumulation; this role of PPK2 is attributed to its ability to serve as a source of GTP, which is a precursor for (p)ppGpp synthesis[60]. However, a *C. jejuni ppk2* mutant accumulated similar (p)ppGpp levels to that of wildtype, suggesting that PPK2 is less likely the link between poly P and (p)ppGpp metabolism in *C. jejuni*. Although PPK1 does not appear to have ppGpp synthetic/hydrolytic activity, it remains to be understood if (p)ppGpp levels are altered in the *ppk1* mutant.

POLY P AND PHOSPHATE METABOLISM

Inorganic phosphate (Pi) is an essential nutrient for many bacterial species. As most natural environments are limiting in Pi, phosphate esters are the preferred source of Pi for bacteria[61]. Phosphate esters are broken down into Pi in the periplasm by alkaline phosphatase. PhoAcj is the only alkaline phosphatase in *C. jejuni*[62]. Drozd *et al*[53] recently demonstrated that a *phoAcj* mutant had significantly reduced intracellular poly P levels compared to its parent, suggesting that PhoAcj is likely the primary source of Pi for poly P synthesis[53,63]. The authors also demonstrated that the *phoAcj* mutant had a significantly reduced ability to survive under nutrient stress, to invade and survive within INT407 cells and to colonize day-old chicks[63]. The phenotypes in the *phoAcj* mutant were in general less severe when compared to those in the *ppk1* mutant. This is likely due to the fact that *phoAcj* mutant only had a modest defect in poly P accumulation compared to the *ppk1* mutant, which had a severe deficiency in poly P accumulation[63]. Unlike *ppk1* mutant, the *phoAcj* mutant had enhanced resistance to antimicrobials, suggesting that this phenotype is less likely due to poly P deficiency[63].

PHENOTYPIC OVERLAP BETWEEN POLY P AND RELATED ENZYMES

Several phenotypes of PPK1, PPK2, PPX/GPPA, SpoT, and PhoAcj enzymes overlap between each other as shown in Table 2[24,25,27,29,34,63,64]. For example, osmotic shock survival and low nutrient stress survival phenotypes were common to PPK1, PPK2, PPX/GPPA, and PhoAcj enzymes. Similarly, intraepithelial survival phenotype was common to all enzymes and, except for PPK1, invasion was also common to all the enzymes. There are two possible explanations for this overlap; the enzymes likely affect the shared phenotypes independently, or the enzymes might impact the phenotypes through a common protein or molecule. As PPK2 is required for poly P-dependent GTP synthesis, it is conceivable that poly P deficiency in the *ppk1* mutant likely also affects GTP levels[25,27]. Thus, the phenotypic overlap between PPK1 and PPK2 may be the consequence of low GTP levels in these mutants. Both *spoT* and *phoAcj* mutants had low poly P levels[34,63]; thus, the phenotypic overlap between PPK1, PhoAcj, and SpoT may be due to low poly P in these mutants. The ability to form biofilms was upregulated in the *ppk1* mutant[24,25], which has low poly P levels, and downregulated in *ppx/gppa* mutants[29], which have high poly P levels; this suggests that the biofilm phenotype is directly associated with poly P. This is further supported by the fact that *spoT* mutant, which has low poly P, also formed more biofilms than its parent[34]. However, the *ppk2* mutant, which has similar poly P levels as its parent, also formed more biofilms compared to its parent[27]; suggesting that the effect of PPK2 on biofilm formation is independent of poly P. Further studies are required to precisely define phenotypic overlap between poly P and related enzymes. For example, if the biofilm phenotype of the *spoT* mutant is due to low poly P levels, supplementation with poly P might rescue the biofilm phenotype in this mutant. Similarly, if the phenotypic overlap between *ppk1* and *ppk2* mutants is due to altered GTP levels, supplementation with GTP might rescue the associated phenotypes in the *ppk1* mutant. Strains containing mutations in multiple of these enzymes accompanied by complementation may also provide insights as to whether these enzymes have a synergistic or antagonistic effect on the overlapping phenotypes.

PPKS AS PROMISING DRUG AND VACCINE TARGETS

PPK enzymes have the potential to be ideal drug targets for controlling *C. jejuni* and other bacterial infections. First, these enzymes are highly conserved across a broad array of bacterial species[13]; thus, the identified drugs will be effective against many bacterial species. Second, homologs of PPKs are absent in higher eukaryotes[13]; thus, it is less likely that the drugs will be toxic to host cells. Third, a recent study by Pina-Mimbela *et al*[45] showed that PPKs modulate outer membrane composition of *C. jejuni*; thus, anti-PPK drugs could expose surface antigens, which are otherwise hidden, and make *C. jejuni* vulnerable to host defense mechanisms. Fourth, deletion of *ppk1* and *ppk2* does not affect growth of bacteria *in vitro*[24,25,27]; thus, there is less selection pressure on the bacteria to develop resistance to anti-PPK drugs. Lastly, *ppk* mutants are more sensitive to several conventional antibiotics[24,25,27]; thus, anti-PPK drugs could be used in conjunction with existing antibiotics for drug-resistant strains.

Mutants of poly P enzymes could also be sought as potential live attenuated vaccine candidates. Strains with deleted virulence genes have been successfully used for controlling *Salmonella* colonization in poultry[65,66]. To have a synergistic effect on virulence/colonization attenuation, mutants containing deletions in *ppk* genes and other established virulence determinants could be used. As discussed before, using *ppk* mutants as live attenuated vaccine candidates not only has the advantage of the strain being attenuated for colonization but also could expose previously hidden antigens, likely inducing a strong protective immunity.

Epidemiological studies have shown that nearly 50%-80% of the human infections originate from ingestion of poultry and poultry products[4]. Both PPK1 and PPK2 are necessary for *C. jejuni* colonization in chickens[4,24,25,27]. These data suggest that PPKs could at least be targeted for controlling *C. jejuni* colonization in chickens, which likely would aid in reducing human infections. A human challenge model is available to study campylobacteriosis using the *C. jejuni* strain CG8421, which lacks ganglioside mimicry, a mechanism known to cause Guillain Barre Syndrome[67-69]. The potential of using PPKs as drug and vaccine targets for human infections warrants that the contributions of these enzymes to human infection be studied using the human challenge model of campylobacteriosis.

CLOSING REMARKS AND PERSPECTIVES

Recent studies have yielded several important insights into the role of poly P in *C. jejuni* biology. Poly P is associated with a plethora of *C. jejuni* phenotypes, which not only impact how *C. jejuni* survives in the environment under different stress conditions but also impact how this organism colonizes poultry and other domestic animals and causes disease in humans. Transcriptome and outer membrane proteomics analyses of the *ppk1* and *ppk2* mutants have provided some valuable mechanistic insights with regards to poly P-associated phenotypes. Nevertheless, our understanding of poly P in *C. jejuni* is still in its first steps. For example, the signals that activate poly P-mediated response are largely unknown. Most bacteria, including *C. jejuni*, contain two PPX enzymes; the reason behind this redundancy is an open question. Although transcriptome and outer membrane proteomic studies have yielded valuable insights into poly P functions, the few genes or proteins differentially expressed in the *ppk* mutants compared to wildtype do not seem to explain the plethora of phenotypes arising from mutants of poly P-associated enzymes. This suggests that poly P may also mediate its functions by affecting its targets posttranscriptionally; global proteomic analyses of the *ppk1* and *ppk2* mutants will likely shed some light on this aspect. It is also not known how poly P is regulated; in other words, what are the upstream components that feed into poly P-mediated response? How poly P feeds into the global regulatory network also remains to be understood. Poly P and ppGpp are linked in *C. jejuni* and other bacteria but the precise mechanisms underlying their interaction are poorly understood.

Given that poly P-related enzymes affect numerous aspects of *C. jejuni* life, these enzymes have the potential to be promising drug and vaccine targets. Therefore, it would be worthwhile to determine the contributions of poly P-related enzymes in a human model of *Campylobacter* infection. Gaynor *et al*[34] demonstrated that *ppk1* was upregulated during *C. jejuni* infection of human intestinal epithelial cells. Thus, studies defining at which stage of human infection and chicken colonization the poly P-mediated response is activated would provide additional insights into its role in *C. jejuni* pathogenesis/colonization. The recent finding that Poly P enzymes modulate IL-8 production in INT-407 cells suggests that host immune response to the mutant strains may be critical to study[45]. Such studies could facilitate development of *ppk* mutants alone or in combination with mutations in other established virulence genes as live attenuated vaccines for reducing *C. jejuni* colonization in poultry and one day for controlling human infections.

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REVIEW

Human papillomavirus and gastrointestinal cancer: A review

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Abstract

Human papillomavirus (HPV) is one of the most common sexually transmitted infections worldwide. Exposure to HPV is very common, and an estimated 65%-100% of sexually active adults are exposed to HPV in their lifetime. The majority of HPV infections are asymptomatic, but there is a 10% chance that individuals will develop a persistent infection and have an increased risk of developing a carcinoma. The International Agency for Research on Cancer has found that the following cancer sites have a strong causal relationship with HPV: cervix uteri, penis, vulva, vagina, anus and oropharynx, including the base of the tongue and the tonsils. However, studies of the aetiological role of HPV in colorectal and esophageal malignancies have conflicting results. The aim of this review was to organize recent evidence and issues about the association between HPV infection and gastrointestinal tumours with a focus on esophageal, colorectal and anal cancers. The ultimate goal was to highlight possible implications for prognosis and prevention.

Key words: Human papillomavirus; Esophageal cancer; Colorectal cancer; Anal cancer; Oncogenesis; Prognosis; Prevention

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Core tip: Human papillomavirus is one of the major causes of infection-related cancer worldwide. Studies on the aetiological role of human papillomavirus (HPV) in colorectal and esophageal malignancies have yielded conflicting results. HPV status has emerged as a possible predictor of treatment response and long-term oncological outcomes for cancer sites where HPV-related and non-related cancers co-exist. Human papillomavirus vaccination is the key to improving HPV-related disease control, and universal vaccination could achieve optimal health benefits.

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INTRODUCTION

Human papillomaviruses (HPVs) are a large family of small double-stranded DNA viruses with established oncogenic potential, and they are recognized as a major cause of infection-related cancer worldwide (along with *Helicobacter pylori* and hepatitis viruses B and C). The International Agency for Research on Cancer (IARC) has found evidence of strong causal relationships between HPV and the following cancer sites: cervix uteri (ICD10-C53), penis (C60), vulva (C51), vagina (C52), anus (C21) and oropharynx, including the base of the tongue and the tonsils (C01, C09, C10)[1]. Recent estimates have attributed approximately 4.8% (610000 cases) of all cancers worldwide to HPV infection[2]. HPV could be responsible for some other cancers, including cancer of the esophagus, lip and oral cavity, but a causal role for HPV has not been established[3]. Therefore, the figures reported above could underestimate the burden of cancer attributable to HPV.

Because the relationship of HPV infection and gastrointestinal carcinogenesis has implications for patient care and cancer prevention, we aimed to provide a review of the recent evidence in the field with a focus on gastrointestinal cancers.

HPV BIOLOGY AND ONCOGENESIS

HPV is a non-enveloped double-stranded DNA virus that infects basal keratinocytes in the skin or mucosal membranes.

HPV infection is almost exclusively acquired from sexual contact. The number of lifetime partners, early onset of sexually activity and co-infection with other sexually transmitted infection, including HIV, have been correlated with risk of HPV infection[4-7].

Exposure to HPV is very common, and an estimated 65%-100% of sexually active adults are exposed to HPV at any anatomic site (oral, genital or anal) in their lifetime. The majority of HPV infections are asymptomatic, and 90% of persons exposed to HPV will clear the virus; the other 10% will have a persistent infection with an increased risk of developing carcinoma[8,9].

HPV types are classified into "high-risk" and "low-risk" groups based on their oncogenesis potential (Table 1). High-risk HPV (hrHPV) types have the ability to cause human cancer. Currently, twelve types are designated by the IARC as carcinogenic, and eight additional types are designated as "probably" or "possibly" carcinogenic[10]. HPV16 has been classified as "high risk" and is also the most prevalent hrHPV type in many regions of the world, followed by HPV18. Non-malignant diseases, such as genital warts, recurrent respiratory papillomatosis and oral papillomas, are attributable to low-risk HPV types, particularly HPV6 and 11[11-16].

Table 1 Human papillomavirus classification

HPV group	Genotypes
High-risk types	
Carcinogenic	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59
Probably carcinogenic	68
Possibly carcinogenic	26, 53, 66, 67, 67, 70, 73, 82
Low-risk types	6, 11, 40, 42, 43, 44, 54, 61, 72, 81, 89

HPV: Human papillomaviruse.

The HPV genome is organized into distinct control regions. The early region codes for viral non-structural proteins called E1, E2, E4, E5, E6 and E7. The late region codes for two capsid proteins for virion assembly called L1 and L2[17].

HPV gene expression and the viral life cycle are tightly controlled by epithelial cell differentiation. The natural history of a high-risk HPV infection involves virus penetration in the basal layer through micro-abrasion and subsequent viral genome replication in basal epithelial cells when the virus is in its episomal form. The progression of a persistent infection is associated with HPV DNA integration into the host genome, and this step has been identified as critical for cancer promotion because it leads to the up-regulation of viral onco-protein expression, especially E6 and E7[18-20]. The E6 and E7 proteins contribute to genetic instability through their inactivation of p53 and retinoblastoma protein (pRb). pRb is a negative regulator of the cyclin-dependent kinase inhibitor $p16^{INK4a}$ (p16), and inactivation of pRb leads to up-regulation of p16[21-23]. p16 is often used as a surrogate biomarker of HPV oncoprotein activity. Over 90% of HPV-positive oropharyngeal cancers overexpress p16, and immunohistochemical evidence of p16 overexpression has been widely used as a correlate for HPV oncogenic activity in cervical cancer and dysplasia[24-26].

HPV infection causes cervical cancer and its precursor lesions at the squamocolumnar junction cells near the transformation zone. These cells appear to be multipotent residual embryonic cells, and an almost identical population was discovered at the gastro-esophageal squamocolumnar junction that was linked to Barrett's metaplasia[27]. The anorectal junction is presumably another squamocolumnar junction similar to the cervix, but comparative microanatomy of the anal and cervical transition zones showed distinct topographical differences[28]. This observation supports the discrepancy in the incidence of cervical and anal carcinoma. Indeed, although HPV DNA was detected at least as frequently in the anus as in the cervix, the

Table 2 Comparison of human papillomavirus detection methods

Methods	Specimens	Advantages	Disadvantages
Southern blotting assay	Fresh/frozen samples	High specificity, ability to differentiate between episomal and integrated DNA	Not easily applied to FFPE samples
ISH	FFPE, fresh samples	High specificity, ability to differentiate between episomal and integrated DNA	Low sensitivity, technically difficult
HPV PCR	Fresh/frozen samples brushing washing any body fluid	High sensitivity, cost effective	Low specificity, provides no quantitative measure of viral load, no confirmation of transcriptionally active virus
Real-time PCR	Fresh/frozen samples, FFPE brushing washing any body fluid	High sensitivity and specificity, ability to differentiate between episomal and integrated DNA	Labour-intensive
Reverse Transcriptase PCR	Fresh/frozen samples, FFPE	High sensitivity	Time consuming, technically difficult
p16 immunostaining	Fresh/frozen samples FFPE brushing washing	High sensitivity, easy and accessible to most laboratories, marker of transcriptionally active virus	Low specificity
Signal amplification methods	Fresh/frozen samples FFPE brushing washing	Easy to perform	False positive products, no typing

FFPE: Formalin-fixed, paraffin-embedded tissues; HPV: Human papillomaviruse; PCR: Polymerase chain reaction; ISH: *In situ* hybridization.

cervical/anal cancer ratio is approximately 17:1[3]. However, HPV infection alone is not sufficient to determine the carcinogenic potential of the lesion, and other cofactors are likely involved, including immune deficiency and host genetic factors. This prediction was confirmed indirectly by the fact that the majority of low-grade squamous intraepithelial lesions do not progress to high-grade lesions or carcinoma[1,29-34].

HPV-related cancers in immunosuppressed patients

The incidence of HPV-related cancers is high in immunocompromised patients. HIV-infected patients show an increased risk of known HPV-related cancers, such as cervical and anal cancer. Moreover, the incidence of other possibly HPV-related cancers is increased in HIV-infected patients[30].

Lifestyle and risk factors other than immune deficiency could be responsible for the heightened risk of several cancers in HIV patients[31]. However, a similar pattern of incidence of HPV-related cancers emerged from studies of transplant patients[32,33]. Transplant patients have generally different lifestyle exposures than HIV patients, but both groups have immune deficiency. Therefore, the immune deficiency is likely responsible for cancer progression and the high incidence of HPV-related cancers in HIV and transplant patients.

The risks of cancer of the penis, vulva, vagina, oral cavity, pharynx, esophagus , larynx and lip are increased in both transplant and HIV patients. The colorectal cancer risk is increased in transplant patients only.

HPV detection methods

Because HPV cannot be grown in conventional cell cultures, virus identification relies on molecular biology techniques.

Several methodologies are available for the detection of HPV infection in tissue and exfoliated cell samples (Table 2). Despite the large number of studies, there is still controversy as to which method or combination of methods is best suited for HPV identification. Common methods include HPV detection by polymerase chain reaction (PCR), *in situ* hybridization (ISH) and the detection of p16 with immunohistochemistry (IHC).

The initial methods of HPV detection included radiolabelled nucleic acid hybridization assays, such as Southern blots and ISH. These methodologies generated high-quality information, but the disadvantages of these direct-probe approaches included low sensitivity, the need for relatively large amounts of purified DNA and time-consuming procedures[35]. Mainstay HPV diagnostic methods involve signal amplification and PCR-based technologies. The PCR-based techniques are highly sensitive, specific and widely used[36,37].

Several studies have also suggested that immunohistochemical detection of p16 overexpression may serve as a surrogate marker of functionally relevant HPV infection[24-26]. It is a simple, fast and inexpensive procedure.

HPV AND ESOPHAGEAL CANCER

Esophageal cancer (EC) is the eighth most prevalent malignant tumour and the sixth leading cause of cancer mortality worldwide, with approximately 500000 new cases diagnosed and an estimated 406000 deaths each year[38]. As reported above, the risk of esophageal cancer is increased in both HIV and transplant patients[32].

The most common tumour type of esophageal cancer is squamous cell carcinoma (ESCC), followed by adenocarcinoma (EAC); there are significant epidemiological and aetiological differences between them. ESCC, which is more prevalent in Eastern countries and in developing countries, is the predominant subtype.

EAC, which is associated with Barrett's esophagus, is less common, but its incidence has been rising by 5%-10% each year, particularly in developed (Western) countries[39]. Incidence rates of esophageal cancer vary greatly by geographic region. The highest ESCC rates occur in the "Asian Esophageal Cancer Belt", which extends from northern Iran east to China and north into Russia. The aetiology of EC remains poorly understood. A multifactorial aetiology may account for the great variability of EC incidence among different ethnic groups and geographic regions[38,40]. Epidemiological studies suggest that tobacco smoking, heavy alcohol drinking, micronutrient deficiency[2,41] and dietary carcinogen exposure may cause ESCC. Gastroesophageal reflux (GER) and Barrett's esophagus (BE) are the most important known risk factors for EAC[42,43].

HPV and ESCC

The role of infection in the development of EC has long been suspected, and recent meta-analyses have assessed the incidence and aetiological role of HPV-ESCC tumour infection. Although HPV has been widely studied, the overall rate of HPV infection in ESCC remains controversial, and many studies have attempted to address this question. According to recent meta-analyses and reviews, worldwide HPV-ESCC infection rates range from 11.7% to 38.9%[44-46]. Syrjänen *et al*[47] reported that the mean prevalence of HPV was 29.0% and ranged from 0% to 78%. Geographic origin was the strongest determinant for the observed disparities in infection rates. While low-incidence countries (*e.g*., Australia and the United States) reported ESCC rates of approximately 2.5 per 100,000 people, the high-incidence countries, such as China and Iran, had rates as high as 250 per 100000 people[47-51]. This asymmetric geographic distribution remains largely unexplained. Several studies have correlated variability in HPV-ESCC infection rates with HPV detection methods. Many techniques were used to find evidence of the involvement of HPV in ESCC[47,50]. To date, PCR and ISH are the most commonly used methods, and most studies use both techniques[52-56]. The prevalence of overall HPV infection varies widely depending on detection method. Infection prevalence ranged from 17.6% for Southern blots to 32.2% for L1 serology, but the two most commonly-used methods demonstrated similar overall HPV-ESCC rates of 27.7% and 24.3%, respectively[51]. In addition to these studies, several meta-analyses have demonstrated that detection method does not account for the variability in reported HPV-ESCC infection rates as variability persists within studies that use the same detection method[47,51,57].

In 1982, Syrjänen *et al*[58] and Syrjänen[59] first suggested the possibility that HPV might play an aetiological role in the progress of both benign and malignant squamous cell carcinoma of the esophagus. The well-known association between HPV and oropharyngeal SCC and the histologic similarities between the oral squamous epithelium and upper esophagus could suggest a similar association. A wide range of studies on this topic have been conducted in various countries, including China, Korea, Iran, India, the United States and Australia[60-62]. HPV16 and HPV18 are the most frequently detected types in HPV-associated cancers, and they are thought to be related to 70% of all cervical cancer cases. Yong *et al*[45] described a significant association between HPV16 and ESCC but not HPV18. In this meta-analysis, they reported an overall HPV16 prevalence rate of 11.7% *vs* 1.8% for HPV18. They also calculated separate ORs for HPV16 (OR = 3.55) and HPV18 (OR = 1.25), and HPV16 was the most frequently observed subtype in ESCC. This outcome agreed with other systematic reviews that showed HPV16 was the most commonly identified strain in HPV-ESCC infections using multiple methods. To date, however, studies examining the potential aetiological role of HPV infection in ESCC have been inconclusive.

Several studies of HPV infection in ESCC have shown little to no agreement between p16 overexpression and HPV-positivity. A recent systematic review suggested that p16 is not a reliable marker of HPV status in ESCC because the odds ratio of HPV-positivity in a p16-overexpressing ESCC lesion was 1.07 (95%CI: 0.70-1.62)[63]. Recent meta-analyses have not evaluated p16 overexpression to characterize the potential aetiological role of HPV infection in ESCC. Studies examining both p16 overexpression and HPV positivity in ESCC have reported a low rate of double-positive ESCC lesions, approximately 5% of all cases[64-71]. The available data are markedly different from oropharyngeal and cervical cancer data, and the association between p16 and HPV in ESCC therefore remains limited. Collectively, p16-overexpression and HPV serological data appear to indicate that, despite the reported rates of HPV infection in ESCC, HPV may not play a significant oncogenic role in ESCC. Nevertheless, a lack of correlation between p16-overexpression and HPV oncoactivity in ESCC would be unusual[72]. This result was complicated by the geographic variation discussed previously because regions with the highest rates of ESCC also had the highest rates of HPV prevalence and HPV-ESCC infection. The geographic correlation between HPV and ESCC may suggest a causal link. However, this correlation must be considered with caution because studies from the same geographic areas report tremendous variability in HPV-ESCC infection rates[66,73].

Therefore, the role of HPV in ESCC remains poorly defined. Geographic variability and methodological heterogeneity complicate the interpretation of current studies and lead to variable conclusions. HPV prevalence correlates strongly with high-ESCC-incidence regions, but in Western countries, such as

the United States, HPV-ESCC infection rates are low (on the order of 5%-15%). The p16 overexpression and HPV serological data do not currently support a definitive aetiological role for HPV in ESCC.

HPV and EAC

EAC is one of the fastest growing cancers in the Western world[74]. GERD, BE, obesity, tobacco use and diet low in vegetables and fruit are the most important known risk factors for EAC[42,43,75-80].

The analogue rise in head and neck cancers in Western countries has led to a suspicion that HPV may also play a role in EAC. Endoscopists and pathologists have often noted warty (papillomatous) lesions in the esophagus, which may indicate a viral infection[81]. Herpes simplex virus, cytomegalovirus and Epstein-Barr virus have all been shown to infect the esophageal epithelium. Rajendra *et al*[82] recently reported for the first time that transcriptionally active hr-HPV was strongly associated with Barrett's dysplasia (BD) and EAC, but HPV was biologically irrelevant in BE. This study was the first to find that HPV positivity, which was detected by PCR, was significantly higher in patients with BD and EAC compared to controls and individuals with Barrett's metaplasia. Even if this study did not prove causality, it suggested that HPV was associated with the transition from BE to dysplastic disease/adenocarcinoma. Biomarkers related to the transcriptional activity of hr-HPV have been investigated for the purpose of identifying the high-risk group of progressors to malignancy[82-86]. When HPV DNA and markers of viral transcriptional activity, *i.e*., p16 and E6/E7 mRNA, were all positive, there was a very strong association with disease severity along the Barrett's metaplasia-dysplasia-adenocarcinoma sequence compared to when the results for all markers were negative. Both an increasing hr-HPV viral load and integration into the host genome were significantly associated with disease severity in the Barrett's metaplasia-dysplasia-adenocarcinoma pathway. Another recent prospective study involving 40 patients reported that persistent biologically active hr-HPV infection (types 16 and 18) and p53 overexpression (assessed by immunohistochemistry and validated by DNA sequencing) were both independently associated with persistent dysplasia/neoplasia after endoscopic ablation of dysplastic BE/EAC[87].

Recent findings from Antonsson *et al*[88] showed the results of DNA quality testing (b-globin) through PCR and evaluation of the presence of HPV DNA in 241 histologically confirmed archived EAC and GEJAC (gastro-esophageal junction adenocarcinomas) tissue specimens from a population-based study in Australia. With a 97% DNA yield and acceptable quality in 233/241 specimens (201 EAC, 32 GEJAC), each sample was tested three times for HPV DNA. None of the 233 tumour specimens tested positive. There was no evidence of HPV DNA in esophageal adenocarcinoma tumour cells, and the researchers strongly stated that: "HPV is unlikely to cause EAC or GEJAC". New studies could investigate persistent hr-HPV infection and p53 as potential dysplasia/EAC risk markers in clinical trials as well as in BE screening/surveillance studies.

Prognostic value of HPV status in esophageal cancer

Several authors have evaluated the prognostic significance of HPV-ESCC infection. In contrast to oropharyngeal lesions, for which several retrospective clinical studies have consistently proven that patients with HPV-positive head and neck squamous cell carcinoma have a better prognosis than patients with HPV-negative tumours[89-92], the outcomes data for HPV in ESCC are still limited and conflicting. Furihata *et al*[93] reported that high levels of p53 protein expression (probably due to p53 mutations) were inversely associated with HPV16 or 18 infection in esophageal cancers, and the prognoses of two groups, one with HPV16 or 18 infection and the other with p53 overexpression, were significantly poorer than the prognoses of the group with neither condition.

Hippeläinen *et al*[94] reported that HPV was involved in 11% of 61 patients with ESCC but lacked prognostic value. Numerous authors have failed to show any significant association between HPV infection and patient survival[95]. In contrast, a recent series of studies showed an improved overall and disease-free survival in ESCC patients with HPV-positive tumours. Cao *et al*[96] reported that HPV-infected patients had better 5-year rates of overall survival (65.9% *vs* 43.4% among patients with HPV-negative tumours; $P = 0.002$ by the log-rank test) when HPV status was considered as an independent prognostic factor for overall survival (OS) and progression-free survival among patients with ESCC.

Because of this variability, clinical data do not suggest a prognostic role for HPV infection in ESCC, which precludes a definitive interpretation. Based on the evidence described above, it currently appears unlikely that HPV is clinically or aetiologically relevant for ESCC. Thus, there is no evidence to recommend that ESCC patients should be tested for HPV infection outside of the context of a research study, and there is no clear indication to change clinical practice or treatment strategy for ESCC lesions based on HPV status. Nevertheless, Oei *et al*[97] examined the response of HPV-positive cells to hyperthermia using cervical cancer cell lines infected with HPV 16 and 18, *in vivo* tumour models and *ex vivo*-treated patient biopsies. They found that hyperthermia at 42 ℃ for a duration of 60 min caused activation of the p53-dependent apoptotic pathway through degradation of E6. This outcome highlights the difference between HPV-negative and HPV-positive cells because the HPV-negative cells induced p53 and caused apoptosis after radiation alone, whereas HPV-positive cells required hyperthermia to promote these effects. Therefore, this finding may lead to further clinical studies of

hyperthermia with other HPV-associated cancers.

HPV AND COLORECTAL CANCER

Colorectal cancer (CRC) is the third most common cancer in men and the second most common in women worldwide. There is a wide geographical variation in incidence across the world, with the highest estimated rates in Australia/New Zealand (ASR 44.8 and 32.2 per 100000 in men and women, respectively) and the lowest in Western Africa (4.5 and 3.8 per 100000 in men and women, respectively)[98]. Although hereditary forms of CRC have been well established, most cases are sporadic[99]. Several epidemiological studies have shown that factors related to lifestyle can contribute to the occurrence of CRC, such as high consumption of red meat or tobacco smoking[100]. However, the role played by environmental factors in the pathogenesis of this malignancy is still under investigation. Due to the vast microbial population in the human intestine and the identification of infectious agents as risk factors for different types of cancers, researchers around the world have recently assessed the relationship between various infectious pathogens and the onset of colorectal neoplasia[101].

HPV could potentially infect the colorectum by an ascending infection from anogenital sites or thorough haematogen or lymphogen spread, and HPV has been hypothesized to be a potential aetiological factor associated with colorectal cancer[102-106].

In recent years, a growing number of studies has investigated the presence of HPV in colonic tissues. The majority of the authors found a much higher prevalence of HPV DNA in malignant tissues compared to controls (disease-free patients or adjacent colic mucosae), which suggested a potential role for HPV infection in colorectal carcinogenesis[107], but the association between HPV and colorectal cancer remains controversial and inconclusive.

There is wide variation in the reported prevalence in the literature, due partly to heterogeneity in HPV detection methods. Although the HPV prevalence was higher in PCR-based than in non-PCR-based studies, there was substantial heterogeneity even between studies using the same PCR-based method. In addition, storage of the specimens might have interfered with virus detection. In fact, viral prevalence tends to be higher in studies in which fresh or frozen tissue samples were analysed than in studies using formalin-fixed and paraffin-embedded tissue[108].

The HPV detection rate in colorectal cancer ranged from 0% to 84%[107-109]. The overall prevalence of HPV was highest in publications from South America, Asia and the Middle East, with ranges from 32% to 45%, whereas the prevalence was 3% or less in studies from North America, Europe and Australia, which was similar to variations in HPV prevalence observed for other non-cervical cancers[108,110-113]. Interestingly, this geographical variation in HPV prevalence in colorectal cancer appeared to be the opposite of the global pattern of colorectal cancer incidence and could reflect differences in HPV prevalence across the region. Furthermore, some populations may be more susceptible than others to HPV-associated colorectal carcinomas. Whereas HPV18 was more frequently detected in colorectal cancer cases from Asia and Europe, HPV16 was more prevalent in colorectal cancer cases from South America[108].

Moreover, Burnett-Hartman[114] analysed the correlation between HPV and colorectal cancer using the Hill criteria[115]. As stated by the author, there was some evidence for analogy, biological plausibility and strength of association but only weak evidence for consistency, specificity and coherence. Thus far, temporality, biological gradient and experimentation have not been evaluated in published studies.

Demonstrating the integration of HPV in the host genome and the expression of viral oncogenes is also important. Lorenzon *et al*[116] investigated the integration status of HPV16 in colorectal tumours by detecting the virus exclusively in its episomal form, in barely traceable quantities and when it was transcriptionally inactive. Previously, Bodaghi *et al*[117] examined 31 of the HPV16 DNA-positive samples for the intact HPV16 E2 gene and frequently found viral integrated DNA in the tumour tissue.

Although two recent meta-analyses have demonstrated a significant increase in colorectal cancer risk associated with the presence of the virus[108,109], the published literature does not provide convincing evidence for a strong association. The sample size in each of the studies was small, and genotyping methods varied across studies. However, because the pathogenesis of colorectal cancer is multifactorial, HPV may play a role in a subset of cancers. Additional large-scale multicentre studies using standardized methodologies and researchers investigating the viral genome integration as well as the molecular basis of HPV-related carcinogenesis are needed to better understand the possible role of HPV in colorectal cancer.

HPV AND ANAL CANCER

Anal cancer commonly occurs in the transformation zone between the squamous and columnar epithelium of the anal canal. Squamous cell carcinoma (SCC) is the most common histological type and accounts for 85% of all anal cancers[118]. SCC is closely related to persistent infection with human papilloma virus. Indeed, HPV is detected in 80%-90% of anal cancers, and HPV16 is the predominant type identified in approximately 80% of patients[119,120] with a frequency much higher than that in other anatomic sites[120-125]. This frequency possibly reflects a differential tropism of HPV 16 or an increased probability of HPV 16 to lead to malignant transformation in the anal mucosa. HPV prevalence in anal cancer varies by geographic region, with the highest prevalence in North America and Europe and the lowest in Africa[119].

Similar to cervical cancer, squamous cell carcinoma of the anus is preceded by a spectrum of precursor lesions of varying cytological and histological severity that are defined as anal intraepithelial neoplasia (AIN). Grades 2 and 3 are grouped together as high-grade anal intraepithelial neoplasia (HGAIN) and are associated with a higher risk of invasive cancer. The natural history of AIN is not well understood. However, it is believed that low-grade AIN may undergo spontaneous regression or progress to high-grade AIN, which is similar to cervical cancer. The rate of progression from anal HGAIN to invasive cancer is unknown but is estimated to be notably lower than the risk of progression to cancer for cervical high-grade squamous intraepithelial lesions (approximately 1 to 80 per year)[126-128]. AIN2/3 is related to HPV in more than 90% of cases, particularly with serotype 16[119,120].

Anal cancer accounts for approximately 2%-3% of gastrointestinal tract malignancies, with 27000 new cases diagnosed worldwide in 2008 and age-adjusted incidence rates approximately 1 per 100000 people[2,119]. Anal cancer is a disease of older individuals, with a peak in those aged 55 to 64 years, and the median age for diagnosis is 60 years. In most countries, women are more commonly affected than men[129].

Although anal cancer is relatively rare, the incidence has been increasing over the past decade in different countries in both sexes[130]. In the United States , the incidence of anal cancer has more than doubled since 1975 and continues to rise by approximately 2.2% per year for both genders[131]. In Denmark, the incidence increased by 2-fold in both women and men in the 30-year period between 1978 and 2008, and the incidence of AIN increased by 1.3-fold in the shorter observation period between 1998 and 2008[132]. Similar patterns have been observed in other European countries and in Australia[130,133]. The reason for the increasing incidence of anal cancer is not well understood but may reflect changes in sexual behaviour, such as the increased adoption of high-risk sexual activities, including receptive anal intercourse and increased sexual promiscuity[134]. Furthermore, most of this increase is attributed to a certain at-risk population, specifically men who have sex with men (MSM)[135] and HIV-positive individuals[118,136] that have a significantly higher risk of anal cancer compared to the general population. MSM are nearly 20 times more likely than heterosexual men to develop the disease[129], and there is a10-fold increase in incidence in HIV-positive women compared to HIV-negative women[131]. There was also an increase of more than 40-fold in men with HIV infection who only have sex with women compared to their HIV-uninfected counterparts, and the increase was even higher among HIV-positive MSM[137,138]. The risk of SCC increases with the duration of HIV infection, and it is not reduced to the use of highly active antiretroviral therapy (HAART)[30,139]. Women with a history of HPV-associated genital (*i.e.*, vulval, vaginal or cervical) high-grade squamous intraepithelial lesions or cancer[140] and people with immune deficiency also have a high risk for this disease[141].

In HIV-infected men who have sex with men, hrHPV is detected in approximately 74% of individuals, and concurrent infections with several HPV types are also common[128,142]. Furthermore, studies have demonstrated that the prevalence of HPV infection of the anal region is common in both heterosexual man and HIV-uninfected individuals. In a study in immunocompetent asymptomatic heterosexual men, the prevalence of overall anogenital HPV infections was 24.8% and, of the men with anal HPV infection, 33.3% had an oncogenic HPV type[143]. In HIV-negative women with no history of HPV-related pathology of the vulva, vagina and cervix, the prevalence of anal HPV varied from 4% to 22% and rose to 23%-36% in women with known HPV-related pathology. In HIV-positive women, the prevalence of HPV in the anus varied from 16% to 85% and was higher than the prevalence in the cervix in the majority of studies[144].

A recent study focusing on the natural history of anal HPV infection in heterosexual women showed that approximately 85% of women cleared low risk-types and non-16 hr-HPV infections within three years, whereas only 76.2% of women cleared HPV16 infections. The slower rate noted for HPV16 in the anus was not surprising because HPV16 appears to be more important than the other hr-HPV types in anal cancer. Interestingly, one of the strongest predictors of anal HPV16 persistence was having a concomitant cervical infection with HPV16. Anal sex behaviours, including anal intercourse, anal touching and lack of condom use during anal sex, were also associated with HPV16 persistence[145].

Several case-control studies have assessed the risk of anal cancer associated with HPV infection and reported odds ratios between 2 and 7 for HPV 16 and HPV 18 seropositivity for both men and women[119].

Anal intercourse is probably the most efficient way of acquiring anal HPV infections, but it is not the only mode of transmission for HPV to the anal canal. Several studies have found anal HPV in women with no history of anal receptive intercourse. Furthermore, most publications have found that HPV infection of the cervix is a significant risk factor for anal HPV[145,146]. In addition, studies have shown a high degree of genotype-specific concordance among women with concurrent anal and cervical infections, which is consistent with a common source of infections or spread from one site to the other[147]. The plausibility that the cervix acts as a source for anal HPV infection is enhanced by the anatomic proximity of the vaginal introitus to the anus, and the likelihood that anal infection acts as a source for cervical infection is somewhat less probable. Several studies have found the same HPV type on the hands or fingertips as in the genital area in men and in women[148-150]. Although HPV on the fingers likely represents DNA deposits from the genitals rather than a true infection, the possibi-

lity of infection through autoinoculation *via* vaginal secretions, digital or fomite transference cannot be excluded[151].

Anal cancer screening

Screening for anal cancer is currently being considered for high risk groups (*e.g.*, HIV-infected men having sex with men). Screening should involve the assessment of anal cytology obtained with a swab and subsequent study with high-resolution anoscopy (HRA) when cytological abnormalities are detected. The sensitivity of a single anal cytology test for detecting histological HSIL (AIN2/3) ranges from 55% to 93%, and the specificity ranges between 32% and 81%[129]. Many studies have found poor correlation between the grade of the cytology report and the grade diagnosed on a subsequent biopsy[152]. Moreover, sensitivity decreases in the highest risk group[129]. For these reasons and given the low specificity of anal cytology as a screening tool, some clinicians have proposed that patients should be referred immediately for an HRA. Additionally, using HPV testing could help reduce the need for HRA. However, HPV testing can have a low specificity for anal HSIL because HIV-infected patients have a high HPV prevalence, and multiple HPV types may be detected in the anal canal.

Although there is currently no evidence of its efficacy, some authors have recommended screening high-risk HIV-positive individuals[152,153], such as MSM, those with a history of condylomata and women with cervical or vulvar dysplasia. This recommendation is based on indirect evidence taken from various epidemiological studies that highlight the high incidence of anal cancer among these subgroups, the availability of the test, the morbidity and mortality associated with anal cancer and studies evaluating the cost-effectiveness of the screening strategy.

Prognostic value of HPV status in anal cancer

Although anal cancer chemoradiotherapy (CRT) has become the standard treatment for patients with loco-regional disease, there has been little improvement in survival over the last few decades[154]. Therefore, there is growing interest in the determination of molecular tumour factors that may predict either a response or resistance to standard CRT.

Recently, in HPV-associated malignancies, particularly oropharyngeal head and neck SCC, most studies have revealed a more favourable prognosis in patients with an HPV-positive tumour compared to HPV-negative patients. Moreover, recent data indicate that treatment response to radiotherapy (RT) or chemoradiotherapy (CRT) is superior for HPV-positive tumours compared to HPV-negative tumours[155]. In patients with cervical cancer, Kim *et al*[156] recently reported that the HPV DNA load was a strong independent prognostic factor for disease-free survival after radical RT. However, the biological basis for this improved outcome in HPV-positive cases was not clear.

However, because the detection rate of HPV DNA in anal SCC commonly exceeds 90%, a comparison between only HPV-positive and HPV-negative cancers with respect to treatment response and long-term outcome is very difficult.

The recent understanding of HPV infection as a causative factor in treatment response and prognosis further encouraged investigators to examine surrogate markers for HPV infection. Among these factors, the cell cycle regulatory protein $p16^{INK4a}$ (p16) has emerged as the best candidate due to its association with high-risk HPV infection. This cyclin-dependent kinase inhibitor is normally repressed by a phospho-retinoblastoma protein (pRB)/transcription factor E2F complex, but this suppression is inhibited by the high-risk HPV oncoprotein E7, which results in overexpression of p16. In line with that finding, several studies have indicated that p16 detection by IHC is predictive for a significantly improved response to treatment with RT/CRT and a more favourable prognosis in patients with HPV-associated malignancies[157].

Serup-Hansen *et al*[158] found that p16 positivity was a strong independent prognostic factor for improved overall survival (OS) and disease-specific survival (DSS) in patients with anal carcinoma. Rödel *et al*[157] showed that a high HPV16 DNA load was an independent prognostic factor associated with improved locoregional tumour control and overall survival, and they also revealed increased locoregional control in patients with a high p16 labelling score compared to patients with low p16 expression. Another recent retrospective study provided an evaluation of the prognostic importance of local control and survival of various combinations of HPV/p16 status in a total of 106 patients treated with CRT. Mai *et al*[159] concluded that p16 positivity alone was not a sufficient marker for HPV-induced transformation because p16 positivity can be caused by an event other than HPV transformation, and that outcome conferred a significantly different prognosis. HPV+/p16+ status only reliably identified HPV-transformed tumours with an extremely favourable prognosis. Tumours with p16 overexpression without HPV infection seemed to have a particularly unfavourable prognosis and may therefore require more treatment to improve results.

However, other studies were unable to find an association between p16 expression and outcomes using conventional IHC staining status[160]. This result may be related to the fact that most of the cases were HPV-positive according to all testing techniques, which made any subset analysis of the IHC score very difficult. Differences in scoring and definition of p16 positivity, the retrospective study design and smaller sample sizes may also explain the discordant results. Therefore, these data need to be validated prospectively in a larger patient cohort. Additionally, the molecular background of tumours that are p16+

without HPV infection has to be investigated further.

HPV VACCINATION IS THE KEY TO IMPROVING HPV-RELATED DISEASE CONTROL

Cervical cancer screening, along with primary prevention measures to control sexually transmitted diseases, has long been the main intervention to reduce the burden of HPV-related disease[161].

With an improved understanding of the role of HPV infection in several malignancies, vaccination has emerged as a central tool for preventing HPV-related disease.

Currently, three vaccines against HPV are approved. The bivalent vaccine (HPV2) is designed to prevent infection from HPV types 16 and 18, and the quadrivalent vaccine (HPV4) is active against HPV types 6, 11, 16 and 18. Data from clinical trials have shown that both vaccines can prevent premalignant genital (cervical, vulvar and vaginal) and anal lesions in females from 9 to 26 years of age. The quadrivalent vaccine was also effective in males ages 9 to 26 for the prevention of genital warts, penile intraepithelial neoplasia and AIN. Although no data from clinical trials are currently available to demonstrate efficacy for the prevention of oropharyngeal cancer, the HPV vaccine is likely to offer protection against these cancers as well because most cases can be attributed to HPV16. Furthermore, clinical trials recently established the safety, efficacy and immunogenicity of the nonavalent vaccine, which includes the next five most commonly detected oncogenic types in HPV-associated cancers (*i.e.*, types 31, 33, 45, 52 and 58)[16,162,163].

By the beginning of 2007, routine vaccination programmes against HPV were implemented in the majority of Western countries. However, the high cost of the vaccine, the diffusion of movements against vaccine use and difficulties in reaching the adolescent population that is the target of vaccination have hindered widespread coverage[164].

Moreover, most existing vaccination programmes target pre-adolescent girls only through school-based or health centres, and these programmes often include catch-up programmes for older girls and women. Therefore, most countries do not currently include boys in HPV vaccination programmes.

Studies comparing the incremental benefits of vaccinating males and females to female-only immunization indicate that gender-neutral vaccination programmes are likely to further reduce the incidence of HPV-related disease in both sexes. Instead, cost-effectiveness analyses indicate that including males in current HPV vaccination programmes may not be cost-effective if high coverage of females is achieved[165-169]. However, in many countries, the goal of vaccination coverage > 80% among females has not been reached.

Failure to achieve high population coverage hampers the additional protection associated with herd immunity such that the potential benefits of vaccination are not fully realized. For instance, significant reductions in HPV infections and genital warts in girls and boys younger than 20 years of age were observed only where high coverage target was reached[170].

In many developed countries, the burden of HPV-associated cancers in men is comparable to the burden in women. In Europe, approximately 30% of all HPV-related cancers are estimated to occur in males[171]. Moreover, men having sex with men receive little benefit from strategies based on the vaccination of women only.

The results of economic analyses have limitations. They critically depend on a modelling approach and assumptions about natural immunity, vaccination strategies, vaccine coverage rate and HPV-related diseases. Furthermore, these studies do not consider the epidemiological trend of HPV-related diseases, such as the observed increase in the incidence of anal and head and neck cancers[172]. Moreover, published cost-effectiveness studies have been criticized for underestimating the benefits of universal vaccination related to several non-health factors, including economic benefits, such as improved patient, family and career productivity. The assessment of the cost-effectiveness of universal HPV vaccination also depends on the estimated costs of vaccination. Strategies to reduce the cost associated with vaccination could make universal vaccination cost-effective. For instance, ongoing studies are exploring the change from a three-dose schedule of HPV vaccination to a two-dose schedule[173,174].

To increase the impact of HPV vaccination, Australia has been the first country to offer free HPV vaccines for boys as well as girls. Canada and the United States now also recommend vaccinating boys. Austria recently became the first European Union country to offer universal HPV vaccination.

Finally, it must be emphasized that the potential population level impact of current prophylactic HPV vaccination on cancers other than cervical cancer is particularly important because there are very few effective screening programmes for most of these malignancies.

Modelling analyses suggest that HPV vaccination can substantially reduce the burden of HPV-associated diseases, with approximately 30%-40% of health gains deriving from the prevention of non-cervical cancers[163].

Optimal implementation of HPV vaccines is an important public health challenge. Targeted efforts are needed to increase vaccine uptake among girls, and universal vaccination should be carefully considered[174]. Indeed, the extension of vaccination to boys is highly desirable and should be implemented in many countries in the next few years.

CONCLUSION

Human papillomavirus is one of the major causes of infection-related cancer worldwide. A clear association has already been established between HPV and genital malignancies, anal and oropharyngeal cancers. Although there is ongoing investigation into the potential aetiological role of HPV in the development of esophageal and other cancers, most studies to date have been inconclusive.

Identification of HPV-related cancers is clinically relevant because HPV status is emerging as a possible predictor of treatment response and long-term oncological outcomes that might help identify patients who are candidates for different treatment regimens.

Further research is required to improve our understanding of the natural history of HPV infection, its oncogenesis and the potential clinical implications. Screening could be introduced to decrease incidence of anal cancer in high-risk groups. Nevertheless, vaccination represents the best available strategy to reduce the burden of HPV-related cancers.

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REVIEW

Prediction of lymph node metastasis and sentinel node navigation surgery for patients with early-stage gastric cancer

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Abstract

Accurate prediction of lymph node (LN) status is crucially important for appropriate treatment planning in patients with early gastric cancer (EGC). However, consensus on patient and tumor characteristics associated with LN metastasis are yet to be reached. Through systematic search, we identified several independent variables associated with LN metastasis in EGC, which should be included in future research to assess which of these variables remain as significant predictors of LN metastasis. On the other hand, even if we use these promising parameters, we should realize the limitation and the difficulty of predicting LN metastasis accurately. The sentinel LN (SLN) is defined as first possible site to receive cancer cells along the route of lymphatic drainage from the primary tumor. The absence of metastasis in SLN is believed to correlate with the absence of metastasis in downstream LNs. In this review, we have attempted to focus on several independent parameters which have close relationship between tumor and LN metastasis in EGC. In addition, we evaluated the history of sentinel node navigation surgery and the usefulness for EGC.

Key words: Early-stage gastric cancer; Sentinel node navigation surgery; Prediction of lymph node metastasis

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Core tip: In this review, we have attempted to focus on several independent parameters which have close relationship between tumor and lymph node metastasis in early gastric cancer. In addition, we evaluated the usefulness of sentinel node navigation surgery (SNNS)

for patients with gastric cancer, in particular technical procedure of SNNS using Infrared Ray Electronic Endoscopes combined with Indocyanine Green injection.

Shida A, Mitsumori N, Nimura H, Takano Y, Iwasaki T, Fujisaki M, Takahashi N, Yanaga K. Prediction of lymph node metastasis and sentinel node navigation surgery for patients with early-stage gastric cancer. *World J Gastroenterol* 2016; 22(33): 7431-7439 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i33/7431.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i33.7431

INTRODUCTION

Newly developing patients with gastric cancer are estimated as 951000 per year and is the fifth most common cancer in the world[1]. The chance of detecting early gastric cancer (EGC) is increasing especially in South Korea and Japan thanks to development in function of endoscopy and the national screening systems[2-5]. In Japan and South Korea, patients with EGC has been blessed with superior prognosis after surgical treatment[5]. In general, lymph node (LN) metastasis in patients with EGC has been reported about 10%-15%, and it is one of the strongest prognostic parameters[1,6-8]. Gastrectomy with D2 lymphadenectomy is recognized as a standard surgical procedure for patients with advanced gastric cancer according to the Dutch trial[9]. Standard D2 lymphadenectomy is appropriate procedure for patients with advanced gastric cancer. On the other hand, more limited lymphadenectomy such as D1 or D1+ for patients with EGC is also available according to the Japanese Gastric Cancer Association[10]. The relationship between pathological parameters and LN metastasis for patients with EGC has been deeply inquired. Meanwhile, the connection between preoperative parameters and LN metastasis of EGC has not been fully investigated[11-14]. Furthermore, the involvement between preoperative and postoperative diagnostic parameters has not been widely evaluated.

The "less invasive" theory behind sentinel LN (SLN) biopsy concept has benefits based on the limitation of morbidity because of avoiding unnecessary LN dissection. At least theoretically, combination of SLN and less invasive surgical procedures such as laparoscopic surgery seems attractive. SNNS has potential to change the current surgical treatment of gastric cancer.

The aim of this review is to clarify the reliability of preoperative prediction of LN metastasis for patients with EGC and evaluate the clinical usefulness of SNNS.

PREOPERATIVE PREDICTION OF LN METASTASIS OF EGC

Mönig *et al*[15] investigated 1253 LNs of 31 specimens and reported that the mean diameter of the LNs which is negative for metastasis was 4.1 mm. On the contrary, mean diameter of LNs which is positive for metastasis was 6.0 mm. Eighty percent of LNs which is negative for metastasis were less than 5 mm in diameter. However 55% of LNs which is positive for metastasis were less than 5 mm in diameter. In addition, 71% of patients who had LN metastasis had at least one node that was 10 mm or greater in diameter. Furthermore, 70% of patients who were free for LN metastasis had at least one node that was 10 mm or greater in diameter. According to these results, they concluded that size of LN is not a reliable parameter for LN metastasis in patients with gastric cancer. In this point, prediction of LN metastasis using CT can't be trustworthy examination for ECG. Fluorodeoxyglucose positron emission tomography (FDG-PET) and EUS are alternative methods for detecting LN metastasis. FDG-PET is a useful preoperative diagnostic instrument to investigate metastasis. However, it has been reported that FDG-PET is not reliable tool to predict LN metastasis because of the low sensitivity[16-18]. In addition, accuracy of prediction for LN presence by EUS was only 64%[19]. These reports suggest that FDG-PET and EUS are not credible tool to predict LN metastasis up to now. Nakagawa *et al*[20] analyzed 1042 patients with EGC who underwent gastrectomy with standard LN dissection. They constructed two receiver operating characteristics (ROC) curves consisting of postoperative independent factors and preoperative independent factors to predict LN metastasis. Comparing with areas produced by the two ROC curves, they investigated which is more reliable factors to predict LN metastasis. As a result, produced areas under the ROC curve made of postoperative parameters including pathological data was 0.824. However, the area under the ROC curve made of preoperative factors obtained from CT or endoscopic examination was 0.660. Hence, they concluded that prediction of LN metastasis for EGC using preoperative parameters is not credible as compared with using postoperative factors[20].

RELATIONSHIP BETWEEN PATHOLOGICAL PARAMETERS AND LN METASTASIS

Many studies have been carried out to evaluate relationship between pathological parameters and LN metastasis in EGC. We have surveyed numerous published articles which describe an association between pathological parameters and LN metastasis in EGC after year 2001. From these articles, we selected 28 articles (Table 1), which investigated relationships between pathological risk factors and LN metastasis in EGC at least using multiple variate analysis. Song *et al*[21] have demonstrated that increased submucosal vascularity, histological differentiation, invasion of tumor cells into the muscularis mucosae had signifi-

Table 1 Published articles which refer to independent risk factors for lymph node metastasis of early gastric cancer after 2001

Ref.	Year	Country	Number of patients	Independent parameters which affect LN metastasis	Odds ratio	95%CI
Folli *et al*[40]	2001	Japan	m: 285, sm: 215	Tumor size	1.34	1.13-1.59
				Tumor depth (pT1b *vs* pT1a)	2.29	1.56-3.36
				Histological differentiation (Diffuse *vs* Intestinal)	5.70	2.88-11.31
				Histological differentiation (Mixed *vs* Intestinal)	4.19	1.89-9.32
				Kodama Type (Pen A *vs* Not Pen A)	1.36	1.17-1.58
Amioka *et al*[26]	2002	Japan	sm: 139	Lymphatic invasion	3.48	1.17-10.40
				VEGF-C (positive *vs* negative)	4.18	1.38-12.70
Abe *et al*[41]	2002	Japan	m: 136, sm: 178	Gender (female *vs* male)	3.23	1.33-7.88
				Tumor size (≥ 20 mm *vs* < 20 mm)	3.39	1.26-9.13
				Tumor depth (pT1b *vs* pT1a)	4.94	1.49-16.27
				Lymphovascular invasion	7.54	3.01-19.04
Matsuzaki *et al*[42]	2003	Japan	sm: 92	Volume of lesions	1.27	2.49-13.51
Abe *et al*[43]	2003	Japan	sm: 104	Gender (female *vs* male)	2.90	1.2-6.9
				Tumor depth (pT1b *vs* pT1a)	29.20	3.9-216.3
				Lymphatic invasion	50.80	8.1-317.3
Song *et al*[21]	2004	South Korea	m: 120	Lymphatic invasion	21.39	10.41-43.95
				Tumor depth (sm massive *vs* sm shallow)	2.56	1.30-5.03
Park *et al*[22]	2004	South Korea	sm: 105	Tumor size (> 40 mm)	4.80	1.05-22.06
				Tumor depth (> 2000 μm)	6.81	1.36-34.17
Hyung *et al*[44]	2004	South Korea	m: 295, sm: 271	Histological differentiation (Undifferentiated *vs* Differentiated)	2.28	1.14-4.56
				Tumor size (≥ 20 mm *vs* < 20 mm)	1.045	1.36-5.93
				Tumor depth (pT1b *vs* pT1a)	3.68	1.67-8.13
				Lymphovascular invasion	26.56	12.77-55.23
Son *et al*[45]	2005	South Korea	sm: 248	Lymphatic invasion	21.39	10.41-43.96
				Tumor depth (sm massive *vs* sm shallow)	2.56	1.30-5.03
Lo *et al*[46]	2007	Taiwan	m: 272, sm: 203	Lymphovascular invasion	8.61	4.43-16.72
				Tumor depth (pT1b *vs* pT1a)	3.05	1.47-6.33
				Tumor size	1.68	1.13-2.51
Kunisaki *et al*[47]	2007	Japan	sm: 615	Tumor size (40 mm < 80 mm *vs* < 20 mm)	2.71	1.31-5.61
				Tumor size (≥ 80 mm *vs* < 20 mm)	3.20	1.02-10.09
				Lymphatic invasion	15.92	9.52-26.63
An *et al*[6]	2007	South Korea	sm: 1043	Tumor size (20 mm < 40 mm *vs* < 10 mm)	1.88	1.03-3.45
				Tumor size (≥ 40 mm *vs* < 10 mm)	1.96	1.34-2.88
				Lymphatic invasion	8.41	5.76-12.29
Yi Kim *et al*[24]	2007	South Korea	m: 9, sm: 51	Lymphatic invasion	8.11	1.61-40.77
				E-Cadherin (abnormal expression *vs* normal expression)	2.62	0.917-7.457
Li *et al*[48]	2008	South Korea	m: 356, sm: 270	Tumor size (≥ 20 mm *vs* < 20 mm)	2.04	1.12-3.73
				Tumor depth (pT1b *vs* pT1a)	2.84	1.48-5.44
				Lymphovascular invasion	15.11	7.41-30.80
Park *et al*[49]	2008	South Korea	Only poorly diff. adenocarcinoma m: 118, sm: 116	Tumor depth (500-1000 μm invasion to submucosa)	14.69	2.54-85.09
				Tumor depth (1000-2000 μm invasion to submucosa)	6.20	1.57-24.52
				Tumor depth (> 2000 μm invasion to submucosa)	6.37	1.35-30.14
				Tumor size (> 30 mm in diameter)	4.53	1.13-18.20
				Lymphovascular invasion	12.63	4.05-39.37
Shen *et al*[50]	2009	China	sm: 144	Histological differentiation (Undifferentiated *vs* Differentiated)	2.70	1.18-6.17
				Tumor size (≥ 20 mm *vs* < 20 mm)	2.93	1.32-6.54
Morita *et al*[51]	2009	Japan	sm: 70	Tumor size	1.04	1.01-1.08
				Lymphatic invasion	5.22	1.84-20.74
				VEGF-C (positive *vs* negative)	3.31	1.00-0.95
Kunisaki *et al*[52]	2009	Japan	m: 269, sm: 304	Tumor size (≥ 20 mm *vs* < 20 mm)	3.34	1.39-8.01
				Tumor depth (SM1 *vs* M)	2.96	1.03-8.52
				Tumor depth (SM2 *vs* M)	4.53	1.69-12.18
				Lymphovascular invasion	9.37	4.78-18.37
Sung *et al*[53]	2010	Taiwan	m: 293, sm: 263	Tumor size (≥ 2 mm in diameter *vs* < 2 cm)	2.28	1.20-4.17
				Lymphatic invasion	27.20	10.3-74.8
				Tumor depth (pT1b *vs* pT1a)	4.91	2.44-9.89
Lee *et al*[54]	2010	South Korea	m: 39, sm: 85	Lymphatic invasion	15.33	5.06-46.44
				Tumor size (≥ 30 mm *vs* < 30 mm)	4.16	1.52-11.45
				Tumor depth (M/SM1 *vs* SM2/SM3)	3.11	1.21-7.98
				Intratumoral vessel density	3.57	1.20-10.64
Lim *et al*[55]	2011	South Korea	sm: 163	Lymphovascular invasion	4.57	1.74-12.24
				Macroscopic type (elevated *vs* flat)	9.09	1.75-50.0
				Macroscopic type (elevated *vs* depressed)	5.89	1.69-20.0
				Macroscopic type (elevated *vs* mixed)	20.00	0.00-2.70

Lee *et al*[56]	2012	South Korea	Only poorly diff. adenocarcinoma m: 510, sm: 495	Tumor size (≥ 20 mm *vs* < 20 mm)	2.47	1.39-4.40
				Tumor depth (pT1b *vs* pT1a)	2.42	1.46-3.99
				Lymphatic invasion	6.50	4.14-10.19
Ren *et al*[57]	2013	China	m: 122, sm: 80	Tumor depth (pT1b *vs* pT1a)	2.74	2.32-3.17
Fujii *et al*[23]	2013	Japan	sm: 130	Lymphatic invasion	8.07	NA
				Lymphocytic infiltration (absent *vs* present)	7.94	NA
Shida *et al*[27]	2014	Japan	sm: 145	Lymphatic invasion	3.11	1.71-5.67
				Vascular invasion	2.44	1.05-5.67
Lee *et al*[12]	2015	South Korea	m: 847	Tumor size	1.36	1.10-1.69
				Lymphovascular invasion	27.52	7.40-102.20
				Ulceration (present *vs* absent)	7.54	1.90-29.90
				Undifferntiated type of component (present *vs* absent)	4.39	1.08-17.89
Park *et al*[25]	2015	South Korea	sm: 756	Tumor size (≥ 2 cm in diameter *vs* < 2 cm)	1.57	1.04-2.36
				Tumor depth (sm2 *vs* sm1)	2.96	1.55-5.64
				Tumor depth (sm3 *vs* sm1)	2.91	1.61-5.29
				Lymphovascular invasion,	7.45	4.93-11.25
				Negative for EB virus	4.24	1.26-14.32
Feng *et al*[58]	2016	China	m: 339, sm: 237	Tumor depth	2.94	1.82-4.77
				Ulceration (present *vs* absent)	2.55	1.21-5.38
				Lymphovascular invasion	4.40	1.19-16.3

cant relation to LN metastasis in intramucosal gastric carcinoma. Depth of tumor invasion and tumor size had also significant correlation with LN metastasis in EGC[22]. Furthermore, An *et al*[6] also demonstrated that lymphatic system invasion and tumor size had strong relationship to LN metastasis in submucosa invading EGC with submucosal invasion. Of the 28 articles, 23 (82.1%) authors concluded lymphatic invasion or lymphovascular invasion as independent risk factors for LN metastasis and tumor depth and tumor size were also confirmed as LN risk factors in 17 (60.7%) articles, respectively. These results are suggesting that lymphovascular invasion, tumor depth and tumor size are strong predictors of LN metastasis for EGC. Furthermore, Fujii *et al*[23] described that lymphatic invasion and absence of clear lymphoid follicle formation at the site of submucosal invasion (lymphocystic infiltration) were independent risk factors for LN metastasis. Immunohistochemical (IH) research is also useful for predicting LN metastasis. Yi Kim *et al*[24] demonstrated that abnormal expression of E-cadherin and lymphatic invasion were independent, statistically significant parameters which is associated with LN metastasis for patients with EGC. Recently, Park *et al*[25] revealed not only larger tumor size (greater than 2 cm), deeper level of submucosal invasion, lymphovascular invasion but also Epstein-Barr virus negativity were independent risk factors for LNM in submucosa invaded EGC using a large series (n = 756) of patients with EGC. In addition, Amioka *et al*[26] investigated clinicopathological relationship between Vascular Endothelial Growth Factor (VEGF) -C expression in submucosa-invading gastric carcinoma and LN metastasis. They demonstrated VEGF-C expression in submucosa-invading gastric carcinoma had significant correlation to LN metastasis.

LN METASTASIS PREDICTING SCORE

One hundred forty-five consecutive patients with submucosa-invaded EGC were analyzed using multivariate analysis, and a formula which predicts LN metastasis was developed by linear discriminant analysis. Additionally, prospective validation study was carried out to estimate if the formula is reliable to predict LN metastasis. Lymphatic system invasion and venous system invasion were selected as independent parameters correlated with LN metastasis for EGC. The LN metastasis predicting formula was developed using these two factors by linear discriminant analysis. The formula is as follows: Y = 0.12 × (venous system invasion: 0, 1, 2 or 3) + 0.19 × (lymphatic system invasion: 0, 1, 2, or 3) - 0.14. If Y > 0, we judge that a patient with gastric cancer is susceptible LN metastasis. Prospective study demonstrated that sensitivity and specificity rates of this formula were 70% and 61.6%, respectively[27]. Flow chart for submucosa-invading gastric cancer after ESD is shown in Figure 1. This flow chart is indicating that if resected tumor through ESD invaded 500 μm below the muscularis mucosae, the LN metastasis predicting score is available. Y > 0 indicates that the tumor in question would be prone to LN metastasis. We defined patients who are satisfied with Y > 0 as a high-risk group (HRG) for LN metastasis and Y < 0 patients as a low-risk group (LRG) for LN metastasis. The flow chart means patients with HRG should undergo additional conventional gastrectomy. However, we think that less invasive treatment like as SNNS is more desirable for patients with LRG.

Fujii *et al*[23] investigated 130 submucosa-invaded gastric cancer. Absence of lymphoid infiltration and lymphatic system invasion were selected as independent significant factors which affect LN metastasis. They also developed LN metastasis predicting score and advocated a scoring system for additional gastrectomy following ESD based on prediction of LN metastasis (Figure 2). Lymphoid infiltration and the presence of lymphatic system invasion were scored as follows: +2 for lymphatic system invasion and -2 for involvement of lymphocystic infiltration, which was considered as a LN

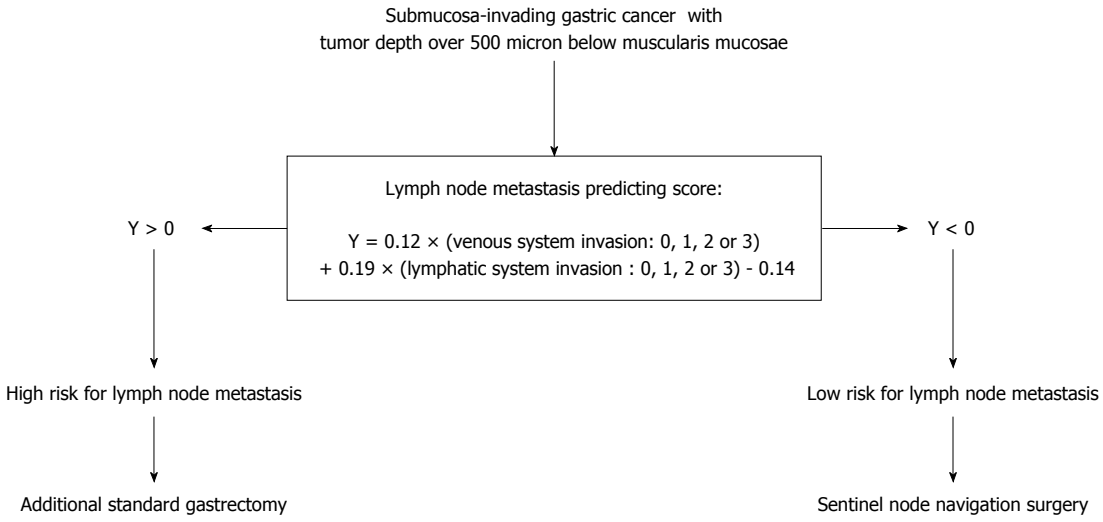

Figure 1 Flow chart for submucosa-invading gastric cancer after ESD. Quoted from Prediction of lymph node metastasis in patients with submucosa-invading early gastric cancer. Adapted from Ref. [27].

Score -2	Score +1	Score +2
Lymphocytic infiltration (+)	Histologically undifferentiated (submucosa)	Lymphatic invasion (+)
	Minor axis ⩾ 2 cm	
	Depth of submucosal invasion ⩾ 2000 μm	
	Ulcer or scar (in cancer lesion)	
	Venous invasion (+)	

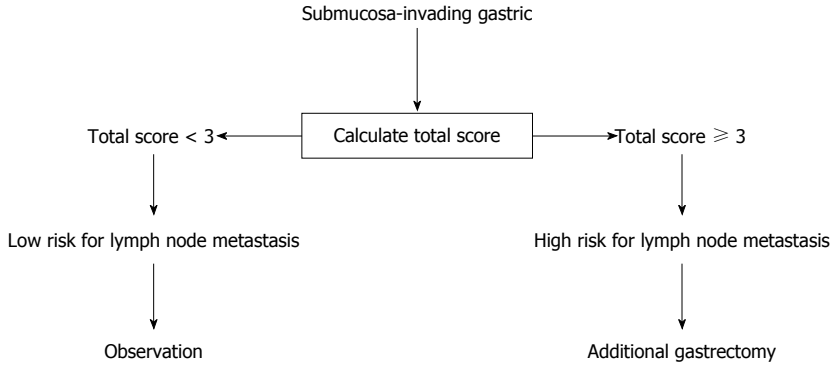

Figure 2 Scoring to predict lymph node metastasis and scoring system for additional gastrectomy following endoscopic resection based on prediction of lymph node metastasis. Quoted from Pathological factors related to lymph node metastasis of submucosally invasive gastric cancer: criteria for additional gastrectomy after endoscopic resection. Adapted from Ref. [23].

metastasis-inhibiting parameter. Next five pathological factors [minor axis length ⩾ 2 cm, submucosal invasion depth ⩾ 2000 μm, histological classification (undifferentiated) of submucosal cancer at the site of invasion, ulceration or scar in the lesion, and venous invasion] were scored +1 each when present. They concluded that a patient with total score 3 and more should be treated as high risk for LN metastasis and such patients are recommended to undergo additional gastrectomy. On the other hand, patients with total score less than 3 should be considered as low risk for LN metastasis and they don't need to undergo additional gastrectomy.

These predictive scores and treatment of flow chart after ESD seem innovative and original strategy for EGC. However, we need further additional clinical trials to validate clinical usefulness of the flow charts.

SNNS AS A SOLUTION TO PREDICT LN METASTASIS OF EGC

In spite of these constant efforts to predict LN metastasis using pathological parameters, it has been still difficult to predict LN metastasis accurately. Meanwhile, SNNS for EGC may become another possible and promising solution to resolve this problem. From early 2000s, articles which focused on SNNS for EGC have been published. Hiratsuka *et al*[28] demonstrated the usefulness of indocyanine green (ICG) for SNNS in particular T1 gastric cancer because of the high successful rate to predict SLNs. Kitagawa *et al*[29]

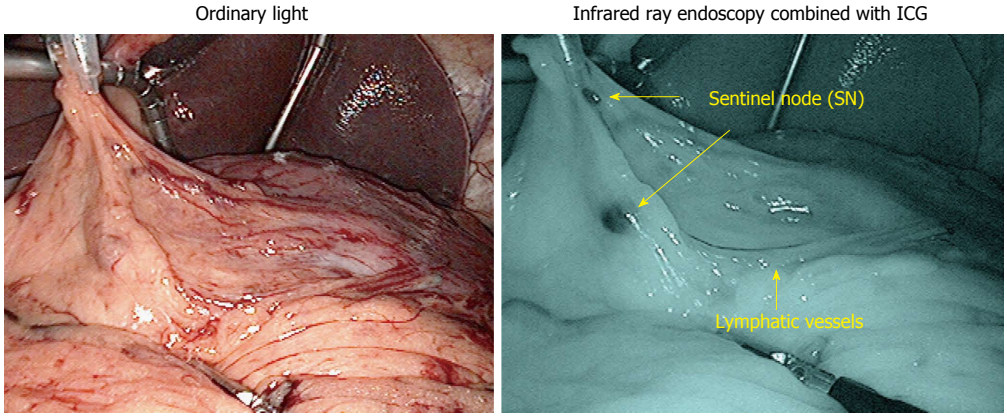

Figure 3 Sentinel node navigation surgery using infrared ray electronic endoscopy. ICG: Indocyanine green.

showed the effectiveness of radio-guided surgery using gamma detection probe technology for SNNS. Technetium-99m-radiolabelled tin colloid was injected endoscopically before the surgery, and radioactive SNs were identified with a gamma probe. They concluded that the radioisotope is useful even for obese patients because it remains for enough time in the SNs after injection. In addition, Miwa *et al*[30] demonstrated the results of a regional multicenter clinical trial of SN mapping for gastric cancer using the dye-guided method. This was the first multicenter trial of SN mapping for gastric cancer. Miwa demonstrated that sentinel lymphatic basins contain truly positive nodes, even in cases with a false negative SN biopsy. Hence, they concluded that the sentinel lymphatic basins dissection are adequate procedure for LN dissection in patients with EGC.

We have reported the clinical usefulness of infrared ray electronic endoscopy (IREE) combined with ICG to detect illuminated SLN in patients with gastric cancer and duodenal tumors as compared with dye alone (Figure 3)[31-37]. Infrared ray has a wave length of around 805 nm. It is able to penetrate fatty tissues up to a depth from 3 to 5 mm. In brief, before the ICG injection, the gastrocolic ligament is opened using ultrasonic coagulation incision device without disrupting the gastro-epiploic vessels. After that 0.5 mL ICG (5 mg/mL; Diagnogreen; Daiichi Phar- maceutical, Tokyo, Japan) is injected endoscopically in four points of the submucosa surrounding the tumor with an endoscopic puncture needle. Twenty minutes after the injection, SN's stained with ICG were observed with the naked eye and with IREE (Olympus Optical, Tokyo, Japan).

Nimura *et al*[31] reported SNNS for gastric cancer by IREE with ICG injection for the first time. They investigated 84 patients with gastric cancer and 11 of the 84 patients had LN metastasis. All of the 11 patients were detected by IREE with ICG injection. However, SLNs detected by ICG injection alone did not include metastasis in 4 of the 11 patients. This result seems to support the usefulness of IREE with ICG injection as compared to ICG injection alone. In addition, Kelder *et al*[33] investigated 212 patients with gastric cancer who underwent SNNS by IREE with ICG injection. The detection rate and sensitivity of SLNs by IREE with ICG injection were 99.5% and 97%, respectively. Meanwhile, those of SLNs with ICG injection alone were 85.8% and 48.4%. Predominance of SLNs by IREE with ICG injection over ICG injection alone is supported by these results.

Ohdaira *et al*[32] focused on lymphatic drainage using IREE with ICG. One of the advantage of this procedure is that SNNS by IREE with ICG injection enables us to detect lymphatic vessels from the tumor easily. They investigated 161 patients with gastric cancer using IREE with ICG and revealed that the most common locations of the SNs, in each of the upper, middle and lower thirds of the stomach, were station No. 7 which is defined as LNs along the trunk of left gastric artery between its root and the origin of its ascending branch by Japanese Gastric Cancer Association. Yano *et al*[34] investigated 130 patients with gastric cancer (3381 LNs) who underwent SNNS by IREE with ICG injection and evaluated LNs by immunohistochemistry (IH) with anti-cytokeratin antibody staining. They reported that 15 patients (27 nodes) were diagnosed without metastasis by hematoxylin and eosin (HE) staining, which turned to metastatic by IH staining. However, all the 27 nodes with micrometastasis were inside the lymphatic basins. They concluded that even if LN micrometastasis is overlooked by intraoperative frozen section with HE staining, micrometastasis can be completely removed by lymphatic basins dissection.

Benefit of SNNS is not only to avoid unnecessary LN dissection but also to enable us performing local resection (LR) of stomach for patients with EGC with curability. Kitaoka *et al*[38], was the first to report the use of LR for treating early gastric cancer. Maintenance of curability and quality of life are essential to introducing LR for early gastric cancer clinically. Kawamura *et al*[39] described the usefulness of partial resection of stomach as compared to conventional gastrectomy. They assessed gastric emptying by 13C-acetate breath

test in 60 patients who underwent distal gastrectomy with Billroth I reconstruction (DGBI) in 26 patients, LR in 34 patients. For the 13C breath test, 100 mg of 13C-acetate sodium salt was mixed in a test meal. Dietary intake and body weight change were significantly more reduced in the DGBI group than the LR group. In addition, significant acceleration of gastric emptying was observed in the DGBI group compared to that in the LR group by 13C breath test. They concluded that LR is an option for selected patients with EGC.

SN mapping concept seems reasonable approach to determine appropriate indications including pylorus-preserving gastrectomy, proximal gastrectomy and LR for cT1N0 gastric cancer. In particular, laparoscopic function-preserving surgeries should be performed for patients with negative for LN metastasis confirmed by SNNS. Earlier recovery after surgery and preservation of quality of life in the late disease phases can be achieved by limited laparoscopic gastrectomy with SNNS.

CONCLUSION

When we predict LN metastasis for ECG, we need at least pathological information derived from resected tumor through ESD. In particular, lymphovascular invasion, tumor depth and tumor size are the strongest LN metastasis predicting parameters for EGC. Basic strategy of additional treatment after ESD for patients with EGC is conventional gastrectomy. However, providing less invasive surgery such as SNNS for patients with EGC has potentiality to improve the quality of life of patients after surgery by preserving gastric function as compared to conventional gastrectomy.

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REVIEW

Immune checkpoint and inflammation as therapeutic targets in pancreatic carcinoma

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Abstract

Pancreatic adenocarcinoma (PAC) is one of the most deadly malignant neoplasms, and the efficacy of conventional cytotoxic chemotherapy is far from satisfactory. Recent research studies have revealed that immunosuppression and inflammation are associated with oncogenesis, as well as tumor development, invasion, and metastasis in PAC. Thus, immunosuppression-related signaling, especially that involving immune checkpoint and inflammation, has emerged as novel treatment targets for PAC. However, PAC is an immune-resistant tumor, and it is still unclear whether immune checkpoint or anti-inflammation therapies would be an ideal strategy. In this article, we will review immune checkpoint and inflammation as potential targets, as well as clinical trials and the prospects for immunotherapy in PAC.

Key words: Immune checkpoint; Therapeutic anticancer target; Inflammation; Randomized clinical trial; Pancreatic adenocarcinoma

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Core tip: Pancreatic adenocarcinoma is recognized as one of the most malignant neoplasms, and more efficacious treatment is desired earnestly. Recent research studies have revealed that the development and progression of pancreatic adenocarcinoma are highly influenced by immune responses, and inflammation is a critical promoter of the disease. In this article, we highlighted the emergence of immunosuppression-related signaling associated with immune checkpoint and inflammation, as a novel treatment target for cancer. Furthermore, the review demonstrated that the current focus on therapeutic strategies involving combination chemotherapy,

immunotherapy, and anti-inflammation therapy might provide considerably more clinical benefits to patients than current therapies.

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INTRODUCTION

Currently, pancreatic adenocarcinoma (PAC) is recognized as one of the most malignant neoplasms. Most patients with PAC are diagnosed at an advanced, incurable stage because of the absence of routine screening and virtually no specific subjective symptoms before tumor progression. Although advances in a variety treatment approaches have improved the therapeutic management, the 5-year survival rate for patients with metastatic and recurrent PAC remains lower than 5%[1].

For over a decade, gemcitabine monotherapy has been a standard regimen for advanced PAC[2]. Recently, two pivotal studies demonstrated that FOLFIRINOX and nab-paclitaxel plus gemcitabine are more effective than gemcitabine monotherapy. However, these combination regimens prolonged the survival by only several months compared to gemcitabine with significantly increased toxicities[3].

Recent research studies have revealed that the development and progression of PCA are highly influenced by immune responses, and inflammation is a critical promoter of the disease. Therefore, targeting immunosuppressive and inflammatory signaling pathways may be a promising strategy. Here, we will review immunotherapy, especially focusing on the immune checkpoint and inflammatory signaling as potential therapeutic targets in PAC.

IMMUNE CHECKPOINT THERAPY IN PAC TO DATE

It is widely known that tumor cells escape from the host immune surveillance, and some tumors are resistant to the host immune responses. This immune tolerance is closely associated with immune checkpoints, which are inhibitory immune-related pathways that are initiated by ligand-receptor interactions between T cells and antigen-presenting cells (APCs) or tumor cells. T cells recognize tumor-associated antigens (TAAs) presented by the major histocompatibility complex class II from APCs or tumor cells through the T-cell receptor. Simultaneously, costimulatory signals are needed to determine whether T cells activate or inhibit immune responses. When the costimulatory signal is an inhibitory pathway, T cells suppress the immune response, and then tumor cells are subsequently shielded from immune surveillance. Immune checkpoint therapy blocks this inhibitory signal and overcomes the immune tolerance[4].

Among the numerous costimulatory molecules, cytotoxic T-lymphocyte antigen (CLTA)-4 and programmed cell death (PD)-1/PD-ligand (PD-L) 1 have been established as treatment targets. Monoclonal antibodies including ipilimumab, nivolumab, and pembrolizumab blockade these molecules. These agents inhibit costimulatory signals, which results in activation of cytotoxic T lymphocytes (CTLs) against tumor cells. These immune checkpoint blockade demonstrated substantial efficacy in melanoma[5], non-small cell lung cancer (NSCLC)[6], and renal cell cancer[7]. Vigorous research studies are currently ongoing in various tumors.

However, immune checkpoint therapy may not be effective in PAC, at least as a monotherapy. A phase 2 trial evaluated the efficacy of ipilimumab in advanced PAC[8], but no response was observed per Response Evaluation Criteria In Solid Tumors at a dose of 3.0 mg/kg. Furthermore, an anti-PD-L1 antibody (BMS-936559) achieved no response (0/14) in a phase 1 trial of patients with PAC[9]. Although several clinical trials are currently ongoing, single immune checkpoint blockade does not a promising option at this time.

The reason why immune checkpoint therapy is not effective in PAC is unclear, but several hypotheses have been proposed.

One possible reason is associated with PD-L1 expression. Several studies suggested that PD-L1 expression in tumor cells, detected by immunohistochemistry, is associated with responses to PD-1/PD-L1 blockade therapy[10]. However, PD-L1 is expressed in approximately 40% of pancreatic cancer cells[11]. In addition, PD-L1 expression can be induced by oncogenic signaling independent of inflammatory signaling[12]. Therefore, the level of PD-L1 expression in tumor cells alone cannot explain their resistance to immune checkpoint therapy in PAC.

The second possible reason is associated with the immune cell population in the tumor microenvironment (TME), which consists of a highly complicated interaction between tumor cells, immune cells, and stromal cells. With regard to immune cells, from the early tumorigenesis phase, myeloid-driven suppressor cells (MDSCs), tumor-associated macrophages (TAMs), and regulatory T cells (Tregs) occur in the TME. These cells deactivate immune response by various mechanisms. For example DMSCs have potent immunosuppressive properties through the production of reactive oxygen species[13], expression of arginase-1[14], depletion of cysteine[15], and suppression of CTLs. TAMs suppress the function of T cells, NKT cells and NK cells by expression of the ligand receptors for PD-1 and CTLA-4, and induce apoptosis by expression of FASL and TRAIL[16]. MDSCs

and TAMs induce Tregs in TME. Tregs suppress APCs *via* CTLA-4, secretion, secrete inhibitory cytokines (IL-10, TGF-β and IL-35), express granzyme/perforin against effector T cells, and inhibit differentiation of effector T cells by IL-2 consumption by CD25[17]. All of these functions provide immune suppressive environment. On the other hand, few effector T cells, which play essential roles in immune checkpoint therapy infiltrate into the tumor tissue[18]. This imbalanced immune cells population tends to enhance immunosuppression and interrupt immune checkpoint therapy.

The third possible reason is associated with stromal cells in the TME. An abundance of desmoplastic stroma is a distinctive feature of PAC. Desmoplastic stroma consists of cancer-associated fibroblasts (CAFs) and extracellular matrix (ECM) and immune cells. It is well known that CAFs promote tumor progression through the Hedgehog, Wnt/β-catenin, Notch, K-ras signaling and the production of growth factors[19]. In addition, CAFs secrete chemokine ligand 12 (CXCL12) and interleukin 17 (IL-17). These mediators suppress T cells *via* chemokine receptor 4 (CXCR4)[19]. The CXCR4-CXCL12 axis may be related to resistance to immune checkpoint therapy because the blockade of this signal has a synergistic effect on anti-PD-1 therapy[20].

As mentioned above, PAC induces a highly immunosuppressive environment regulated by immune cells, stromal cells, and mediators. This condition may contribute to its resistance to immune checkpoint therapy.

OVERCOMING RESISTANCE TO IMMUNE CHECKPOINT THERAPY

Comprehensive research studies have revealed strategies to overcoming the resistance to immune checkpoint therapy. One approach involves the establishment of a positive predictive biomarker for immune checkpoint therapy. Expression of PD-L1 may be one candidate predictive marker. In NCSLC, several trials have reported that the objective response rate is associated with PD-L1 expression, although conflicting evidence exists[21]. However, as mentioned above, it is unclear whether the expression of PD-L1 could serve as a useful biomarker in patients with PAC. In addition, the standard immunohistochemical tests for the determination of PD-L1, and the cut off for PD-L1 positive status have not been established yet.

Another candidate predictive marker is the mismatch repair (MMR) status of DNA. MMR deficiency leads to a high number of somatic mutations in tumors. Theoretically, accumulation of these somatic mutations can be recognized by the patient's immune system. Le *et al*[22] hypothesized that tumors with MMR deficiency are sensitive to immune checkpoint therapy, and initiated a phase 2 trial in which pembrolizumab was administered to 41 patients with or without MMR deficiency. In that trial, MMR status was assessed by microsatellite instability (MSI) analysis. Microsatellite is region of repetitive DNA, where hundreds to thousands of somatic mutations are occurred in tumor with MMR deficiency. Condition of accumulation of somatic mutations in microsatellite is referred to as MSI, and MSI reflects MMR deficiency. Of the 41 patients, 32 had colorectal cancers, which were regarded as immune resistant tumors. Eleven patients had MMR-deficient colorectal cancer and 21 had MMS-proficient forms. The remaining nine patients had MMR-deficient noncolorectal cancer. PAC was not included in the trial. They reported that the immune-related objective response was 40% in the patients with MMR deficiency, while patients without MMR deficiency did not achieved any response. Therefore, MMR status can be a useful predictive marker for pembrolizumab therapy. That is why PAC with MSI expects to be sensitive to PD-1 therapy. However, PAC with MSI is extremely rare[23] and, therefore, this enrichment strategy based on MSI examination may not be realistic.

Another approach to overcome the resistance to immune checkpoint therapy is establishment of more potent treatment. Combination immunotherapy is currently emerging as a promising treatment. However determining the most effective combinations is a challenge. Candidates for combination therapy with immune checkpoint inhibitors are (1) cytotoxic agents; (2) other immune checkpoint inhibitors; (3) direct cytotoxic T cell stimulators; (4) cancer vaccines; and (5) radiation (Tables 1 and 2). Rationales and problems of each combination therapy are discussed here.

Combination therapy with cytotoxic regimens

Originally, it was thought that cytotoxic agents impaired immunity due to myelosuppression, and combination of cytotoxic agents and immunotherapy was not desirable. Accumulating evidences suggest that cytotoxic agents affect the immune system differentially; sometimes they act on the activate tumor immunity and other times on the inactivate[24]. Gemcitabine, oxaliplatin, irinotecan, 5-FU, and paclitaxel (nab-paclitaxel) are key drugs in the treatment of PAC. Preclinical data showed that these agents have immune effects such as enhancement of cellular immunity, augmentation of dendritic cell maturation, and reduction of MDSC and Tregs (reviewed by Duffy *et al*[25]). These findings suggest cytotoxic agents may be good partners of immune checkpoint therapy. In fact, a synergistic effect was observed with the combination of ipilimumab and cytotoxic agents in a preclinical tumor model[26]. In addition, randomized phase 2 trial showed that combination ipilimumab and CP (paclitaxel and carboplatin) improved immune-related progression-free survival (irPFS) compared to CP alone in NSCLC[27].

The results of the phase 1 trial of the combination of ipilimumab and gemcitabine (NCT01473940)

Table 1 Rationales of each combination therapy

Treatments	Rationales	Concerns
Checkpoint inhibitor plus cytotoxic agents	Enhance cellular immunity	Efficacy may be influenced by timing when cytotoxic agents add
	Augment dendritic cell maturation	Severe myelosupression may interrupt immune checkpoint therapy
	Reduce MDSC and Tregs	
	Decreases CAF	
Combination with checkpoint inhibitors	Activate tumor immunity by different mannar	ir AE will increase
	Provide synergy efficacy even in immune resistant tumor	
Checkpoint inhibitor plus T cells stimulate agents	Activate tumor immunity by different mannar	Severe AE including cytokine storm may occur
	Deactive Tregs	
Checkpoint inhibitor plus cancer vaccine	Increase the presentation of taas	
	Enhance PD-L1 expression	
Radiotherapy	Enhance cross priming of ctls	Optimal schedule and dose are not established
	Enhanse abscopal effect	

MDSC: Myeloid-driven suppressor cell; CAF: Cancer-associated fibroblast; Tregs: Regulatory T cells.

Table 2 Problems of each combination therapy

Treatment	Disease	Phase	Clinical trial number	Status
Checkpoint inhibitor plus cytotoxic agents				
Ipilimumab (anti-CDLA-4)	PC	1	NCT01473940	Ongoing
Gemcitabine				
Nivolumab (anti-PD-1)	PC	1	NCT02309177	Ongoing
Nab-PTX ± gemcitabine				
Combination with checkpoint inhibitors				
Nivolumab (anti-PD-1)	TNBC, GC, PC,	1, 2	NCT01928394	Ongoing
Ipilimumab (anti-CTLA-4)	SLCL. BC, OC			
MEDI4736 (anti-PD-1)	Solid tumor	1	NCT02261220	Ongoing
Tremelimumab (anti-CTLA-4)				
Nivolumab (anti-PD-1)	Cervical cancer, BC, CRC, HN, GC,	1, 2	NCT01968109	Ongoing
BMS-986016 (anti-LAG-3)	HCC, melanoma, NSCLC			
PDR001 (anti-PD-1)	Solid tumors	1, 2	NCT02608268	Ongoing
MBG453 (anti-TIM-3)				
Ipilimumab (anti-CDLA-4)	B7-H3 expressing tumors (melanoma,	1, 2	NCT02381314	Ongoing
MGA271 (anti-B7-H3)	HN, NSCLC)			
Pembrolizumab (anti-PD-1)	B7-H3 expressing tumors	1, 2	NCT02475213	Ongoing
MGA271 (anti-B7-H3)	(melanoma, HN, NSCLC)			
Checkpoint inhibitor plus T cells stimulate agents				
Nivolumab (anti-PD-1)	Solid tumors, B-cell NHL	1, 2	NCT02253992	Ongoing
Urelumab (anti-4-1 BB)				
Pembrolizumab (anti-PD-1)	Solid tumors	1	NCT02179918	Ongoing
Urelumab (anti-4-1 BB)				
Tremelimumab (anti-CTLA-4)	Solid tumors	1, 2	NCT02205333	Ongoing
MEDI6469 (anti-OX-40				
MEDI4736 (anti-PD-L1)	Solid tumors	1, 2	NCT02205333	Ongoing
MEDI6469 (anti-OX-40)				
Tremelimumab (anti-CTLA-4)	Melanoma	1	NCT01103635	Ongoing
CP-870,893 (anti-CD40)				
Checkpoint inhibitor plus cancer vaccine				
Ipilimumab (anti-CTLA-4) + GVAX	PC	2	Ref 58	Terminated
FOLFIRINOX followed by	PC	2	NCT01896869	Ongoing
Ipilimumab (anti-CTLA-4) + GVAX				
Nivolumeb (anti-PD-1) + GVAX	PC	2	NCT02243371	Ongoing
Checkpoint inhibitor plus raditaion				
Ipilimumab + radiation	Melanoma	1	NCT01557114	Terminated
	Melanoma	2	NCT016899747	Terminated
	NSCLC	2	NCT0221739	Ongoing
	Melanoma	2	NCT01970527	Ongoing

BC: Bladder cancer; CRC: Colorectal cancer; HN: Head and neck cancer; NSCLC: Non-small cell lung cancer; OC: Ovarian cancer; PC: Pancreatic cancer; SCLC: Small cell lung cancer.

and combination therapy with tremelimumab (CTLA-4 blocking IgG2 antibody) and gemcitabine for advanced PAC were reported that both regimens were well tolerated. However, tremelimumab and gemcitabine achieved responses in only 10.5%, which appeared comparable to the effects of gemcitabine monotherapy[28]. Nab-paclitaxel, which decreases CAFs in preclinical PAC model[29], is also a promising combination candidate. A phase 1 study of combination therapy with nivolumab and nab-paclitaxel with or without gemecitabine is currently ongoing (NCT02309177). Phase 2 trial of FOLFIRINOX followed by ipilimumab with tumor vaccine for metastatic PAC is also ongoing (NCT01896869).

To develop this combination therapy, there are two challenges that need to be overcome; determining what cytotoxic agents to combine and when to use the cytotoxic agent combination. Several studies reported that the absolute lymphocyte count had a positive relationship with improved OS in patients with melanoma who were treated with ipilimumab[30]. Thus, cytotoxic agents with high myelotoxicity may not be desirable. In addition, the timing of the combination may affect efficacy. Phased combination improved the irPFS, but the concurrent combination did not, in NSCLC phase 2 trial[27].

Combination therapy with immune checkpoint signaling blockers

Although both PD-1 pathway and CTLA-4 pathway are related to the regulation of T cell function, the mechanisms of action of each are different[31]. Das *et al*[32] reported that CTLA-4 blockade leads to a proliferative signature predominantly in a subset of transitional memory T cells, whereas PD-1 blockade leads to changes in genes implicated in cytolysis and NK cell function *in vivo*. Thus, dual blockade therapy is a reasonable strategy and may have synergistic efficacy. In a melanoma model, combination blockade was related to prolonged survival, proliferation, and enhanced function of CD8+ and CD4+ cells, and increased ratio of effector T cell /Treg and MDSCs[31]. Das *et al*[32] also reported that combination blockade is associated with changes in plasma chemokine and cytokine compared to mono blockade. Lussier *et al*[33] showed that combination blockade with CTLA-4 and PD-L1 (ligand of PD-1) antibody could achieve complete control of an osteosarcoma model whereas mono blockade could not. These preclinical data suggest that combination blockade with CTLA-4 and PD-1 may have more potent efficacy, even for immune resistant cancer including PAC, than monotherapy.

Several clinical trials showed the efficacy of combination blockade therapy. A phase 3 trial of combination therapy with ipilimumab and nivolumab showed that combination therapy provided greater clinical benefits than ipilimumab monotherapy did in melanoma[34]. The same combination regimen was evaluated in a phase 1 trial for NSCLC and renal cell cancer[35,36], and phase 3 trials are currently ongoing. For other tumors including PAC, a phase 1/2 trial to evaluate the tolerability and efficacy of the same regimen tumors is currently ongoing (NCT01928394). Phase 2 trial of durvalumab (MEDI4736, anti-PD-1 IgG1 mAb) with tremelimumab (anti-CTLA-4 IgG2 mAb) for advanced PAC is also ongoing (NCT02558894).

A critical issue in this combination therapy is increased toxicity. Compared to monotherapy, combination therapy was associated with increased serious adverse events (AEs) and discontinuation of treatment due to AEs[34]. Whether this combination therapy has a good risk/benefit balance or not in PAC is unclear.

Lymphocyte-activation gene 3 (LAG-3) and T-cell immunoglobulin and mucin domain 3 (TIM3) signaling are other immune checkpoint targets. Preclinical studies showed that dual blockade of PD-1 and LAG-3[37], and PD-1 and TIM3[38] induce a synergistic effect in controlling tumor growth. These combination therapies have been evaluated in phase 1 trials such as nivolumab plus BMS-986016 (anti-LAG-3) (NCT01968109 (PAC was not included) and PDR001 (anti-PD-1) plus MBG453 (anti-TIM-3) (NCT02608268). Furthermore, recent studies implicated the B7-H3 and B7-H4 pathways in the progression of pancreatic cancer and the blockade of these signaling pathways is a novel treatment target[39]. In future, B7-H3 or B7-H4 inhibitors may become combination partners with other immune checkpoint inhibitors.

Combination therapy with direct cytotoxic T cell stimulators

The immune system is regulated both by immuno-suppressive signaling and by immunoreactive signaling. Immune checkpoint signaling is an example of immunosuppressive signals. On the other hand, active costimulatory signaling activates an immune response, which is contrary to immune checkpoint signaling. This costimulatory signaling is associated with the tumor necrosis family receptors superfamily (TNFRSF). TNFRSF proteins play an important role in B and T cell development, survival, and antitumor immune response[40]. In addition, some TNFRSFs are involved in the deactivation of Tregs[41]. Therefore, TNFRSF agonists activate tumor immunity, and their combination with immune checkpoint therapy is promising.

TNFRSF include 4-1BB, OX-40, GITR, CD27, GITR, TNFRSF25, and CD40. Several antibodies that act as TNFRSF agonist have been evaluated in clinical trials, combined with immune checkpoint therapy. Urelumab (BMS-663513) and PF-05082566 are agonistic 4-1 BB-specific antibodies. In a preclinical model, a combination of a 4-1BB agonist and PD-1antagonist enhanced the antitumor effector/memory T-cell activity. This activity was observed in a poorly immunogenic melanoma

model without severe toxicity[42]. Thus, the combination of uretinib with nivolumab or PF-05082566 with pembrolizumab is reasonable. These combinations have been investigated in phase 1 trials (NCT02253992 and NCT02179918).

OX-40 agonists can be good partners with both CTLA-4 antagonist and PD-L1 antagonist. The combination of OX-40 agonist with CTLA-4 antagonist enhanced therapeutic efficacy[43], whereas the combination of an OX-40 agonist with a PD-L1 antagonist, restored the functions of exhausted CD8+ T cells in preclinical models[44]. MEDI6469, an OX40-specific antibody, in combination with tremelimumab or MEDI4736 (an anti- PD-L1 antibody) were investigated in a phase 1 trial (NCT02205333). This study was completed in April 2016, and the results are currently awaited.

The monoclonal CD40 agonist antibody, CP-870,893 in combination with gemcitabine was well tolerated and associated with antitumor activity in patients with PAC in a phase 1 trial[45]. The combination of tremelimumab and CP-870,893 demonstrated safety and clinical activity in patients with melanoma reported in a 2015 American Association for Cancer Research (AACR) annual meeting. As far as we know, there is no clinical trial of investigating the combination of a CD40 agonist and PD-1/PD-L1 antagonist.

Targeting TNFRSF requires close patient monitoring to avoid overstimulating the immune system. Notably, treatment with TGN1412, an antibody against the CD28 receptor, led to a cytokine storm in a phase 1 trial[46].

Combination therapy with cancer vaccines

Cancer vaccines stimulate the immune system to produce tumor-specific T and B cells[47] by increasing the presentation of TAAs to the immune system. In general, cancer vaccine therapies are well tolerated because the vaccines are very specific. The most promising vaccine is GVAX, which is a whole cell vaccine composed of two irradiated cancer cell lines and engineered to express GM-CSF. GVAX induces movement of effector T cell to the TME and PD-1/PD-L1 mediated signaling. Thus, immune checkpoint therapy and GVAX have synergistic antitumor effects. This combination was compared to single ipilimumab for advanced PAC in a randomized phase 2 trial. The combination therapy achieved CA19-9 biochemical response and prolonged the patient survival, although not significantly[48]. Multicenter phase 2 study is ongoing, in which FOLFIRINOX followed by ipilimumab with GVAX is being compared to FOLFIRINOX alone.

PD-1 blockade therapy and GVAX may be more a desirable combination than ipilimumab and GVAX, regarding efficacy and toxicity. Preclinical data showed that GVAX enhances PD-L1 expression, PD-1/PD-L1 blockade with GVAX (with cyclophosphamide) overcomes the immunosuppressive situation including Treg and CTLA4 expression on T cells[49]. In a neoadjuvant setting, the efficacy of GVAX (with cyclophosphamide) with or without nivolumab was compared in phase 1/2 trial (NCT02451982). In addition, a phase 2 trial of GVAX vaccine (with cyclophosphamide) and CRS-207 with or without nivolumab in advanced PAC is ongoing.

Moreover, triple therapy, PD-1 and CLTA-4 blockade with GVAX was better than dual blockade therapy was in a preclinical setting[50]. As far as we know, this triple therapy has not been translated to the clinical setting.

Combination therapy with radiotherapy

Radiotherapy is a good candidate combination partner for immune checkpoint therapy for two reasons. First, radiation facilitates the cross-priming of CTLs, and secondly, radiation has an abscopal effect.

Radiation enhances cross-priming of CTLs in two ways. When radiation induces tumor cell apoptosis, calreticulin is displayed on the surface of tumor cells, which acts as an "eat me" signal to DCs. This signal facilitates the cross-priming of CTLs. In addition, radiation induces the release of danger-associated molecular patterns (DAMPs) including ATP and HMGB-1. These DAMPs are endogenous immune adjuvants that stimulate DC activation, facilitating cross-priming of CTLs[51].

The abscopal effect is a phenomenon in which a primary tumor is irradiated and a response is observed at distant metastatic sites outside of the radiation field. The mechanism of this phenomenon is not understood completely, but it may be mediated by immunologic mechanisms, especially T cells[51]. The abscopal effect was reported in several types of cancer including melanoma, lymphoma, and renal cell carcinoma. In PAC, the abscopal effect was reported in a xenograft model[52].

Synergistic effects of immune checkpoint blockade and radiation were reported by Dewan *et al*[53]. CTLA-4 blockade acts synergistically with radiation to induce an abscopal response in preclinical models of poorly immunogenic cancers. In addition, Postow *et al*[54] reported a case of the abscopal effect in an advanced melanoma patient treated with ipilimumab and radiotherapy. The efficacy of radiotherapy with immune checkpoint therapy has been evaluated in melanoma, castration-resistant prostate cancer, and NSCLC. A randomized phase 3 trial compared radiation with ipilimumab to radiation alone was conducted in prostate cancer, but there was no significant difference in OS between the two arms[55]. Other trials are ongoing. Optimum radiation schedule, dose, or risk benefit balances of combination therapy remain unknown.

INFLAMMATION IN PANCREATIC CANCER

Inflammation contributes to carcinogenesis and tumor progression. It is widely known that PAC is

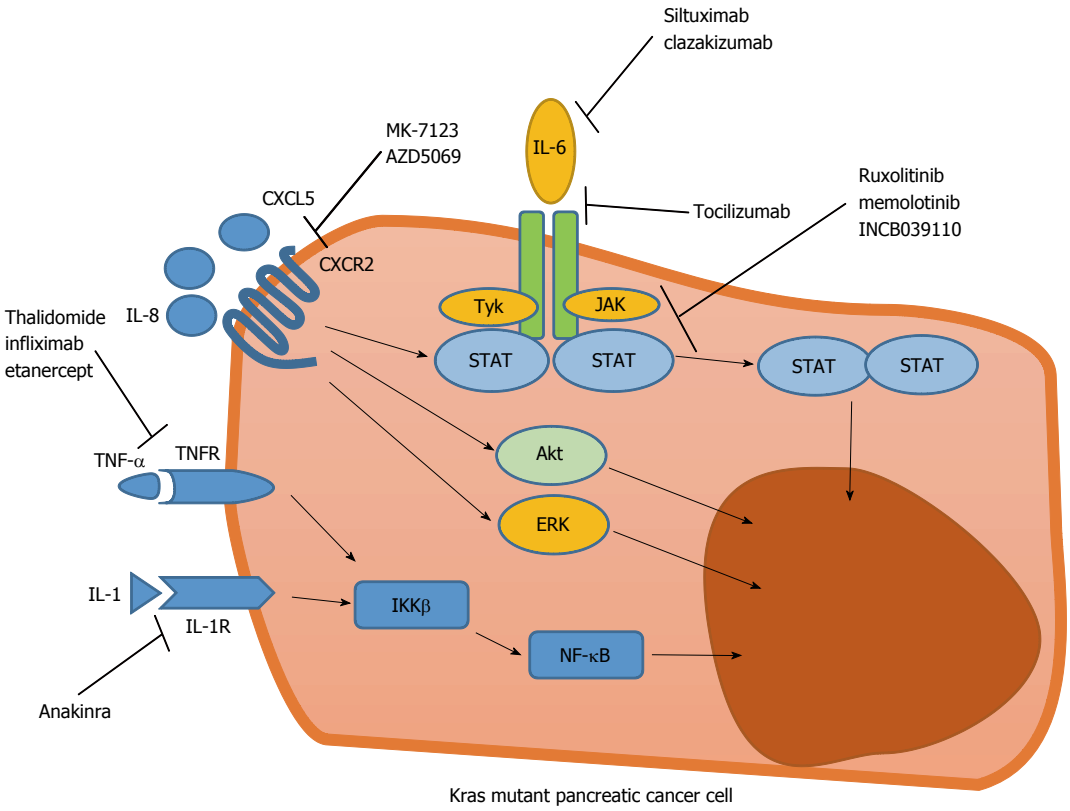

Figure 1 Various inflammation-associated signaling pathways activated in Kras mutant pancreatic cancer cells, as novel treatment targets. CXCL5: C-X-C motif chemokine ligand 5; ERK: Extracellular signal-regulated kinase; IL: Interleukin; JAK: Janus kinase; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; STAT: Signal transducer and activator of transcription; TNF: Tumor necrosis factor; TNF-R: TNF-receptor.

associated with both systemic and TME inflammation. The influence of systemic inflammation has been investigated in the clinical setting. On the other hand, the importance of inflammation in the TME has been implicated mainly in preclinical models.

Multiple large studies demonstrated that elevated systemic inflammatory markers are a negative prognostic factors in various cancers. This negative impact is particularly strong in patients with PAC. We previously reported that elevated C-reactive protein and pentraxin 3 were prognostic factors in of advanced PAC[56]. In addition, CRP and hypoalbuminemia are the defining measures used by the modified Glasgow Prognostic Score (mGPS)[57]. The validation of mGPS has been examined in more than 60 studies and over 30000 patients across multiple tumor types and clinical settings[58]. Systemic inflammation is also related to malignancy-associated symptoms including cachexia, muscle loss, poor performance status, fatigue, cognitive dysfunction, and reduced quality of life[59].

On the other hand, inflammation in the TME is associated with numerous tumor-promoting effects including enhancement of proliferative signaling, resistance to apoptosis, enhancement of angiogenesis[60], and modulation of antitumor immunity to support immune evasion[61]. Inflammation in the TME consists of crosstalk between tumor, stromal, and immune cells. Crosstalk is conducted by inflammatory mediators. Thus, inflammatory mediators are considered potential treatment targets. These target include the activating oncogene (Kras), tumor suppression gene (tumor protein p53 [*TP53*] and mothers against decapentaplegic homolog 4 [*SMAD4*]), chemokines (CXCR2 ligands), cytokines (interleukin [IL]-6 and IL-1), and downstream effectors (STAT3 and nuclear factor kappa-light-chain-enhancer of activated B cells [NF-κB], Figure 1).

CXCR2 and CXCLs

Chemokines are small-molecular-weight cytokines. Chemokines and their receptors play a role as inflammatory mediators. In malignant conditions, CXCLs and CXCR2 are of particular importance. CXCR2 is a G-protein-coupled cell surface chemokine receptor commonly found on neutrophils. In normal physiology, CXCR2 signaling is important for neutrophil migration in acute inflammation or wound healing. Ligands of CXCR2 (CXCLs) are ELR motif positive chemokines. CXCLs include CXCL-1, 2, 3, 5, 6, and 8 (IL-8).

CXCR2 signal is upregulated during the primitive stage of PAC development, and this signaling maintains the feed-forward loop in PAC cells and this autocrine effect of CXCR2 signaling promotes transformation and progression of PAC[62]. In addition to inducing direct effects, CXCR2 signaling also promotes the progression of PAC indirectly to affect the TME[63].

CXCLs also play important roles. CXCL5 and

CXCL8 expression in tumors are elevated[64], and overexpression of CXCL5 is associated with poor survival[65]. CXCL5 also activates several pathways including the protein kinase B (Akt), extracellular signal-regulated kinase (ErK), and STAT in human endothelial cells[65].

Moreover, Kras mutation is the most important oncogene-related factor in PAC, and Kras presents in more than 90% of PAC from the early stage[66]. Kras signaling involves various downstream effectors including Raf/Mek/Erk, PI3K/Pdk1/Akt, and the Ral guanine nucleotide exchange factor pathway[67]. Kras also plays important roles in inflammatory responses. When mutant Kras expression remains activated, the stimulation of the hedgehog signaling pathway, upregulation of inflammatory mediators IL-6, STAT3, and cyclooxygenase (COX)-2 are observed. In addition, when Kras becomes inactivated, the expression of these inflammatory mediators decreases[68]. These findings suggests that Kras mutation is related to the coordination of the inflammatory response in PAC.

Although oncogenic Kras is required for initiation, maintenance and progression in PAC, pharmacological inhibition of KRAS continues to be challenging. Recent studies have revealed the association between CXCR2 signaling, Kras mutation, and tumor progression. Activated Kras enhances CXCLs in pancreatic epithelial cells[69]. Preclinical models also showed that inhibition of CXCR2 signaling regulates Kras-induced autocrine growth of PAC and disrupts the tumor-stromal interaction, suppresses metastases and improves survival in preclinical models[62]. These findings suggest CXCR2 signaling is a surrogate target of Kras in PAC.

Interestingly, CXCR2 inhibition may enhance the efficacy of PD-1 blockade therapy. Steele *et al*[70] and Highfill *et al*[71] reported that combination therapy with anti-CXCR2 and anti-PD-1 improved survival compared to anti-PD-1 monotherapy in a preclinical model. The reason for this synergistic effect is thought to be the inhibition of CXCR2, which prevents MDSCs trafficking to the TME and increases activated CD8+ cells within the tumor.

However, no clinical trial has evaluated the efficacy of CXCR2 signaling blockade in malignant tumors. Several CXCR2 antagonists were evaluated in clinical trials for chronic obstructive pulmonary disease[72].

IL-6

IL-6 is a proinflammatory cytokine produced by various cells including macrophages, hepatocytes, and pancreatic cancer cells[73]. In PAC, IL-6 plays a multifunctional role in the development and progression of PAC by directly affecting the tumor cells as well as modulating the TME[74]. Although several molecules including mesothelin and receptor for advanced glycation end products influence IL-6 expression, the most important enhancer of IL-6 is Kras[75]. IL-6 signaling activates Janus kinase (JAK)-1 and JAK2, which leads to the phosphorylation of STAT-1 and 3, which plays an important role in tumor growth, survival, angiogenesis, and metastasis. In addition, IL-6 signaling activates the Ras-mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)-Akt signaling cascades[76], which is associated with antiapoptotic and tumorigenic functions. Furthermore, IL-6 contributes to creating a pro-tumorigenic TME by enhancing the expression of IL-10, IL-13, IL-5, IL-7, and GM-CSF[77]. In patients with PAC, high serum IL-6 was related to cachexia, progression to an advanced stage, and poor survival[78]. Thus, IL-6 signaling has emerged as a treatment target of PAC.

Siltuximab (CNTO328) is a chimeric anti-IL-6-monoclonal antibody, which binds strongly to IL-6 and neutralizes its activity and, thereby, promotes tumor cell death. The safety and efficacy of monotherapy with siltuximab were evaluated in phase 1/2 trials. Because IL-6 has a significant role in mutant Kras-driven tumorigenesis, patients with Kras-mutant tumors including PAC, NSCLC, colorectal, head, and neck cancers received siltuximab in the phase 1 expansion and phase 2 cohorts. Unfortunately, no objective response was observed in any of the 84 patients. Therefore, no further trial is planned with this drug for solid tumors[79]. However, siltuximab was approved for the treatment of human immunodeficiency virus negative and human herpes virus (HHV)-8 negative multicentric Cattleman's disease by the United States Food and Drug Administration (FDA) in 2014[80]. In addition, siltuximab is a promising agent for the treatment of B-cell non-Hodgkin's lymphoma and multiple myeloma.

Tocilizumab is a humanized antibody that acts as an antagonist of the IL-6 receptor while clazakizumab is a glycosylated anti-IL-6 monoclonal antibody. Both agents have shown efficacy in the treatment of rheumatoid arthritis[81]. Currently, there are no ongoing clinical trials to evaluate the efficacy of both agents in solid tumors. However, anti-IL-6 and anti-IL-6R antibodies may be useful in cancer-related cachexia. Ando *et al*[82] reported a case of a patient with lung cancer who had cachexia, and whose symptoms were rapidly palliated by the use of tocilizumab[82]. Clazakizumab was well tolerated in a phase 2 trial (NCT00866970), and it improved the tumor-related symptoms of patients with NSCLC[83].

In PAC, the sources of IL-6 are myeloid cells[84] and CAFs[76]. These cells are components of the TME. Recent studies revealed that a somatostatin analog SOM230 (pasireotide) inhibits protein synthesis in activated CAFs, and decreases IL-6 secretion[85]. Thus, targeting CAFs is another strategy to regulate IL-6. At the ASCO 2015 annual meeting, the results of a phase 1 trial were reported showing that the combination of SOM230 with FOLFIRI was well tolerated for gastrointestinal malignancies including PAC. In addition,

combination of SOM230 LAR and gemcitabine was also well tolerated and achieved a disease control rate of 68% for patients with advanced PAC[86] .

NF-κB

NF-κB is a transcription factor that plays critical roles in inflammation, cell production and differentiation, immune responses, and cancer[87]. In cancer cells, the oncogenic role of NF-κB includes the promotion of cell proliferation, control of apoptosis, stimulation of angiogenesis, and metastasis. NF-κB transcriptional factor is constitutively activated in most patients with PAC[88]. Kras mutation in PAC induces the secretion of the cytokine IL-1α, which further leads to the ubiquitination of the TNF receptor-associated factor 6 (TRAF6) and activation of inhibitor of nuclear factor kappa-B kinase subunit-β. This further activates NF-κB to induce its target genes including the protein p62, which in turn positively regulates TRAF6 ubiquitination and promotes the constitutive NF-κB. This feed-forward loop leads to PAC development[89]. In addition, NF-κB functions as a crucial link between pancreatic inflammation and PAC.

Several previous trials examined the role of anti-TNF directed therapy in the treatment of pancreatic cancer and cachexia, and the efficacy of anti-TNF targeted therapy in PAC was controversial. Thalidomide is an immunomodulatory agent known to decrease TNF levels. A randomized and double blind phase 2 study of thalidomide demonstrated an improvement in weight and lean body mass at 8 wk compared to the placebo[90]. In contrast, a placebo-controlled randomized phase 2 study of gemcitabine and infliximab, a monoclonal antibody that blocks TNF, did not show a benefit in preserving lean body mass or survival[91]. Etanercept is a recombinant human TNF receptor that specifically binds to soluble TNF and biologically inactivates it by blocking its interaction with cell surface TNF receptors. Recently, the safety and efficacy of etanercept combined with gemcitabine were evaluated in a phase 1/2 study[92]. This combination was well-tolerated; however, etanercept did not show a significant enhancement of the activity of gemcitabine[92]. These results imply that targeting TNF alone is not sufficient for an antitumor response or reversal of cachexia.

Another proposed target is IL-1. Aanakinra is a recombinant, non-glycosylated synthetic form of the human IL-1β receptor antagonist. This agent has been approved by the FDA for the treatment of neonatal-onset multisystem inflammatory disease and rheumatoid arthritis. In a preclinical model, anakinra significantly decreased NF-κB, and its co-administration with gemcitabine reduced the tumor burden[93]. Several early clinical trials to evaluate the safety and efficacy of anakinra are currently ongoing. For PAC, treatment with anakimura plus a conventional cytotoxic regimen are under investigation (NCT02550327, NCT02021422) as well.

JAK/STAT

The JAK/STAT pathway is an emerging and promising treatment target of PAC. JAKs are a family of cytoplasmic tyrosine kinases that consist of four members, JAK1, JAK2, JAK3, and Tyk2. In addition, the STATs, which are a family of downstream transcription factors for JAKs[94], have dual roles as cytoplasmic signal transduction molecules and nuclear transcription factors. Both intrinsic and extrinsic pathways activate JAK/STAT signaling. Abnormalities of the JAK/STAT pathway contribute directly to cellular transformation, increased cell proliferation, apoptosis, and angiogenesis in cancer. In addition, STAT3 induces the expression of various cytokines, chemokines, and other mediators including IL-6 and COX-2, which are associated with cancer-promoting inflammation. Importantly, the receptors for numerous cytokines, chemokines, and mediators in turn further activate STAT3, thereby forming autocrine and paracrine feed-forward loops that result in a stable change that promotes cancer-related inflammation[95].

Among the several JAK/STAT signaling targeted drugs, the development of ruxolitinib is the most advanced. Ruxolitinib is a potent JAK1/JAK2 inhibitor and deregulator of JAK/STAT signaling. In a randomized phase 2 study, 127 patients with metastatic gemcitabine-refractory PAC were administered capecitabine plus either ruxolitinib or a placebo. In the intent-to-treat population, the addition of ruxolitinib to capecitabine did not demonstrate any significant improvement in the OS or PFS. However, in a prespecified subgroup analysis of patients whose serum CRP level was greater than that of the study population median, the OS was significantly longer in the combination arm compared to the monotherapy arm. A post hoc analysis indicated that ruxolitinib achieved a longer OS in patients with high mGPS, representing a more severe systemic inflammation. Two randomized, double-blind phase 3 trials with ruxolitinib or placebo in combination with capecitabine in patients with PAC patients who failed to respond to first-line chemotherapy (the JANUS 1 and JANUS 2 Studies) are currently ongoing. Based on the previous phase 2 trial, the selection criteria in these studies included an mGPS of 1 or 2 (NCT02119663, NCT02117479). A phase 1b study of the safety and tolerability of ruxolitinib in combination with gemcitabine with or without nab-paclitaxel in advanced solid tumors is also currently ongoing. (NCT01822756). Treatments with other novel JAK inhibitor are also being investigated.

CONCLUSION

Various research studies have revealed that immunosuppression and inflammation play critical roles in oncogenesis, development, invasion, and metastasis in

PAC. Understanding the complicated crosstalk between the immune and stromal cells would certainly lead to the development of effective treatment strategies. However, single immune checkpoint therapy may not achieve the desired clinical benefits. Combination therapy with immune chokepoint blockers and other agents or anti-inflammation targeted therapy are expected to provide considerable clinical benefits to patients with PAC.

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REVIEW

New devices and techniques for endoscopic closure of gastrointestinal perforations

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Abstract

Gastrointestinal perforations, which need to be managed quickly, are associated with high morbidity and mortality. Treatments used to close these perforations range from surgery to endoscopic therapy. Nowadays, with the development of new devices and techniques, endoscopic therapy is becoming more popular. However, there are different indications and clinical efficacies between different methods, because of the diverse properties of endoscopic devices and techniques. Successful management also depends on other factors, such as the precise location of the perforation, its size and the length of time between the occurrence and diagnosis. In this study, we performed a comprehensive review of various devices and introduced the different techniques that are considered effective to treat gastrointestinal perforations. In addition, we focused on the different methods used to achieve successful closure, based on the literature and our clinical experiences.

Key words: Gastrointestinal perforations; Devices; Techniques; Endoscopic closure; Treatment

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Core tip: We introduce and discuss endoscopic devices and techniques used to treat gastrointestinal perforations, based on the literature and our clinical experiences. Endoscopists should avoid causing perforations, especially during therapeutic procedures. Sometimes, an intentional perforation is necessary for the complete removal of a tumor. However, the integrity of the mucosa should be considered, and the retained mucous membrane could contribute to the

effective closure of the perforation after full-thickness resection. We also provide advice for choosing the appropriate method to close perforations effectively.

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INTRODUCTION

Gastrointestinal perforation is defined as the presence of gas or luminal contents outside the gastrointestinal tract. In recent years, the high incidence of gastrointestinal perforations has received more and more attentions. The absolute number of perforations is likely to increase because of the widespread implementation of endoscopic screening programs and the expansion of the indications for endoscopic therapy[1]. Gastrointestinal perforations can be caused by a number of factors, such as iatrogenic factors, spontaneity, foreign body, trauma and surgery[2-4]. Among these etiologies, iatrogenic factors contribute most to the increased incidence of perforations. These iatrogenic factors include endoscopic examination, endoscopic mucosal resection (EMR), endoscopic submucosal dissection (ESD), peroral endoscopic myotomy, endoscopic retrograde cholangiopancreatography (ERCP), stricture dilation, foreign body removal and malignant tumors[2,5-9]. The incidence of iatrogenic perforations varies in diagnostic and therapeutic endoscopy, ranging from 0.029% to 5%[10,11]. With the developments of new devices and techniques, endoscopic closure has been considered as the primary method and should be a priority option compared with surgery or conservative treatments[12-14]. Endoscopic closure has advantages, such as high success rate, minimally invasion, short hospitalization and low medical expense. Based on the latest studies, endoscopic devices and techniques are considered to be a safe and effective measure to close gastrointestinal perforations[15-27]. In our previous work, we used multiple-band ligators to repair successfully a lateral duodenal ERCP-related perforation[27]. The overall rate of successful endoscopic closure has been reported as approximately 89.9%, with different devices and techniques having success rates ranging from 87.5% to 100%[4]. Many studies have described various kinds of endoscopic devices and techniques. However, there is no systematic introduction to the latest developments in devices and techniques. Recently, we reported two new techniques to close a perforation and used them successfully. In this study, we introduce and summarize different endoscopic devices and techniques based on the latest research and our previous experience. We hope to provide advice for physicians that allows them to choose the appropriate method to close perforations effectively and improve the success rate of endoscopic closure.

NEW DEVICES AND TECHNIQUES

Endoclip techniques

Clipping techniques for the endoscopic closure of gastrointestinal perforations are the most common treatment methods[11,28,29]. From treating gastrointestinal bleeding to perforations, endoscopic clips have an increasingly important role. Conventional endoscopic clips, also known as through-the scope clips (TTSC), can effectively close perforations of the esophagus, stomach, duodenum and colon[5,6,9,19,30-32]. There was no failure in three patients with esophageal perforations following EMR managed by endoclips, as reported by Shimizu *et al*[5]. Sekiguchi *et al*[33] had also reported complete endoscopic closures of gastric perforations[34,35]. Attention should be paid conservatively to those patients with a medical history of laparotomy, because closure failed in one such patient[33]. Yang *et al*[36] demonstrated a success rate of 95.5% with effective clipping for colonoscopy-associated perforations. When the tissues around the edge of defect were inflamed or indurated, closing the perforation using endoclips may be difficult.

A combination of clips and other devices and techniques is used common to close certain special perforations. Tanaka *et al*[37] applied clips and a detachable snare to close a large esophageal perforation that was difficult to manage using alone[37,38]. Using a two-channel scope, clips were placed at equal distances to fix the detachable snare around the defect. When the rubber stopper was tightened, the perforation was closed successfully[38]. Endoloop and metallic clip interrupted sutures have also been used to close gastric perforations. Shi *et al*[39] proved that using an endoloop and metallic clip interrupted suture to repair gastric defects resulting from endoscopic full thickness resection (EFTR) was safe, easy and feasible. The endoloop was anchored to both sides of the defect using two clips. After the maneuver was repeated sufficiently around the defect, the endoloop was tightened, closing the defect. This method proved to be safe and quick, with the only side effects being slight abdominal pain and fever in the early days of recovery.

Large mucosal defect areas usually occur during ESD. A new closure device, named the loop clip, was designed to close large mucosal defect after ESD[40]. The loop clip is anchored to the edge of the mucosal defect at the distal side, and then a normal clip is inserted and attached at the proximal side, after which the nylon loop attached to the loop clip is first grasped. The first conventional endoscopic clip is placed beside the deployed loop clip to bring the distal edge to the proximal side. Next, a second clip is placed beside

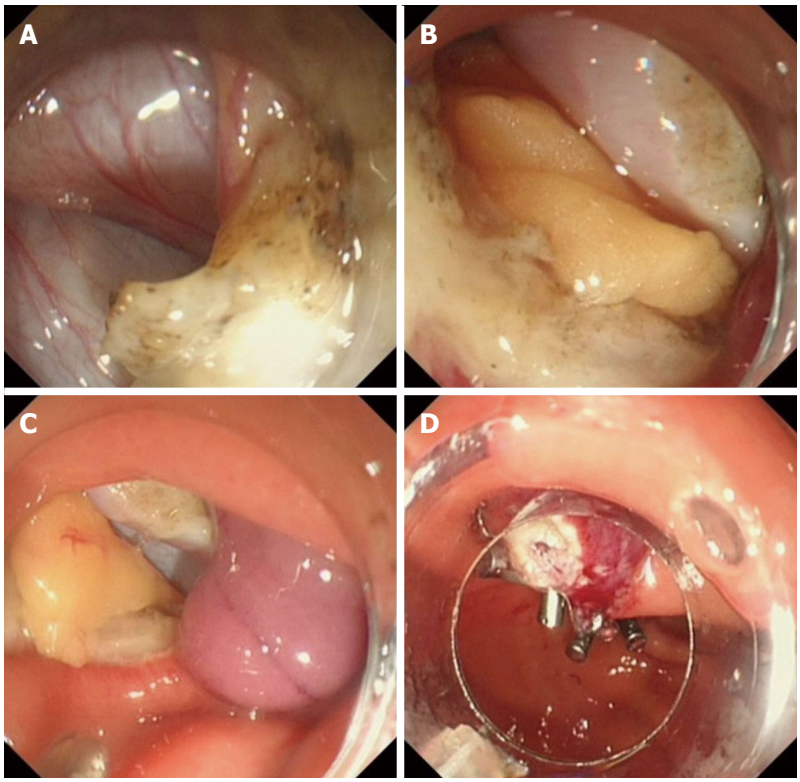

Figure 1 Over the scope clip system and clips applied to make a successful closure. A: When endoscopic submucosal dissection (ESD) was performed for a lesion located in gastric antrum, a large perforation occurred, which was about 30 mm × 15 mm; B: The omentum majus could be seen through the perforation; C: It was difficult to make complete closure using only endoclips, and the over the scope clip (OTSC) system was applied to close the perforation. However, only partial closure was achieved because the perforation was too large; D: Eight endoclips were then used to make a complete closure.

the loop clip on the opposite side of the first clip. This operation is repeated until the whole defect is closed.

Over the scope clip system

Unfortunately, gastrointestinal perforations are sometimes too difficult to manage using endoclip techniques or nylon loops. The over the scope clip (OTSC) system can be more effective than TTSC in an emergency or in a complex situation. OTSC is more suitable for larger defects and is more effective for closing a fistula. The principle of OTSC system depends on its twin grasper. When the graspers are released to grasp the sides of the lesion, they are retracted completely and the perforation is closed. The advantages of OTSC system were demonstrated in the case report by Ono *et al*[8]. Using the OTSC system, they closed a esophageal perforation that involved esophageal stenosis. Although the OTSC system is useful to solve difficult cases, it does have certain limitations. It is hard to remove the clips once they are placed, and the grasper is not flexible enough to rotate. We usually apply the OTSC system in complex situations, for example if the perforation is large or is difficult to manage by endoclips. Recently, we closed a large perforation successfully during ESD. The submucosal tumor (SMT) was about 20 mm and originated from the deep muscularis propria layer. To remove the tumor completely, we made a full thickness resection and a large perforation occurred. OTSC and clips were then applied to achieve a successful closure (Figure 1).

All of these new devices and techniques have good efficacy for closing different large and complicated perforations. However, whether they can be used widely in the clinic treatment or whether further clinical studies are required remains a mater of debate.

How to choose an appropriate clipping technique to treat a perforation? Normal endoclips are suggested for use when the diameter of the perforation is less than 10-20 mm. OTCS clips are suitable to close a perforation with a diameter less than 30 mm or when the edge of mucosa is swollen and invaginating. Larger perforations can be closed effectively by a combination of clips and other devices or techniques.

In a conclusion, compared with other endoscopic devices and techniques, clipping techniques are used much more commonly to close perforations. They have become the first choice for most situations that involve closing a perforation, instead of surgery or conservative treatment[41].

Self-expanding metal and plastic stents

Recently, temporary stent placement has emerged as another endoscopic treatment for patients with perforations. There are two main types of stents with different shapes: fully covered self-expanding metal/

plastic stents (fSEMS/ fSEPS) and partially covered self-expanding metal stents (pSEMS)[2,20,23,42]. Fully covered ones have the advantages of good drainage and closure of the perforation without obvious complications[23,43]. Unfortunately, they nearly all of these types of stents have a high migration rate that delays the recovery[43-45]. pSEMS have a very low migration rate compared with the fully covered ones. Nevertheless, it is a great challenge to remove them because of tissue embedding[46]. Nevertheless, they provide a further choice of method for patients and in some situations they function better than other devices and techniques when used correctly. From the literature and our clinical experiences, stents are a good choice for esophageal perforations with stenosis, where the defect's diameter is more than 20-30 mm and there are malignant lesions around the defect. Gastric perforations near the pylorus, perforation caused by dilating an anastomotic stricture and perforation that are not close to the duodenal ampulla are suitable for stent treatment.

Kumbhari *et al*[20] reported that an iatrogenic pharyngoesophageal perforation was closed by fSEMS after three days. The lack of working space and risk of pulmonary aspiration made it almost impossible to apply endoscopic clips, OTSC or endoscopic suturing. This case highlighted the importance of using stents instead. Ribeiro *et al*[21] closed a large fistula perfectly using a combination of fibrin glue, a partially covered stent and a biological patch. Stents are much more effective for esophageal perforations, having a 100% success rate from a technical aspect. Generally speaking, when stents were used to treat upper gastrointestinal perforations, anastomotic leaks and fistulas, the success rate ranged from 65% to 88%, with different migration rates[23].

Endoscopic band ligation

Endoscopic band ligation (EBL) is one of the first-line choices for the management of gastroesophageal varices and variceal hemorrhage in cirrhosis[47]. EBL is also safe and effective to treat dieulafoy lesions and diverticular bleeding[48-50]. Furthermore, EBL is also shows promise as an effective and safe treatment for gastric small gastrointestinal stromal tumors[51-53]. In recent years, EBL has been used to close GI perforations, such as gastric, duodenal, colonic and rectal perforations. In most cases, EBL is an alternative choice to close those perforations after failure of metal clips. According to studies worldwide, perforations could be closed with a very high success rate when EBL was applied. EBL is easy, safe, quick and effective. Lee *et al*[26] compared the EBL technique with endoclips for the closure of colonic perforations. Closure by EBL was faster than closure by endoclipping (3.2 ± 1.7 min *vs* 6.8 ± 1.3 min, $P < 0.01$). Our group reported the successful closure of a lateral duodenal perforation by EBL after endoscopic clipping failed because of the fragile edge of the tear[27]. Although the endoscopic management of a duodenal perforation is much more difficult than the others, the patient's perforation was closed perfectly by the EBL technique, without any symptoms after six months. We revealed that EBL might be easier and faster than endoclipping, and could be considered as the primary repair method for duodenal perforations[27].

Han *et al*[54] carried out a case study to evaluate the clinical efficacy and safety of EBL in gastric perforations when endoclips closure failed. Successful closures were achieved in all cases. However, the number of patients was limited and the study was not a comparative study. Han *et al*[55] also reported similar case studies for colon perforations and obtained the same results. Moon *et al*[56] also used the EBL technique to close a rectal perforation caused by ESD, with reasonably good results.

There is no doubt that new devices and techniques have limitations. EBL may prolong the hospital stay by binding together more tissue than required. In addition, it can cause injury to adjacent organs[25,26,54]. Further studies and developments are needed to expand EBL's clinical use.

Biological glue

Among numerous tissue sealants, biological glue, a mixture of fibrinogen and thrombin, is used widely[2,3,57]. Originally, the fibrin glue was used in the area of gastroduodenal ulcer bleeding, wound healing and bleeding caused by resections of the gastrointestinal tract[58]. Fibrin glue can form a clot *in vivo* and can be fully reabsorbed by macrophages after approximately two weeks[59,60]. The fibrin glue can promote the growth of proliferating cells and increase the number of microvessels[61]. In addition, the use of fibrin glue induces the upregulation of growth factors' expressions, which contribute to healing defects and stopping bleeding[61,62].

Many recent studies have verified the efficacy of biological glue to close GI defects. Kotzampassi *et al*[57] gained a 96.8% success rate among 63 patients with anastomotic leaks. Mutignani *et al*[59] obtained similar results using for fibrin glue to treat GI perforations. All six patients in his study had refractory post-ERCP bleeding and were treated with fibrin glue. The study revealed that fibrin glue might provide a new therapeutic choice to cure ERCP-related type 1 perforations after the failure of clipping techniques[59]. Biological glue is mainly applied to close fistulas and leaks. However, its use to close GI perforations has been rarely reported and deserves further exploration.

To improve the efficacy of biological glue, Doyama *et al*[63] adopted a treatment comprising polyglycolic acid (PGA) sheets, fibrin glue and clips. This technique solved the problems of gravitational influence on PGA and the weakness of clips to close larger defects of more than 30 mm in diameter[32]. Although biological glue seems inspiring and easy to apply, it does have disadvantages. Whether biological glue is suitable

for retroperitoneal perforations and how to handle emergencies involving fibrin clot infection remain unknown. In addition, the frequent use of fibrin glue might increase surgical difficulties[59,60].

Innovative endoscopic devices for suturing

At present, there are a number of innovative endoscopic devices for suturing. The Bard EndoCinch suturing device (Davol, Cranston, RI, United States)[64] is still used commonly. However, most of the suturing devices developed in last two decades are cumbersome and expensive, and more and more physicians are searching for simple but useful suturing devices[65].

Bergström *et al*[65] conducted a clinical study using a new, simple stitching technique. The technique relied on two threads in the tissue on each side of a defect, and then the stitching technique locked the threads and finally the defect was closed perfectly[65-67]. It was used successfully to treat three patients with a duodenal perforation, an upper-GI vessel leak and an anastomosis leak. This technique does not need multiple and complicated devices to close the perforation and is more effective[65,66]. Nevertheless, its limitations are obvious. It takes more time to finish the operation and it is clumsy to suture in the gastric fundus, suggesting that this device needs further improvement. Moran *et al*[68] designed a cap-type suturing device based on natural orifice transluminal endoscopic surgery (NOTES)[69,70]. This suturing device could have a great ability to close full-thickness perforations effectively and efficiently compared with most of the other endoscopic devices[68,71,72]. The device consists mainly of a dual channel, a tissue retractor or a grasping forceps, and a detachable needle tip attached with suture material, making it easy to use. After the forceps grasp one side of the defect, the reloaded needle tip is passed through the tissue surface and then the same operation is done on the other side of the defect. When the grasping forceps are retracted, the suture ends are pulled tight to close the defect. In Moran *et al*[68]'s study, it was effective and universally adaptable to almost all kinds of endoscopes, providing additional suture choices. This technique requires considerable technical skill, for example, having a good command of the methods for tying a knot. Mori *et al*[73] performed a study on 30 excised swine stomachs to investigate properties of their innovative devices termed the double-arm-bar suturing system (DBSS). The results showed that the efficacy of DBSS was nearly equal to hand-sewn sutures. Although better efficacy was achieved in the OTSC group, according to the statistical results, DBSS performed much better at closing perforations larger than 20 mm.

Although many useful and innovative endoscopic devices for suturing have been reported, large-scale clinical applications have not yet been carried out and the long-term safety and efficacy of these devices require further evaluation.

As mentioned above, most of gastrointestinal perforations are iatrogenic and therapeutic endoscopy-related perforations are the most important factors. The integrity of the gastric mucosa is not consciously emphasized in the current endoscopic resection, which usually leads to mucosal defects and perforations. Recently, we proposed a new method, termed endoscopic mucosa-sparing lateral dissection, to remove SMTs, which not only retains an intact mucous membrane, but also provides a good operating field during the procedure. In addition, the retained mucous membrane contributed to the effective closure of the perforation after full-thickness resection for a tumor that originated from the deep layer[74]. In our experience, endoscopists should avoid perforation consciously, especially during therapeutic procedures. Sometimes, intentional perforation is necessary to completely remove the tumor. However, the integrity of the mucosa should be considered because a retained mucous membrane contributes to successful closure. To date, we have applied this method to close large perforations in four patients, and all the perforations were successfully managed (Figure 2).

CHOICES OF ENDOSCOPIC DEVICES AND TECHNIQUES IN PATIENTS WITH DIFFERENT GASTROINTESTINAL PERFORATIONS

Esophageal perforations

The European Society of Gastrointestinal Endoscopy (ESGE) suggests that endoscopic dilations, mucosal resection/submucosal dissection and foreign body removal should be considered to carry an increased risk of esophageal or gastric perforation[11]. Most esophageal perforations are iatrogenic. As mentioned above, there are many methods to close esophageal perforations. TTSC is suitable for closing defects with a diameter less than 10 mm and OTSC performs well in closing perforations with swollen and everted edges or those with a diameter less than 30 mm. In addition, esophageal stents show great advantages in handling perforations with malignant lesions or stenosis. Endoscopic devices for suturing can be applied for lesions less than 20 mm. Particular attention should be paid as follows: (1) The use of endoscopic techniques may be challenging in the proximal esophagus because of space constraints and patient intolerance; a conservative treatment should be considered in stable patients; (2) stent fixation with clip application or suturing techniques may be useful to prevent migration of the stent; and (3) fibrin glue application has been reported for the closure of esophageal perforations, but experiences are limited.

Gastric perforations

Gastric perforations are most often related to

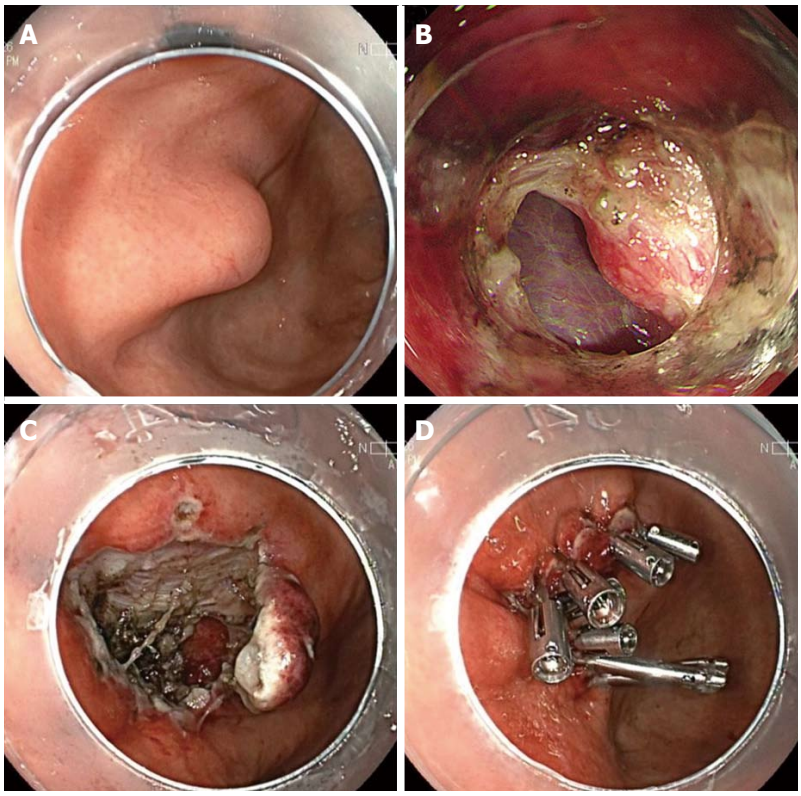

Figure 2 Successful closure of large perforations in a patients. A: There was a submucosal tumor located in the gastric fundus, which was about 15 mm × 12 mm and originated from the deep muscularis propria; B: An intentional perforation occurred during the procedure for full-thickness resection; C: A larger perforation was left after complete removal of the tumor. However, the mucosa over the tumor was kept intact; D: We use the retained mucosa to cover the perforation and the wound was successfully covered by the intact mucosa combined with several endoclips.

therapeutic procedures, including gastroenteric anastomosis dilation (2%)[75]; overdistension during argon plasma coagulation or cryotherapy (< 0.5%); standard snare polypectomy; EMR (0.5%) and more frequently, ESD[11,76,77]. Compared with esophageal perforations, more methods are available for closure of gastric perforations. TTSC clips alone are not recommended for perforations of more than 10 mm. In the case of perforations measuring 10-30 mm, the OTSC system has been the most evaluated and should be recommended. If the OTSC technique is unavailable, the combined technique using TTSC clips plus endoloop can be recommended. Techniques combining omental patches or nylon rope with clips are also good choices to close defect greater than 10 mm. Endoscopic suturing is required to close post-ESD defects. Stents are an option for perforations near the pylorus, or caused by dilating an anastomotic stricture. Evidence supporting the use of endoscopic band ligation for gastric perforations is scarce, and requires further exploration. Particular attention should be paid as follows: (1) most perforations of the stomach are small defects that occur during EMR, ESD procedures; (2) intentional perforation during endoscopic resection is becoming more frequent, and the integrity of the mucosa should be emphasized; (3) closing perforations in the proximal stomach might be challenging; and (4) EBL for gastric perforation closure has been reported, but experiences are limited.

Duodenal perforations

In the case of the immediate recognition of a perforation, an endoscopic closure should be attempted; however, this is effective in a minority of cases only (22%)[11]. Reports about new devices and techniques for the endoscopic closure of duodenal perforations are relatively rare. TTSC clips alone are recommended for perforations less than 10 mm. In the case of perforations measuring 10-30 mm, TTSC clips combined with endoloops or the OTSC system should be considered. EBL could be attempted when clips fail, but is not recommended routinely. If the iatrogenic perforation is diagnosed several hours after endoscopy and the patient shows symptoms of generalized peritonitis and/or sepsis, the only option is surgery. Particular attention should be paid as follows: (1) the use of a transparent cap might be helpful in difficult locations; (2) closure of medial duodenal wall defects with clips may be challenging because of the risk of clipping the ampulla and anatomic location; and (3) a nasoduodenal drain to divert pancreatic and biliary secretions may be beneficial.

Colonic perforations

ESGE recommends that complex EMR, ESD and balloon dilation procedures should be considered to

carry increased risk of colorectal perforation. Risk factors include female gender, presumably related to pelvic adhesions; major co-morbidities; inflammatory bowel disease; and older age[78-81]. TTSC is suitable to close small holes and the OTSC system is useful for larger ones. Clipping plus endoloops can also close large colonic perforations. EBL is verified to be useful for this type defects, but more evidence is needed. Particular attention should be paid as follows: (1) asymptomatic patients with retroperitoneal air alone require no additional treatment; (2) the success rate of endoscopic closure is higher when the perforation is recognized and managed during the same procedure; and (3) large vertical perforations should be closed from top to bottom, and horizontal perforations should be clipped from left to right.

CONCLUSION

It is sometimes difficult to decide which device or technique is the best method for endoscopic closure of gastrointestinal perforations. In general, the decision to attempt endoscopic closure of an iatrogenic perforation depends on multiple factors, including the location, size and the cause of the perforation, the endoscopist's experience and the accessories available at the time. The devices and techniques discussed in this study may not apply in all situations and should be interpreted in the light of specific clinical situations. With the rapid development of new endoscopic devices and techniques, more and more perforations can be managed well by endoscopy. However, more attention should be paid to avoid perforation during endoscopic procedure. Occasionally, intentional perforation is necessary to completely remove a tumor. However, the integrity of the mucosa should be considered: the retained mucous membrane can contribute to successful closure. We introduced and discussed endoscopic devices and techniques in this review, with the aim of providing more information about choosing the appropriate method to close perforations effectively and perfectly. Undoubtedly, further, large, randomized, controlled trials are needed to compare the clinical efficacies of the different endoscopic techniques in every situation.

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REVIEW

Role of bile acids in carcinogenesis of pancreatic cancer: An old topic with new perspective

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Abstract

The role of bile acids in colorectal cancer has been well documented, but their role in pancreatic cancer remains unclear. In this review, we examined the risk factors of pancreatic cancer. We found that bile acids are associated with most of these factors. Alcohol intake, smoking, and a high-fat diet all lead to high secretion of bile acids, and bile acid metabolic dysfunction is a causal factor of gallstones. An increase in secretion of bile acids, in addition to a long common channel, may result in bile acid reflux into the pancreatic duct and to the epithelial cells or acinar cells, from which pancreatic adenocarcinoma is derived. The final pathophysiological process is pancreatitis, which promotes dedifferentiation of acinar cells into progenitor duct-like cells. Interestingly, bile acids act as regulatory molecules in metabolism, affecting adipose tissue distribution, insulin sensitivity and triglyceride metabolism. As a result, bile acids are associated with three risk factors of pancreatic cancer: obesity, diabetes and hypertriglyceridemia. In the second part of this review, we summarize several studies showing that bile acids act as cancer promoters in gastrointestinal cancer. However, more question are raised than have been solved, and further oncological and physiological experiments are needed to confirm the role of bile acids in pancreatic cancer carcinogenesis.

Key words: Bile acids; Pancreatic adenocarcinoma; Pancreatitis; Metabolic syndrome

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Core tip: Bile acids bridge the gap between risk factors and pancreatic cancer, providing a new horizon in pancreatic cancer carcinogenesis.

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INTRODUCTION

Pancreatic cancer mortality is the fourth leading cause of cancer deaths in males and females and accounts for 7% of all deaths in cancer patients[1]. Therapeutic strategies for these cancers are well developed, but the death rates of pancreatic cancer have remained stable from 1930 to 2011 due to delayed diagnosis and elusive mechanisms of cancer initiation and progression. Pancreas is a retroperitoneal organ, located behind the stomach and in front of the spine. Because of the relatively large space around this organ, pancreatic tumors do not generally cause obstructive symptoms or pain. Moreover, the pancreas contains two types of cells, exocrine and endocrine cells. Most pancreatic tumors are pancreatic duct adenocarcinomas, which originate in exocrine cells, with no changes in hormone secretion. Therefore, early diagnosis of pancreatic cancer is difficult due to a lack of symptoms. Most pancreatic cancer is diagnosed at a late, inoperable, and incurable stage. Scientists have sought to identify early diagnostic markers and to elucidate the underlying mechanisms of pancreatic cancer initiation and progression. Etiological studies have identified a number of risks for developing pancreatic cancer, including (1) alcohol intake; (2) smoking; (3) diet (high-fat and red meat); (4) obesity; (5) diabetes; (6) gallstones; (7) long common channel of the biliary duct and the pancreatic duct; (8) chronic pancreatitis; (9) hypertriglyceridemia; and (10) other risks, including age and sex, race (black population), non-O blood type, autoimmune disease, hereditary pancreatitis, and infectious disease[2]. Notably, 60% of pancreatic cancers occur in the head of the pancreas[3], which is close to the bile tracts, suggesting that bile acids may play a role in pancreatic cancer formation[4,5]. Bile acids were first proposed as a carcinogen in the 1940s[4]. Since then, increasing evidence has shown that bile acids, particularly secondary bile acids, play important roles in the carcinogenesis in gastrointestinal cancers[4] and breast cancer[6]. We review the systemic and local effects of bile acids in pancreatic cancer initiation and progression and propose that bile acids have key roles in different metabolic and oncogenic pathways (Figure 1).

SYSTEMIC EFFECT OF BILE ACIDS

Bile acids and alcohol intake

A large body of evidence has shown that alcohol intake significantly increases blood and intestinal bile acids levels[7,8]. Alcohol induces bile acid secretion *via* two pathways[9]. First, alcohol increases cholesterol 7α-hydroxylase synthesis, rather than directly activating the enzyme[10]. Second, alcohol has an inhibitory effect on gallbladder contraction, leading to a decrease in the amount of bile acid moving into the duodenum. Subsequently, enterohepatic circulation of bile acids is interrupted, resulting in reduced feedback inhibition of bile acid synthesis. Long-term alcohol intake results in prolonged low-dose exposure of the pancreatic epithelial cells to bile acids, which activate intracellular signaling pathways. Equilibrium of the alcohol-bile acids-microbiome axis must be taken into account in the relationship between bile acids and alcohol intake. After consumption of alcohol, fecal deoxycholic acid (DCA), one type of secondary bile acid, increased 3-4 times that of the control groups[7]. Secondary bile acids play an important role in shaping the gut microbiome[11], which is critical for the gut barrier. Additionally, the acute effects of alcohol administration directly impair the duodenum and jejunum barrier[12]. Gut barrier injury leads to changes in gut permeability, resulting in an increase in serum DCA levels and systemic inflammation[13].

Bile acids and smoking

Epidemiological and clinical studies have indicated that smoking is a risk factor for pancreatic cancer. Recent reports have shown that nicotine stimulates mutated K-ras activation, as well as other mutations associated with pancreatic cancer, including those in p53, COX-2, SMAD4 and p16INK4A[14,15]. However, little is known about the mechanisms of how smoking causes gene mutation and pancreatic cancer formation. Bile acid concentration in the stomach of smokers is significantly higher than that in non-smokers, and this trend is found even when not actually smoking[16]. Additionally, nicotine induces gastric acid secretion, leading to a significant drop in the pH of the stomach[17]. Gastric acid is a strong regulator of the secretion of bile acids. However, bile acid reflux into the pancreatic duct is associated with intraductal papillary carcinoma in the pancreas[18]. Further investigations are still needed to determine whether smoking also leads to bile acid reflux to the pancreatic duct. Overall, the above findings are not convincing evidence of the association between smoking and pancreatic cancer initiation. The local effects of bile acids, which are induced by smoking, on pancreatic cancer formation may be overestimated, but nicotine may act on pancreatic cells *via* blood circulation delivery.

Bile acids and diet

Little is known about how diet is associated with cancer formation, partly because there is high variation in diets. The basic function of bile acids is to promote the absorption of dietary fat and help absorb fat-soluble vitamins, as well as to regulate cholesterol metabolism. Dietary fat, which is the strongest regulator, induces secretion of bile acids into

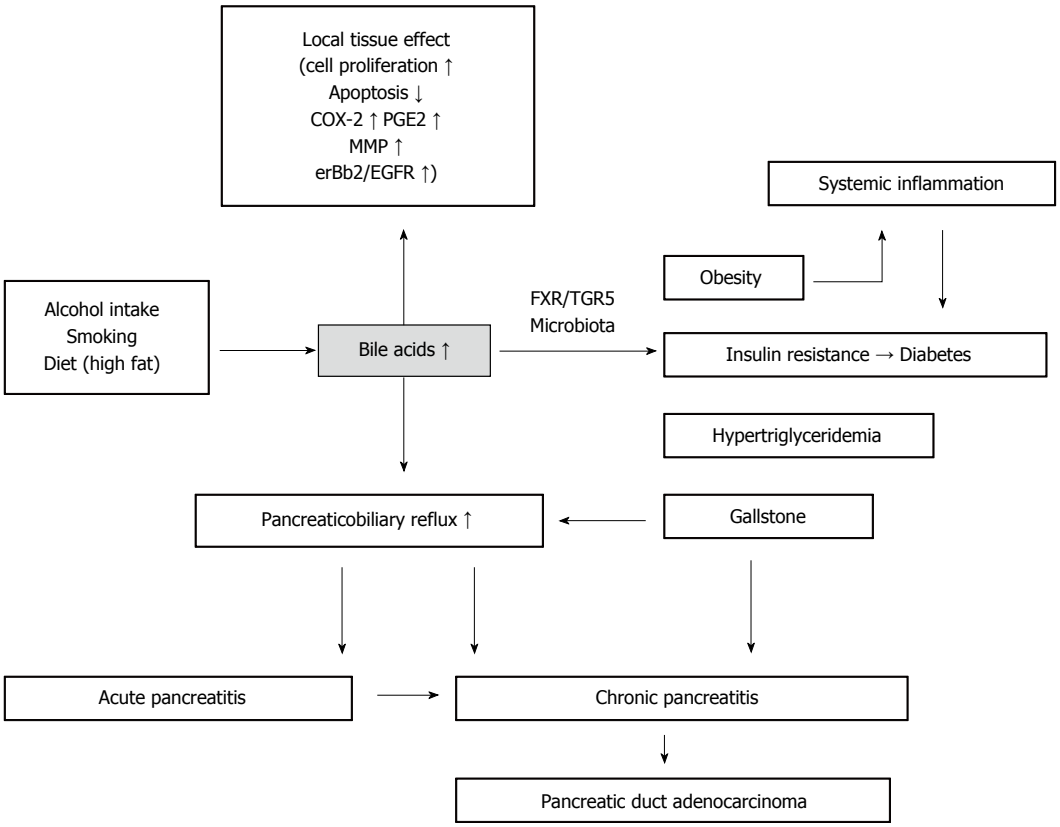

Figure 1 Bile acids are in the central position of oncogenic and metabolic pathways. MMP: Matrix metalloproteinase; FXR: Farnesoid X nuclear receptor; TGR5: Takeda G-protein receptor 5.

the duodenum, resulting in an elevated fecal bile acid concentration. Vegetables and carbohydrates, which do not induce secretion of bile acids, are not associated with pancreatic cancer[19]. Approximately 95% of bile acids are reabsorbed into the intestine and transported to the liver. During this process, bile acids also escape into blood circulation. Studies have shown that the plasma bile acid concentration is correlated with the fecal concentration[20] due to intestinal epithelial cell exposure to bile acids. Accumulating evidence has shown that excess bile acids are associated with colon cancer initiation. However, the pancreas does not directly contact bile acids. How diet-induced bile acids promote pancreatic cancer formation, through local effects (by bile reflux) or though systemic effects (by circulation), remains unknown.

Bile acids and obesity, diabetes, and hypertriglyceridemia

Metabolic syndrome includes the following disorders: abdominal obesity, hypertension, hyperglycemia, hypertriglyceridemia, and low serum high-density protein. Metabolic syndrome and prediabetes share the same disorders. Thus, we here discuss obesity, diabetes and hypertriglyceridemia at the same time. Possible mechanisms linking obesity and cancer include: (1) Insulin or insulin-related growth factors (IGF); (2) microbiome; (3) chronic inflammation; (4) sex hormones; (5) circulating adipokines; and (6) white adipose tissue-derived progenitor cells[21]. Type 2 diabetes is cause by insulin resistance, with hyperinsulinemia. Approximately half of individuals with diabetes are obese[22], and up to 60% of diabetes cases are caused by obesity[23]. A recent study revealed that type 2 diabetes results from chronic inflammation caused by obesity[24]. Above all, when deeply studying the mechanism of these two diseases, it is difficult to identify which is the original metabolic defect, hyperinsulinemia or insulin resistance, and which is secondary. We hypothesize that hyperinsulinemia is the original defect[25]. In parallel, obesity is a complex and multifactorial metabolic disease. Here, we only discuss diet-induced obesity and review several bile acid-related factors.

In addition to the important role of bile acids in nutrient absorption, accumulating evidence indicates that bile acids play key roles in glucose and lipid metabolism. The concentration of deoxycholic acid, a secondary bile acid, is elevated in type 2 diabetes, along with elevation of the hydrophobic 12a-hydroxylated bile acids[26]. *In vivo,* ob/ob mice also had elevated plasma bile acids[27]. Bariatric surgery[28] and bile acid binding resins improve insulin resistance[29] and ameliorate obesity and type 2 diabetes, indicating that changes in bile acid flow or compositions promote remission of metabolic

disorders. The dominant type of bariatric surgery is Roux-en-Y gastric bypass (RYGB)[28], which alters bile acid flow and re-absorption by changing the anatomy of the intestine. Plasma primary bile acids, including chenodeoxycholic acid (CDCA), and cholic acid (CA), increased after surgery, along with increased taurine-conjugated and glycine-conjugated bile acids[30], which indicated that re-absorption increases in the upper intestine. Consequently, fewer bile acids reached the distal intestine, resulting in decreased secondary bile acid pools. Bile acid binding resins predominantly function by decreasing bile acids in the intestine and by blocking re-absorption of bile acids, which limits the total bile acid pool. In other words, both bariatric surgery and bile acid binding resins promote primary bile acid synthesis and re-absorption and limit secondary bile acid synthesis and their concentration in plasma.

Farnesoid X nuclear receptor and bile acid synthesis

Farnesoid X nuclear receptor (FXR) is a nuclear receptor, and its major ligands are bile acids[31]. A primary bile acid, CDCA, is the strongest agonist of FXR. Secondary bile acids, such as lithocholic acid (LCA) and deoxycholic acids (DCA), are also activators of FXR but have a lower affinity. In contrast, hydrophilic bile acids do not activate FXR[31]. In addition to FXR, bile acids activate other nuclear receptors, such as pregnane-X-receptor, constitutive androstane receptor and vitamin D receptor, inducing different signaling pathways[32]. FXR is predominantly expressed in the liver, intestine, kidney, adrenal gland, pancreas, and reproductive tissues[33]. In the liver, primary bile acids bind to FXR in hepatocytes after re-absorption, leading to increased expression of small heterodimer partner 1 (SHP-1), which is a DNA-binding domain. SHP-1 inhibits expression of cholesterol 7 α-hydroxylase (CYP7A1) *via* liver receptor homologue 1 (LRH-1) and liver X receptor α (LXRα), resulting in decreased synthesis of bile acids[34]. This is the major mechanism of bile acid re-absorption feedback inhibition of bile acid synthesis. Furthermore, SHP-1 can also inhibit expression of CYP8B1 (cytochrome P450, family 8, subfamily B, polypeptide 1) *via* hepatocyte nuclear factor 4 (HNF4). CYP8B1 regulates the synthesis of cholic acid (CA), which is hydrophilic. Thus, composition and hydrophobicity of the primary bile acids is determined by CYP8B1[35]. In the intestine, activation of FXR induces the secretion of fibroblast growth factor-19 (FGF-19), FGF-15 in mouse, which binds to fibroblast growth factor receptor 4, to decrease the expression of CYP7A. This is a SHP-1-independent pathway in the regulation of bile acid synthesis[36]. Enterohepatic circulation of bile acids leads to feedback inhibition of bile acid synthesis. Any problems in the steps in this cycle will lead to metabolic diseases, including cholestatic liver disease, gallstones, fatty liver, diabetes and obesity.

Bile acids regulate metabolism via FXR

Bile acids and FXR are regulators of glucose homeostasis and insulin resistance. Gene encoding phosphoenolpyruvate carboxykinase, glucose-6-phosphatase, and fructose-1,6-biphosphatase (FBP1) are target genes of FXR[37]. All of these are rate-limiting enzymes in glucose metabolism. Activation of FXR or overexpression FXR in the liver reduces the plasma glucose level. FXR deficiency, in the liver not in the intestine, leads to glucose metabolism disruption and results in insulin resistance[37]. However, expression of FXR in the intestine has a negative effect on human disease development. In FXR$^{-/-}$ mice, enhanced glucose clearance and insulin sensitivity were observed, but hepatic insulin sensitivity was not altered[38], indicating that the effect of intestine FXR overcomes the effect of the liver in regulating glucose metabolism. A recent study was also consistent with these findings. In high-fat-induced nonalcoholic fatty liver disease mouse models, changing the composition of bile acids by administration of antibiotics, which results in gut microbiota alternation, led to nonalcoholic fatty liver disease development. This study demonstrated that bile acids or gut microbiota (which will be discussed in a later section) regulate nutrient metabolism in a FXR-dependent manner in the intestine but not in the liver[39]. A intestine-selective, high-affinity FXR inhibitor, glycine-β-muricholic acid (Gly-MCA), improved metabolic parameters, high-fat diet-induced and genetic obesity, insulin resistance and hepatic steatosis in mice[40].

Bile acids and FXR also regulate lipid metabolism. FXR regulates lipogenesis by inhibiting LRH-1 and LXRα[41]. In addition, activation of FXR induces expression of Apolipoprotein C-II and Apolipoprotein A-V (apoA-V) and suppresses expression of Apolipoprotein C-III, which results in an increase in lipoprotein synthesis and a decrease in plasma triglycerides[42]. Peroxisome proliferator-activated receptor α, which is involved in lipid, lipoprotein and fatty acid metabolism, is also regulated by bile acids *via* FXR[43]. Taken together, these results show that bile acids and FXR regulate lipid metabolism in direct and indirect manners.

Bile acids and insulin resistance and hyperinsulinemia

Although the regulation of bile acid synthesis and bile acid metabolism is complex, clinical evidence suggests that adjusting the flow rate and composition of bile acids can improve metabolic disorders. Bypass surgery and bile acid sequestrant improve insulin resistance, obesity and hyperlipidemia, although the mechanism of these two treatments is unclear. Bile acid binding resins function by sequestering bile acids, which suppresses absorption and increases excretion

of bile acids in the feces. Despite the complexity of the regulation of bile acids and their receptors, bile acid binding resins improve insulin resistance in diet-induced rat models of obesity[44]. In bile acid binding resin-treated groups, plasma glucose levels decreased to baseline values throughout the oral glucose tolerance test, a parameter of insulin resistance, and insulin levels declined to baseline as well. These findings indicated that bile acids modulate glucose metabolism and insulin sensitivity. Another approach for improving metabolic disorder is bariatric surgery, as mentioned above. In contrast to bile acid binding resins, bariatric surgery increases plasma bile acids to improve metabolic parameters, although the underlying mechanism remains to be determined. However, bariatric surgery did not decrease the standardized incidence of obesity-related cancers but increased the incidence of colon cancer with time after the surgery[45,46]. This study included a large sample size cohort, 15095 and 62016 in the surgery and control cohort, respectively, and long-term follow up (up to 30 years). It provided a very convincing result, that bariatric surgery provided a short-term benefit for metabolic disorders but increased colorectal cancers instead over time. It is still unclear why and how bariatric surgery changed the incidence of colorectal cancer. We hypothesize that changing the anatomy of the intestine leads to bile acid flow and composition alteration and results in turnover of the population of gut microbiota. Studies have shown that gastrointestinal bypass surgery may lead to changes in the intestinal and fecal microbiota, resulting in colonic mucosa exposure to increased toxicity of the feces and increased incidence of colon cancer[47].

Takeda G-protein receptor 5

Takeda G-protein receptor 5 (TGR5) is a membrane receptor of bile acids, and it belongs to the superfamily of G-protein coupled receptors. TGR5 is expressed in the gallbladder, intestine, human spleen and mononuclear and white blood cells, as well as in liver cells, brown adipose tissue, skeletal muscle and the nervous system[48]. Bile acids activate TGR5 with different potency, and LCA > DCA > CDCA > CA[49]. In TGR5$^{-/-}$ mice, the bile acid pool was decreased by increasing fecal bile acid excretion[50]. Because TGR5 is expressed in the gallbladder, it also regulates bile composition by induction of chloride secretion[51]. In addition, TGR5 regulates contraction of smooth muscles of the gallbladder, participating in gallstone disease development[52]. However, the exact mechanism of how TGR5 regulates synthesis and the bile acid pool is still unknown. Similar to FXR, TGR5 also plays a role in glucose metabolism. By binding to TGR5, bile acids induce intestinal glucagon-like peptide-1 (GLP-1) and GLP-2 release, which results in secretion of insulin[53]. One possible mechanism is that GLP stimulates oxidative phosphorylation, resulting in an increase in the ATP/ADP ratio, membrane depolarization and Ca^{2+} mobilization, leading to insulin secretion from pancreatic β-cells. Hyperinsulinemia is associated with insulin resistance and type 2 diabetes. Interestingly, in female TGR5$^{-/-}$ mice, insulin sensitivity increases but not in male mice[54], indicating that alternative regulatory pathways exist and that TGR5 regulates glucose metabolism and insulin sensitivity.

Insulin, insulin-like growth factor 1 and pancreatic cancer

Insulin regulates the production and activity of insulin-like growth factor 1 (IGF1) by down-regulating insulin-like growth factor-binding protein 1 (IGFBP1) and IGFBP2, which inhibit the activity of IGF1[55]. High plasma concentration of IGF1 and low concentration of IGFBP1 are observed in type 2 diabetes. The main function of IGF1 is to promote cell proliferation and to inhibit cell apoptosis[56]. Both the IGF1 receptor and insulin receptor belong to the family of transmembrane receptor tyrosine kinases. They are structurally and functionally related in cancers[57]. The insulin receptor is highly expressed in insulin-sensitive tissues, such as the liver, skeletal muscle and white adipose tissue, and shows low expression in other tissues, such as the brain, heart, kidney, lung, pancreatic acini, platelets, endothelial cells, monocytes, megakaryocytes and fibroblasts. Insulin does not activate the insulin receptor in these tissues at normal concentrations[58]. Insulin abnormally activates these receptors due to hyperinsulinemia in diabetes. Moreover, in cancer patients, the tumor cells often highly express the insulin receptor, which results in non-metabolic effects. The non-metabolic effects include promotion of cell mitosis, proliferation, and metastasis[59]. The PI3K pathway and MAPK pathway play important roles in pancreatic cancer formation. Appleman *et al*[60] showed that the insulin receptor and IGF receptor could be activated by their ligands and in turn activated MAPK signaling and PI3K signaling. The insulin receptor and IGF receptor, along with the kras mutation, facilitate pancreatic cancer development[61]. Additionally, another study revealed that there was cross-talk between the insulin receptor and IGF receptor with G-protein coupled receptors, which further activated mTOR signaling and promoted DNA synthesis[62].

Bile acids and gut microbiota

Primary bile acids are converted into secondary bile acids by structural modification by the gut microbiota (Figure 2). The gut microbiota has an important impact on the composition of bile acids, and vice versa, as bile acids re-shape the population of bacteria in the intestine. The role of intestinal flora in

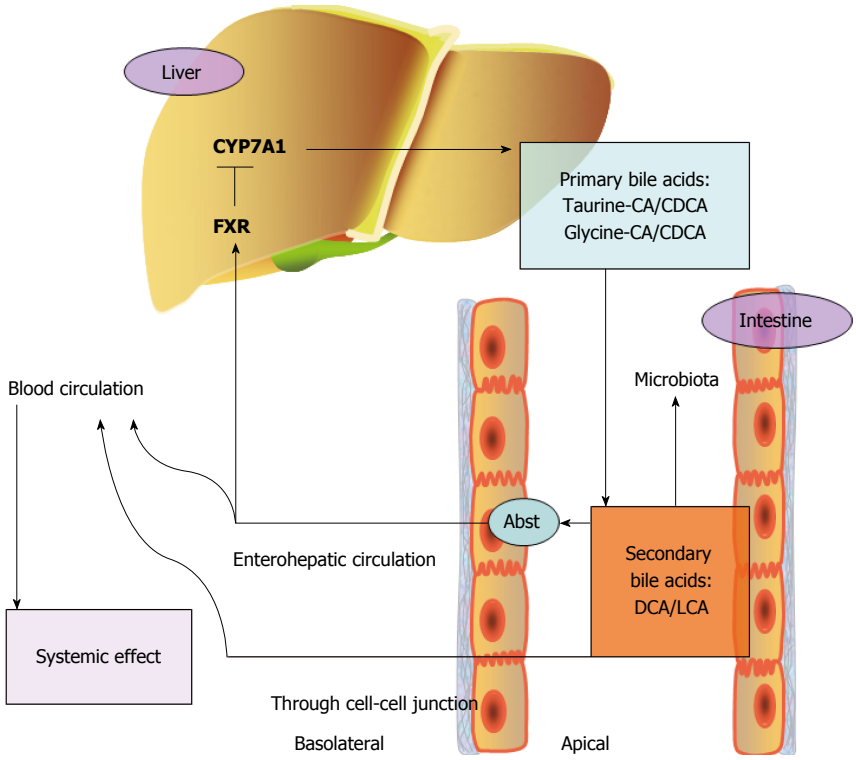

Figure 2 Bile acids metabolism. FXR: Farnesoid X nuclear receptor; DCA: Deoxycholic acid; LCA: Lithocholic acid; CDCA: Chenodeoxycholic acid; CA: Cholic acid.

modulating the host metabolism has received much attention after it was revealed that diabetic patients had changes in intestinal flora, with increases in the Firmicutes to Bacteroidetes ratio. Then, obese patient were also found to have a similar composition shift[63]. Gram-negative bacteria, which belong to Bacteroidetes and Proteobacteria, are enriched in type 2 diabetes[64]. Organic acids decrease luminal pH and damage bacterial cell membranes, which strongly affect the bacterial composition, especially after a high-fat diet[65]. Rats were fed a high-CA diet, which mimicked bile acids induced by a high-fat diet, and it was found that the fecal DCA concentration was much higher, with the CA/DCA ratio reversed, compared to the control diet group[66]. DCA is ten times more toxic to intestinal bacteria than CA[67]. Firmicutes and Bacteroidetes, which are the two major types of intestinal flora, accounted for 54.1% and 30.7%, respectively, in the control group. In contrast, the proportion of Firmicutes increased to 98.6% in the high-CA group[66]. However, the total number of bacteria decreased in the feces, with an increased bile acid concentration, up to 50% that of the control diet group. Taken together, the results showed that a high-fat diet regulates intestinal flora by affecting bile acid composition. Additionally, bile acids change with the gut microbiota composition shift. A recent study found that oral administration of antibiotics led to changes in the gut microbiota and subsequently, changes in bile acids and glucose metabolism *via* FGF-19 signaling[68]. Vancomycin had the strongest effect on the Firmicutes and Proteobacteria phyla, with Firmicutes decreasing and Proteobacteria increasing. The Firmicutes phylum, which consists of Gram-positive bacteria, plays a crucial role in primary bile acid modification. Researchers have attributed the promotion of insulin sensitivity to the decrease in the Firmicutes phylum and the increased primary bile acids (CA), which are an activator of intestinal FXR. However, to our knowledge, as mentioned above, activation of intestinal FXR may have negative effects on metabolic disorders. Therefore, the mechanism remains to be confirmed.

Increasing gut permeability, which is controlled by microbiota, is associated with many metabolic diseases[69] and chronic low-grade inflammation[70]. All surfaces of the body, including the skin and the intestinal, oral and vaginal mucosa, are covered with microorganisms that maintain human health, rather than cause diseases. These microorganisms interact with the host to maintain the body's health. However, when there are changes in the density or species composition of these organisms, it may result in disease. The majority of microorganisms exist in the human intestine as an essential part of mucosal immunity. A long-term, high-fat diet affects intestinal flora density through bile acids, resulting in higher mucosal permeability. The integrity of tight junctions in the intestine and trans-epithelial permeability are regulated by the normal intestinal flora, through redistribution of Toll-like receptor 2 protein[71], a toll-like receptor on epithelial cells, and expression of

tight-junction proteins in cell to cell contacts[72]. High permeability has two results: bile acid as a metabolism-regulating molecule will enter the blood circulation, and gut microbes and their products will translocate to the bloodstream, leading to chronic local and systemic inflammation[70].

A large number of experiments confirmed that inflammation provides a suitable environment for tumor initiation and progression. Tumor-associated inflammatory cells and tumor stromal cells work together to promote tumor cell metastasis. Chronic inflammation induces bone marrow-derived mesenchymal stem cells to migrate to the tumor site and inhibits tumor suppressor T cells, thereby inhibiting the body's anti-cancer immunity[73]. Intestinal polyp patients had higher intestinal permeability compared with normal subjects. IL-6, IL-11, IL-17, IL-22, and IL-23 secreted by ectopic bacteria are required for the development of intestinal polyps[70,73,74]. Intestinal flora also affect tumor formation in distant organs by modulating tumor necrosis factor, oxidative stress and DNA damage repair[70]. A more recent study revealed that a long-term, high-fat diet first affected visceral adipose tissue. This effect was caused by damage of the intestinal mucosal barrier function. The local pro-inflammatory response led to the accumulation of fat due to distant and systemic inflammation[75].

Bile acids and gallstones, pancreaticobiliary maljunction, chronic pancreatitis

Several risk factors for pancreatic cancer, such as gallstones, pancreaticobiliary maljunction (long common channel) and chronic pancreatitis, share a common pathophysiological feature of bile acid dysmetabolism and bile acid reflux. Consequently, these three are causative factors of pancreatitis. The sphincter of Oddi loses function with a long common channel, resulting in communication of the bile duct and pancreatic duct[76]. The reflux of pancreatic juice into the bile duct leads to a higher incidence of biliary cancer, whereas the reflux of bile juice into the pancreatic duct results in pancreatitis. It is still debatable whether the reflux of pancreatic juice into the bile duct actually occurs. Because pressure in the bile duct is higher than that in the pancreatic duct, and even in the long common channel, there is a greater possibility that pancreatic juice refluxes into the bile duct[77]. However, among the causal factors of acute pancreatitis, pancreatic juice reflux or duct obstruction is the most convincing one. Bile reflux into the pancreatic duct is known to be necessary for the induction of acute pancreatitis[78,79]. Additionally, it has been known for a long time that bile infusion can be used to establish pancreatitis animal models[80]. After a high-fat diet, the secretion pressure of bile may increase to a level high enough to reflux into the pancreatic duct, leading to mild or chronic pancreatitis. Chronic pancreatitis develops from recurrent acute pancreatitis, and it involves pancreatic exocrine and endocrine dysfunction and gradually progresses to malignant tumors and diabetes[81].

Due to different cell sources, pancreatitis and pancreatic cancer were once considered two unrelated disease because pancreatitis predominantly affects pancreatic acinar cells, and pancreatic cancer originates from ductal cells[81]. However, a recent lineage tracing study questioned this hypothesis. Chronic inflammation induces dedifferentiation of acinar cells into progenitor duct-like cells, and the latter could be the source of pancreatic cancer[82]. Whether the bile acids reflux into the pancreatic duct and reach the acinar cells to induce pancreatitis is still controversial. There are two possible ways for bile to reach acinar cells: through bile duct epithelial cells and through cell-cell contacts, with tight junction impairment[83]. Bile acids were originally identified as detergents. Now, they are studied as regulatory molecules. Gpbar1 (the other name of TGR5 mentioned above), a G-protein coupled receptor, is expressed on acinar cells and mediates bile acid-induced pancreatitis. Deletion of this gene reduced hyperamylasemia, edema and inflammation[84]. Acinar cell exposure to bile acids and activation of Gpbar1 cause cell injury mediated by Ca^{2+} signaling and downstream NF-κB translocation[85]. Ca^{2+} signaling also mediates intra-acinar cell zymogen activation and in turn damages the acinar cells. In addition to NF-κB signaling, oxidative stress, which is related to bile acid injury[86], is also indispensable in acinar cell necrosis and fibrosis. All these processes produce inflammatory cytokines and chemokines, which activate the immune system. Inflammatory mediators generate secondary oxidative injury and damage cells[81]. Surprisingly, insulin-producing cells develop a malignant phenotype in inflammatory circumstances[87]. Chemokines are inflammatory cues for mesenchymal stem cells from different types of tissues, which can regulate tissue immune response[88]. Mesenchymal stem cells differentiate into fibroblasts or leucocytes infiltrating in the inflammatory lesion. As in the old hypothesis - cancer is just likes a wound that does not heal[89] - duct epithelial cells and acinar cells, as well as stroma and immune cells, function as intrinsic and external factors, respectively, to promote cancer formation (Figure 3).

LOCAL TISSUE EFFECTS OF BILE ACIDS

Bile acids induce cell membrane perturbations

Removal of cholesterol on the cell membrane can inhibit apoptosis induced by DCA. After staining with filipin, it was found that DCA could cause redistribution of membrane phospholipids. Similarly, DCA could also affect the distribution of plasma membrane

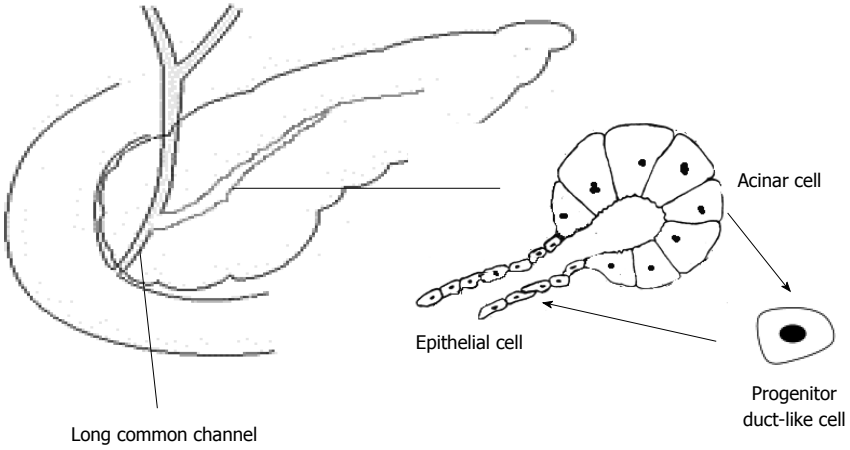

Figure 3 Oncogenic process of bile acid reflux.

caveolin and reduce membrane fluidity. Radiolabeled DCA showed that bile acids are located in the cell membrane microdomains, and different reactions depends on the physical and chemical properties of bile acids. These findings suggest that redistribution of membrane cholesterol is the initial stage of bile acid-induced signaling activation[90]. Additionally, whether the bile acids enter the cell depends on the critical micelle concentration, which is the lowest concentration of surfactant in the solvent molecules to form micelles[91].

Bile acids increase cell proliferation and mitotic events

Treatment of colonic epithelium with bile acids leads to phospholipid turnover, thereby increasing the release of diacylglycerol, which is a protein kinase C (PKC) activator. Bile acid activation of PKC is mediated by activator protein-1 (AP-1)[92]. PKC activation increased synthesis of DNA and promoted cell proliferation[93]. In addition, ornithine decarboxylase (ODC) activity and DNA synthesis varied with different types of bile acids in an *in vitro* study. Compared with 12-O-tetradecanoylphorbol-13-acetate (TPA), a tumor-promoting agent, deoxycholic acid (DCA) was a more potent activator of ODC. DCA and TPA both stimulated DNA synthesis within 2 d of treatment, with a peak at 2 h and a decline after 4-12 h. Moreover, the stimulatory activity of bile acids with different structures is different. By analyzing 26 types of bile acid component, bile acids, which are 5β-cholanic acids with two α-hydroxy groups in 3α, 7α, and 12α position and 5β-cholanic acids with a 3 α-hydroxy group, had the strongest activities. Therefore, the composition of bile acids plays an important role in cell proliferation and DNA synthesis in colonic epithelial cells[94].

Bile acids reduce susceptibility to apoptosis

An in vivo study showed that rats fed a diet containing 2% CA for 18 wk had significantly decreased apoptotic bodies in the normal intestinal epithelium and aberrant crypt foci (ACF) compared to those in the normal diet group (P = 0.0034), and the number of apoptotic bodies in ACF was significantly lower than those in normal intestinal epithelium (P = 0.012). In conclusion, CA simultaneously reduced apoptotic bodies in normal intestine ACF, and ACF are more susceptible to bile acids than normal intestinal mucosa. Bile acids promotes colorectal cancer formation and progression[95], which was consistent with another clinical study that also found the same phenomenon. In patient biopsy specimens, after co-culturing with bile acids, intestinal mucosal cell apoptosis was significantly reduced[96].

Bile acids stimulate COX-2 and PGE2 production

DCA and CDCA were found to induce COX-2 expression in the pancreatic cancer cell lines BxPC3 and SU86.86[97] and colon cancer cell lines[98]. Both studies found that bile acids acted in a dose-dependent manner, but the strongest effect was induced by different concentrations (100 μmol/L and 250 μmol/L, respectively) and different reaction times (6-12 h and 24 h, respectively). Glinghammar *et al*[98] also revealed that bile acids induced COX-2 expression mediated by AP-1, PKC and p38.

Bile acids induce MMP7 mRNA expression

The main function of matrix metalloproteinase (MMP) proteins is decomposition of the extracellular matrix proteins, which are involved in cancer metastasis and inflammatory responses. MMP proteins are expressed in a wide range of cancers, including esophageal cancer, stomach cancer, liver cancer, pancreatic cancer, and kidney cancer[99]. Tumors with high expression of MMP7 are more aggressive and have a greater metastatic ability. Apical sodium-dependent bile acid transporter (Asbt)-deficient mice, which show a 10–fold increase in bile acids in the intestinal tract, have 54% more aberrant crypt foci than that in wild-type mice, and the probability of colon cancer development

is twice as high as that in the wild-type mice. The study found that increasing the content of bile acids significantly increased MMP7 expression, which is mediated by muscarinic receptors (a G-coupled protein receptor)[100]. This indicates that bile acids also play a role in cancer invasion and migration[101].

Bile acids induce overexpression and activation of the erbB2 and EGFR signaling cascades

Epithelial cells in the gall bladder and bile tract, which directly contact bile acids, highly express erbB2 in their malignant lesions[102,103]. EGFR expression in intrahepatic cholangiocarcinoma, extrahepatic cholangiocarcinoma and gallbladder cancer was 100%, 52.6% and 38.5%, respectively, and HER2 is overexpressed in 10% and 26.3% of gallbladder and extrahepatic cholangiocarcinomas, respectively, suggesting that EGFR and HER2 may contribute to the initiation and development of these cancers[103]. However, whether bile acids directly induce the expression of EGFR and HER2 and activate these receptors was not clear. A recent study confirmed that bile acids induced the expression of EGFR and HER2 directly and activated the EFGR/HER2 pathway and downstream pathways, contributing to cancer formation and progression[104]. The study found that secondary conjugated bile acids, such as taurochenodeoxycholic acid, induced gallbladder cancer cell EGFR/erbB2 expression and activation of EGFR/erbB2 and downstream signaling: bile acid → src → TACE → EGFR/erbB2 → downstream signaling cascade. This induction and activation have also been verified in skin cells. This study was consistent with previous studies showing that in hepatocytes, the bile acids activated the MAPK and PI3K/Akt signaling pathways[105,106], indicating that bile acids have a prolonged effect on the activation of EGFR/erbB2 signaling and finally led to intranuclear effects, rather than acute effects. HER2 is overexpressed in 61.2%-90% of pancreatic cancer patients[107,108], and survival of patients with HER2 overexpression is significantly lower compared with patients with low expression, 14.7 mo and 20.7 mo respectively. In a multivariate analysis, HER2 overexpression is an independent prognostic factor. These findings suggested that a HER2 monoclonal antibody may be beneficial for this subtype of patients[107].

The kras mutation plays an important role in pancreatic cancer initiation. Kras induces endogenous EGFR expression and activation. EGFR inhibitors eliminated kras mediation of pancreatic cancer. In other words, without EGFR activation, kras cannot activate the MEK/ERK pathway and promote tumorigenesis[109]. However, unlike gallbladder and bile tract cancer, whether bile acids induce expression of EGFR and HER2 in pancreatic duct epithelial cells remains unknown. A recent study revealed that there was crosstalk between a bile acid membrane receptor, TGR5, and EGFR signaling. The cell surface protease TACE/ADAM-17, which is required in EGFR activation by its ligands amphiregulin (AREG) and TGF-α, is highly expressed in colorectal cancer and pancreatic cancer. Exposure of colorectal cancer cells and pancreatic cancer cells to bile acids activates EGFR in an AREG-dependent manner. Furthermore, this effect was mediated by a G-protein coupled receptor, TGR5[5,104]. RNA silencing of TGR5 inhibited EGFR, MAPK and STAT3 signaling induced by bile acids.

QUESTIONS REMAIN

Toxicity of bile acids

Although we discussed bile acids as a molecular regulator in metabolic and cancer signaling, we still must note that bile acids are a type of detergent. Exposure of cells or tissues to bile acids at high concentrations (Table 1) primarily causes cell death, whereas activation of signaling pathways is secondary. This may be a reason for the contradictory findings. A study[110] found that DCA and CA increased the proportion of cells in G0 and G1 phase, while GCA and TDCA increased the proportion of cells in S phase. Effective biological effects could not be found, even with different concentrations and different times. After 48 h of treatment, Panc-1 cells showed cell structural damage. Therefore, the researchers concluded that increases in bile acid concentration in the serum might inhibit the progression of pancreatic cancer. The conclusion might be far-fetched. In previous studies (listed in Table 1), the concentration of bile acids and the processing time varied substantially, indicating that bile acid concentration and treatment time are critical factors in research on the biological role of bile acids in cancer. Moreover, even if there is a clear biological effect in an *in vitro* study, how to simulate the in vivo environment is another issue. In the study of bile acids and pancreatic cancer, determining how bile acids reach the pancreatic duct epithelial cells or acinar cells is a prerequisite for all studies. If the bile acids do not reflux at high concentrations (for example, 500 μmol/L) into the pancreatic parenchyma, how can these studies determine how bile acids affect the development of malignant tumors? Bile acid retrograde infusion into the pancreatic ducts are widely used to induce pancreatitis *in vivo*[111]. It has been shown that 37 mmol/L of taurocholate acids or 3 mmol/L of tauro-LCS induces maximally severe, acute necrotic pancreatitis but not chronic pancreatitis. For studies on chronic pancreatitis, due to the duration of the study, duct ligation models with bile acid reflux are often used[112]. However, a profile of bile acids is missing in these cases. Therefore, the concentration of bile acids is a crucial factor for both *in vivo* and *in vitro* studies. Additionally, the method of contact of bile acids with cells is also important, whether it is by contacting the cell surface (luminal or basal surface) or by

Table 1 Review of the biological effects of bile acids

Ref.	Year	Cell/tissue	Bile acid	Dose	Time	Biological effect
Jean-Louis *et al*[90]	2006	HCT 116	DCA	500 μmol/L	5, 15, 30 min 1, 2, 4 h	Cholesterol aggregation at membrane
			DCA	500 μmol/L	1 h	Internalization of caveolin-1
Hirano *et al*[92]	1991	Gastric mucosal primary culture	DCA		30 min	PKC activation
DeRubertis *et al*[93]	1987	Colonic epithelial cells	DCA			DAG ↑
Takano *et al*[94]	1984	Colonic epithelial cells	DCA		2 d	DNA ↑ ODC ↑
Magnuson *et al*[95]	1994	*In vivo*	CA	2% in diet	18 wk	Apoptosis ↓
Garewal *et al*[96]	1996	Biopsies	DCA	1 mmol/L	30 ℃ 3 h	Apoptosis ↓
Tucker *et al*[97]	2004	BxPC3 SU86.86	CDCA, DCA	100 μmol/L	6-12 h	COX-2 ↑ PGE-2 ↑
Glinghammar *et al*[98]	2001	HCT 116	Tauro-CDCA DCA, CDCA, CA Butyric acid	200-1200 μmol/L 250 μmol/L 0.1-4 mmol/L	15 h	AP-1 ↑, COX-2 ↑, PKC(+), P38(+)
		HT 29	DCA	500 μmol/L	24 h	COX-2 ↑, PCNA ↑
Raufman *et al*[100]	2015	*In vivo* (Asbt-deficient)				Aberrant crypt foci
Cheng *et al*[101]	2007	H508	DCT	50 μmol/L	24 h	MMP ↑
Kitamura *et al*[104]	2015	Primary culture (BK5 erbB2 mice)	TCDC	100 μmol/L	72 h	Cell proliferation ↑, EGFR MAPK Cyclin D1 ↑
		Sk-Ch-A-1	TCDC	10-200 μmol/L	72 h	Cell viability ↑
			CDCA, DCA, TC, TDC	0.5 mmol/L	30 min	p-erbB2, p-EGFR, p-MAPK, p-Akt ↑
			TCDC	500 μmol/L	3 h	HB-EGF ↑
			TCDC	200 μmol/L	60 min	TACE activity ↑
		In vivo (BK5 erbB2 mice)	TCDC	2.5 mmol/L 200 μL	Twice/wk for 20 wk	Skin tumor ↑
Qiao *et al*[105]	2001	hepatocytes	DCA	50 μmol/L	5 min	EGFR/Ras/MAPK activation
Rao *et al*[106]	2002	Primary rat hepatocytes	TDCA, TCA, DCA	50 μmol/L	20 min	p-raf-1↑, MEK ↑, ERK ↑
Nagathihalli *et al*[5]	2014	HCT116, HCA-7, BxPC3, AsPC-1, Capan 2	DCA	300 μmol/L	4 h-6 h	TACE co-localization, TGF-α mRNA ↑

DCA: Deoxycholic acid; CA: Cholic acid; MMP: Matrix metalloproteinase.

disrupting cell-cell connections to enter the pancreatic parenchyma. However, the solution to these questions cannot be determined from *in vitro* experiments. *In vitro* studies focus on one type or a few types of cells, and they cannot simulate tissue or organ structures. For example, apical or basolateral membranes of pancreatic ductal epithelium have different cell surface receptors and ion channels[77]. Thus, they have different biological effects caused by contact with bile acids. These effects are different and may even be opposite.

Systemic effects or local tissue effects

After reviewing the role of bile acids in pancreatic cancer formation and progression, more questions are raised. Bile acids enter the bloodstream by enterohepatic circulation. Bile acid receptors, including cell surface receptors and nuclear receptors, are widely distributed in the organs and tissues, including the pancreas. Bile acids regulate endocrine and exocrine functions of the pancreas, and they may be involved in pancreatic cancer formation and progression. We cannot assume that the role of bile acid-induced pancreatic cancer is just due to local effects (reflux); it is more likely to function *via* systemic effects. Moreover, bile acid receptors in different organs and tissues have different effects (liver FXR and intestine FXR). They simultaneously play a pathogenic role and a protective role, which makes studying these processes very complex.

Proportion of different fractions in bile acids

Bile is composed of a mixture of ingredients, and bile acid is the main component of bile. Bile acid itself has different ingredients, including conjugated bile acids and free bile acids, which have different hydrophilic properties, and their ability to cross the cell membrane is different. Glycine-conjugated bile acids have pKa values of 4.3-5.2, and they constitute greater than 60% of the bile, while taurine-conjugated bile acids have pKa values of 1.8-1.9, accounting for approximately 20% of the bile[113]. Therefore, the ratio of glycine-conjugated bile acids and taurine-conjugated bile acids is approximately 3:1. Taurine-conjugated bile acids are soluble and contact cells with a high frequency. They contribute to the role of bile acids as a carcinogen. Gastrointestinal inflammation and tumorigenic effects caused by different components of bile acids, glycine-conjugated or taurine-conjugated, conjugated or free, are not the same. Non-conjugated bile acids have more significant carcinogenic effects[110]. A novel function of UDP glycosyltransferase

8 (UGT8) has been found; it galactosylates bile acids up to 60-fold more efficiently than its activity towards ceramide[114]. This finding suggested that UGT8 might be involved in modulating bile acid signaling. In contrast, UDCA has anti-neoplastic effects but is also commonly used for clinical treatment of biliary tract disease[90,115]. Therefore, we need to understand the variety in the composition and concentration of bile acids in pancreatic cancer patients to further clarify the role of bile acids in pancreatic cancer.

CONCLUSION

Bile acids are associated with most risk factors of pancreatic cancer, including alcohol intake, smoking, a high-fat diet, gallstones, a long common channel, and chronic pancreatitis, as well as obesity, diabetes and hypertriglyceridemia. In addition to systemic effects, bile acids have local tissue effects, and they directly activate cancer signaling pathways. Bile acids are likely to be recognized as signaling molecules in pancreatic cancer in the future.

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REVIEW

Regional but fatal: Intraperitoneal metastasis in gastric cancer

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Abstract

Peritoneal carcinomatosis appears to be the most common pattern of metastasis or recurrence and is associated with poor prognosis in gastric cancer patients. Many efforts have been made to improve the survival in patients with peritoneal metastasis. Hyperthermic intraperitoneal chemotherapy remains a widely accepted strategy in the treatment of peritoneal dissemination. Several phase Ⅱ-Ⅲ studies confirmed that the combined cytoreducitve surgery and hyperthermic intraperitoneal chemotherapy resulted in longer survival in patients with peritoneal carcinomatosis. In addition, proper selection and effective regional treatment in patients with high risk of peritoneal recurrence after resection will further improve prognosis in local advanced gastric cancer patients.

Key words: Gastric cancer; Intraperitoneal metastasis; Regional metastasis; Cytoreductive surgery; Hyperthermic intraperitoneal chemotherapy

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Core tip: The recurrence rate of gastric cancer after surgery within 2 years remains at 79%. Gastric cancer patients with peritoneal metastases have a median survival of only 3.1 mo. Understanding the influence of peritoneal metastasis on survival in gastric cancer patients, the potential molecular mechanism of peritoneal metastasis, and individualized treatment of patients with high risk of peritoneal metastasis is essential for selecting effective treatment strategies in advanced gastric cancer. In this review, we summarized

translational and clinical researches on peritoneal carcinomatosis, providing comprehensive information to better understand the fatal role of peritoneal metastasis in gastric cancer.

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INTRODUCTION

Gastric adenocarcinoma is the fourth most common cancer and the second leading cause of cancer-related death worldwide[1]. Apart from countries with national screening programs such as Japan and South Korea, most gastric cancer patients present with advanced disease because early-stage tumors are usually asymptomatic and often develop metastatic recurrences even after curative resection. Despite improvements in the surgical treatment of gastric adenocarcinoma, a high recurrence rate persists, with a 5-year overall survival rate for all diagnosed patients of only 24.5% in Europe[2] and 40%-60% in Asia[3,4]. The most frequent cause of treatment failure following surgery for gastric cancer is peritoneal dissemination, mainly caused by the seeding of free cancer cells from the primary gastric cancer, which is the most common type of spread. Gastric cancer patients with evidence of macroscopic peritoneal carcinomatosis have very poor prognoses, with a median overall survival of 3-6 mo[5,6].

In this review, we aim to summarize the influence of peritoneal metastasis on survival in gastric cancer patients, the potential molecular mechanism of peritoneal metastasis, and individualized treatment of patients who have high risk of peritoneal metastasis.

PERITONEAL METASTASIS IS THE MOST IMPORTANT FACTOR FOR PROGNOSIS IN GASTRIC CANCER

The Recurrence rate of gastric cancer remains high, particularly in patients with advanced stage disease. Among patients receiving R0 resection, 79% have documented recurrences within 2 years, and the median time to death from the time of recurrence is 6 mo[7]. Many patients, especially those with stage Ⅲ disease, develop locoreginal recurrence, peritoneal recurrence, or distant metastasis[8]. Many investigators have analyzed recurrence patterns of gastric cancer after curative surgery, but the data have shown variable incidences of these patterns. Schwarz *et al*[9] found that the most common pattern was distant metastasis while Eom *et al*[10] found that hematogenous metastasis was most common among patients with early recurrence and locoregional and peritoneal recurrence among patients with late recurrence using a cutoff time of one year after curative resection for patient subgroups. This disagreement was attributed to differences in patient cohorts undergoing evaluation, the cutoff at which recurrence was determined, and the methods for determining recurrence patterns. In addition, autopsy studies revealed only end-stage disease, but not early recurrence patterns, and re-operation series probably reflect early locoregional and peritoneal recurrence. Laparoscopy and peritoneal cytology have been shown to detect occult metastatic disease not seen on conventional imaging[11].

A recent study of 1178 patients with metastatic or recurrent gastric cancer showed that about 46% of patients had peritoneal metastases and about 30% had liver metastases[12]. Several other clinical studies have reported recurrence patterns in a population of patients with early stage to advanced disease[7,9,13-15], showing that 30%-54% of patients had peritoneal recurrence alone or in combination.

Our unpublished data showed in a total number of 349 patients with stage Ⅲ and Ⅳ gastric cancer, peritoneal metastasis was detected in 62.8% of the patients. And 81.1% of the patients developed metastasis in peritoneal cavity (peritoneal and liver) at the time of recurrence or diagnosis. Furthermore, peritoneal cancer involvement is associated with poor prognosis and quality of life compared with metastasis to other organs. Our research showed that stage Ⅳ patients with peritoneal metastasis had shorter survival (7.5 mo *vs* 14 mo) and a higher risk of mortality (HR = 2.026, P = 0.004).

MOLECULAR MECHANISMS OF PERITONEAL METASTASIS

Cancer cells are thought to undergo the following sequential steps to form peritoneal metastases: (1) penetration of cancerous tissues into the visceral serosa; (2) exfoliation of the cancer cells from the primary tumor; (3) dissemination and survival of the cancer cells within the abdominal cavity; (4) adhesion of cancer cells to the peritoneum; (5) invasion of cancer cells through the peritoneal membrane; and (6) formation of the peritoneal metastasis[16]. However, the mechanisms governing the formation of peritoneal metastasis remain poorly understood. A global expression profile of 21168 genes was analyzed in a gastric cancer cell line established from a primary main tumor and other cell lines established from the metastasis to the peritoneal cavity. They found that 24 genes of cell adhesion, epithelial markers, drug metabolism and signal transduction were up-regulated and 17 genes of immune response, cell cycle and adhesion were down-regulated[17] (Table 1). Loss of hypoxia inducible factor-1α may accelerate

Table 1 Twenty-four up-regulated and 17 down-regulated genes in gastric cancer cells from malignant ascites compared with those from primary lesions

Gene expression level	Gene name	Gene function
Down-regulated		
	Nucleobinding 2	Signaling (apoptosis)
	Acyl-Coenzyme A dehydrogenase	Signaling
	Chaperonin containing TCP1	Signaling
	FKBP54	Signaling
	Histone deacetylase 3	Signaling
	p27kip	Signaling
	PAK-interacting exchange factor alpha	Signaling
	CD4	Immune response
	IL4 stat	Immune response
	L2 receptor gamma	Immune response
	IGFBP2	Growth and metabolism
	RAD51 homologue C	Chromosome stability
	Heterogenous nuclear ribonucleoprotein	Cell adhesion
	Integrin β4	Cell adhesion
	Tubulin beta-1 chain	Cell adhesion
	Death associated protein	Apoptosis
	H2A histone family member L	Apoptosis
Up-regulated		
	Dopa decarboxylase	Signaling or progression
	Caveolin-3	Signaling (modification)
	CD9	Signaling
	Dystroglycan1	Signaling
	Inositol triphosphate receptor	Signaling
	LMO 7	Signaling
	Sodium/hydrogen exchanger, isoform 1	Signaling
	Cystein protease (legumain)	Invasion
	Myosin 6	Intracellular organelle transport
	Destrin (actin depolymerising factor)	Interaction with extracellular matrix
	Renal tumor antigen RAGE1	Immune response
	Aldehyde dehydrogenase	Drug metabolism
	Aldo-keto reductase family 1	Drug metabolism
	Keratin 14	Cell adhesion, invasion
	Keratin 7	Cell adhesion, invasion
	Keratin 8	Cell adhesion, invasion
	CD44	Cell adhesion
	Desmoplakin (DPI, DPII)	Cell adhesion
	Galectin 3 (lectin)	Cell adhesion
	Integrin alpha3	Cell adhesion
	Occludin	Cell adhesion
	S100 A10 (ligand of Annexin II)	Cell adhesion
	Leukocyte elastase inhibitor	Apoptosis
	TGFb-induced anti-apoptotic factor	Apoptosis

the development of peritoneal dissemination *via* the upregulation of matrix metalloproteinases (MMP) -1 in gastric cancer cells, which was manifested in a mouse model[18]. MMP-7-positive gastric cancer patients have significantly poorer overall survival and die more frequently of peritoneal recurrence than those patients with MMP-7-negative tumors in a Japanese cohort[19]. Another comparative analysis between the parental cell line GC9811 and its highly metastatic peritoneal counterpart, cell line GC9811-P revealed and confirmed that recombinant human S100 calcium binding protein A4 (S100A4) and cadherin-associated protein beta 1 (CTNNB1) were upregulated and phosphatase and tensin homolog deleted on chromosome ten was downregulated in GC9811-P cells. Identification of these differentially expressed genes could disclose the molecular mechanisms involved and provide new targets for therapeutic intervention to avoid peritoneal dissemination of gastric adenocarcinoma[20]. A recent study revealed that intraoperative hemorrhages were strongly correlated with peritoneal recurrence, probably due to an increased ability of cancer cells and mesothelial cells to adhere to each other in the presence of factors in plasma[16]. Zinc protoporphyrin IX[21] androquoishomeobox protein 1 (IRX1)[22] was reported to inhibit peritoneal metastasis *via* neovascularization. Identification of these differentially expressed genes could disclose the molecular mechanisms involved and provide new targets for therapeutic intervention to avoid peritoneal dissemination of gastric adenocarcinoma. At present, chemokinereceptor 5 antagonism can reduce the potential for gastric cancer cell dissemination[23]. P38- mitogen-activated protein

kinase inhibition by targeted small molecule inhibitor was demonstrated to be beneficial in preventing the peritoneal dissemination in poorly differentiated gastric cancer[24]. Nevertheless, the molecular mechanisms of peritoneal dissemination need to be further clarified to provide more information for peritoneal dissemination therapy.

EFFECTIVE TREATMENTS FOR PATIENTS WITH PERITONEAL METASTASIS

Patients with peritoneal carcinomatosis of gastric origin have an extremely bad prognosis. Systemic chemotherapy improves median survival in metastatic gastric cancer to 7-10 mo, but in patients with peritoneal carcinomatosis from gastric cancer, the same improvement has not been reported[25]. And 20%-50% of patients treated with radical surgery will develop postoperative peritoneal recurrence[26], and intraperitoneal spread of tumor cells was observed in 54% of patients who died of recurrence after surgery for advanced gastric cancer[27].

At present, hyperthermic intraperitoneal chemotherapy (HIPEC) is the most widely accepted strategy among the treatment options for peritoneal dissemination which is the most frequent metastatic pattern in gastric cancer[28]. The theoretical advantage of the HIPEC is to add the direct cytotoxic effects of heat to a high local concentration of used cytostatic drug[29,30]. In addition to the mechanical washing effect, HIPEC also has the theoretical advantage of delivering a higher anticancer drug concentration into abdominal lavage with reduced systemic toxicity. There are many molecular explanations for the effect of HIPEC. For example, induction of apoptosis, alterations of cell membrane property, changes in intracellular proteins and in their synthesis and heat inhibition of DNA repair enhanced by inhibitors of the cellular heat-shock response[31,32].

In gastric cancer patients with peritoneal carcinomatosis, surgical treatments aiming at removing the primary lesion of peritoneal dissemination is palliative. The combination of cytoreductive surgery (CRS) and HIPEC was first described in 1980 by Spratt *et al*[33]. In the following years, Sugarbaker and his colleagues applied and introduced this innovative technique for peritoneal carcinomatosis[34]. Phase Ⅱ-Ⅲ studies revealed that patients who received CRS plus HIPEC obtained better survival results only if completeness of cytoreduction (CCR-0) resection was achieved. However, the survival benefit of HIPEC remains extremely low if cytoreductive surgery can not accomplish sufficient down-staging of the carcinomatosis burden[35-37]. The largest experience published so far was a retrospective French study involving 159 patients which confirmed this combination advantage in a selected CCR0 group of patients[35]. The dismal effect of HIPEC in patients with extensive peritoneal carcinomatosis not amenable to downstaging to CCR-0 may be explained by limited drug penetration leading to no anti-tumor effect on the deeply invasive microfoci[38]. Thus, drug delivery system with high permeability has the potential perspective role in the treatment of extensive peritoneal carcinomatosis cases[39].

OPTIONAL AGENTS FOR INTRAPERITONEAL TREATMENT

Even though multimodal treatment strategies have been used to improve the prognosis of gastric cancer patients with peritoneal recurrence, the results remain unsatisfactory[40]. The oral anticancer drug S1 is a fluoropyrimidine derivative, combining tegafur with two modulators. A recent meta-analysis showed that the use of S1 monotherapy was associated with a significant survival benefit (HR = 0.48, 95%CI: 0.32-0.70, P = 0.0002)[41]. The advantage of S1 over other chemotherapeutic agents is its ability to attain higher concentrations intraperitoneally, due to the higher concentrations of 5-FU and CDHP achieved in peritoneal tumors than in plasma[42,43].

In addition to S1, paclitaxel and docetaxel, which binds to tubulin, leading to microtubule stabilization, and mitotic arrest, also have high sensitivity against diffuse-type adenocarcinoma, which is a common type of peritoneal tumor. And some of these compounds are transported into the peritoneal cavity when administered intravenously[44].

There have been numerous studies evaluating intraperitoneal drug delivery in gastric cancer. Intraperitoneal administration of anticancer drugs enables an extremely high concentration of drugs to directly contact the target cancer lesions in the peritoneal cavity. However, intraperitoneal administration of mitomycin C or cisplatin yielded no apparent therapeutic effects against peritoneal metastasis of gastric cancer due to immediate absorption through the peritoneum[45]. In contrast to these drugs, intraperitoneal administration of paclitaxel was developed to enhance antitumor activity against peritoneal metastasis by maintaining a high concentration of the drug in the peritoneal cavity over a long period, and its clinical effects have been verified by a number of convincing clinical trials in ovarian cancer with peritoneal metastasis.These superior results were due to the pharmacokinetic advantage of taxanes after regional delivery[46]. Taxanes are absorbed through the openings of lymphatic system, such as the milky spots and the stomata which are important sites for the formation of peritoneal dissemination[47], due to their large molecular weight and fat solubility[48]. A phase Ⅰ/Ⅱ study of intraperitoneal docetaxel plus S1 for the gastric cancer patients with peritoneal carcinomatosis demonstrated a superior 1-year overall survival rate of 70%, and peritoneal cytology turned negative in 81% of the patients[49]. Fujiwara *et al*[50] also reported a median survival of 24.6 mo ingastric

cancer with peritoneal carcinomatosis treated with intraperitoneal docetaxel combined with S1.

Although intraperitoneal paclitaxel showed a profound pharmacokinetic advantage 1000 times higher than systemic administration, the main problem of intraperitoneal chemotherapy is the limited depth of penetration of anticancer drugs directly into the tumor. Accordingly, optimum use of paclitaxel may consist of intraperitoneal and intravenous administration, because intraperitoneal paclitaxel reaches the systemic circulation in only a small amount[51]. Actually, Ishigami *et al*[48] established intraperitoneal paclitaxel with S1 plus intravenous paclitaxel as systemic chemotherapy. The phase Ⅱ study showed an overall response rate of 56% of patients with target lesions and decrease or disappearance of malignant ascites in 62% of the patients.

Another recent phase Ⅱ trial in serosa-positive gastric cancer patients showed a higher similar response rate of 71.4%, and the 3- and 5-year OS rates of 78.0% and 74.9%, respectively[52].

In addition, the efficacy of intraperitoneal irinotecan has been demonstrated in several animal studies. The AUC ratio of SN-38 varied between 3.7 and 14.8 depending on the concentration of administered irinotecan[53]. Moreover, pemetrexed has been proven to be an option when used intraperitoneally in a phase Ⅰ trial in ovarian cancer[54].

Except for chemotherapeutic agents, catumaxomab, a rat-mouse hybrid monoclonal antibody, was registered for the treatment of malignant ascites of various epithelial cell adhesion molecule (EpCAM) positive malignancies, including ovarian, gastric, breast and colorectal cancer. Two studies[55,56] demonstrate that this drug seems to improve progression-free survival in patients with gastric cancer (median 71 d *vs* 44 d, $P = 0.03$) and that it seems to improve the survival of patients with gastrointestinal anti-EpCAM positive tumors in intraperitoneal use.

SELECTED POPULATION FOR INTRAPERITONEAL CHEMOTHERAPY

Positive peritoneal cytology was classified as metastatic disease (M1) in the 7th edition of the American Joint Committee on Cancer tumor node metastasis staging system for gastric cancer[57]. Intraperitoneal free cancer cells isolated during peritoneal washing in patients with gastric cancer have been demonstrated to be significantly and independently related to the prognosis, influencing both recurrence and survival. It is important to prevent peritoneal recurrence after curative surgery to improve the prognosis of gastric cancer patients. However, to apply this modality, selection of patients who are at high risk for peritoneal recurrence is crucial. And, the recent trend in treatment is the administration of adjuvant intraperitoneal chemotherapy immediately after resection in patients who are at high risk of peritoneal recurrence[58,59].

Although the precise mechanism driving peritoneal recurrence remains unclear, the presence of malignant cells in the peritoneum at the time of surgery can lead to peritoneal recurrence[60,61]. Therefore, examination of peritoneal fluids has emerged as an option for identifying patients who are at high risk for peritoneal recurrence after curative resection.

Although conventional peritoneal cytology is the standard and reliable method for detecting free cancer cells in the peritoneal wash and for predicting peritoneal metastasis, in large-sample studies, approximately 4%-11% of patients will have cytology positive and therefore it is not practical or cost-effective to perform it in all patients[62]. Furthermore, it lacks sensitivity for the detection of residual cancer cells and prediction of peritoneal spread[63,64]. A recent prospective clinical study demonstrated that conventional cytology is not beneficial for predicting peritoneal recurrence after curative surgery for gastric cancer, because peritoneal washing cytology was not able to predict peritoneal recurrence or survival in gastric cancer patients[65].

A study including 655 patients indicated that intraoperatively assessed macroscopic serosal changes confer a poor prognosis and increased peritoneal recurrence for patients with curatively resected gastric cancer. Macroscopic serosal changes were defined as changes in color or nodular texture of the serosal surface on inspection and palpation. Macroscopic assessment of serosal changes may be a useful indicator that allows better risk stratification of patients with resected gastric cancer in terms of prognosis and peritoneal recurrence[66].

Recently, genetic detection using reverse transcriptase polymerase chain reaction analysis has been found more sensitive than conventional cytology. The target genes of carcinoembryonic antigen (CEA), heparanase, matrix metalloproteinase-7, cytokeratin 20, telomerase, zinc-finger E-box binding homeobox 1 and melanoma-associated gene in single or in combination were used as potent molecular markers[67-69].

However, the amplified mRNA may be derived from dead cells or phagocytes that have engulfed tumor cells, and can be released from hematopoietic cells in an inflammatory context[70]. Therefore, the clinical issue of false-positive cases remains to be addressed. Using DNA methylation or flow cytometry to identify intraperitoneal tumor cells is another valuable alteration for selecting patients who might have a high risk of peritoneal metastasis[71,72].

CONCLUSION

Gastric cancer is the second leading cause of cancer death worldwide and more than half of the gastric cancer patients show disease progression and

die of peritoneal carcinomatosis. Proper selection of intraperitoneal chemotherapy in patients with peritoneal metastasis or patients with potential risk of peritoneal recurrence may be a promising approach to improve the prognosis of advanced gastric cancer patients. Administration of chemotherapeutic agents with a maintaining high concentration and a high permeability in the peritoneal cavity is an ideal choice for intraperitoneal chemotherapy. Moreover, the study of potential biomarkers from peritoneal washing could provide valuable information for a better selection of subsequent treatment combinations.

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REVIEW

Aberrant regulation of Wnt signaling in hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is one of the most lethal malignancies in the world. Several signaling pathways, including the *wingless/int-1* (Wnt) signaling pathway, have been shown to be commonly activated in HCC. The Wnt signaling pathway can be triggered *via* both catenin β1 (CTNNB1)-dependent (also known as "canonical") and CTNNB1-independent (often referred to as "non-canonical") pathways. Specifically, the canonical Wnt pathway is one of those most frequently reported in HCC. Aberrant regulation from three complexes (the cell-surface receptor complex, the cytoplasmic destruction complex and the nuclear

CTNNB1/T-cell-specific transcription factor/lymphoid enhancer binding factor transcriptional complex) are all involved in HCC. Although the non-canonical Wnt pathway is rarely reported, two main non-canonical pathways, Wnt/planar cell polarity pathway and Wnt/Ca^{2+} pathway, participate in the regulation of hepatocarcinogenesis. Interestingly, the canonical Wnt pathway is antagonized by non-canonical Wnt signaling in HCC. Moreover, other signaling cascades have also been demonstrated to regulate the Wnt pathway through crosstalk in HCC pathogenesis. This review provides a perspective on the emerging evidence that the aberrant regulation of Wnt signaling is a critical mechanism for the development of HCC. Furthermore, crosstalk between different signaling pathways might be conducive to the development of novel molecular targets of HCC.

Key words: Hepatocellular carcinoma; Wingless/int-1; Catenin β1; Crosstalk; Canonical wingless/int-1 signaling; Non-canonical wingless/int-1 signaling

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Core tip: The development of hepatocellular carcinoma (HCC) is regarded as a multistage process in which multiple genetic alterations are necessary. The wingless/int-1 (Wnt) pathway is a signaling mechanism that is frequently activated in HCC, especially the canonical Wnt pathway. Moreover, two main non-canonical pathways are also involved in the regulation of hepatocarcinogenesis. Interestingly, the non-canonical Wnt pathway could antagonize the canonical Wnt pathway in HCC. Crosstalk between other signaling pathways and the Wnt pathway has also been shown to promote tumorigenesis. This review highlights the details regarding the Wnt pathway in HCC, which might provide new potential targets for HCC prevention and therapy.

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INTRODUCTION

Hepatocellular carcinoma (HCC), the fifth most common malignancy in men and ninth among women worldwide, is the second leading cause of cancer deaths[1]. There are over half a million new cases diagnosed per year[1]. The pathogenesis of HCC involves a complex multistep process that derives from the accumulation of aberrant genetic and epigenetic changes and the dysregulation of certain signaling pathways[2-4], including the *wingless/int-1* (Wnt) signaling pathway.

Wnt signaling plays crucial roles in the regulation of diverse processes, including cell proliferation, survival, migration and polarization, embryonic development, specification of cell fate, and self-renewal in stem cells[5]. Aberrant activation of Wnt signaling may contribute to numerous malignancies, such as colon cancer[6,7], gastric cancer[8], esophageal cancer[9], HCC[10], and others. Approximately 95% of observed HCC cases showed deregulation of the Wnt signaling cascade[11].

The Wnt signaling pathway is activated *via* both catenin beta 1 (CTNNB1)-dependent (also known as "canonical") (Figure 1) and CTNNB1-independent (often referred to as "non-canonical") pathways (Figure 2). It is suggested that abnormal regulation of the canonical Wnt signaling pathway is a major and early carcinogenic event[12]. The role of the non-canonical Wnt signaling pathway in HCC is also uncertain. Some studies have shown that non-canonical Wnt signaling is activated in HCC[11,13]. However, others have demonstrated that non-canonical Wnt ligands antagonized canonical Wnt signaling[14,15] and inhibited HCC cell proliferation and migration[15]. Here, we present the general molecular pathology of both the canonical and the non-canonical Wnt signaling pathways, and also the crosstalk between distinct signaling cascades and the Wnt signaling in HCC. This will provide potential clinical implications in finding effective therapeutic targets.

WNT SIGNALING PATHWAY

Canonical Wnt signaling

Wnt proteins, which are highly conserved in metazoan, are a family of 19 secreted glycoproteins[16]. The canonical Wnt signaling pathway is operated by stabilizing the transcriptional co-activator CTNNB1 through preventing its phosphorylation-dependent degradation. In a normal steady state, there are two pools for CTNNB1 in cells. One is known to interact with the cell adhesion molecule cadherin 1 (CDH1) at the cell-cell junction. The second is present in the destruction complex in cytoplasm, which is assembled by the scaffold proteins AXIN, the human tumor suppressor adenomatous polyposis coli (APC), glycogen synthase kinase 3 beta (GSK3B, also known as GSK3β), and casein kinase 1 alpha 1 (CSNK1A1)[17].

The second pool assembly maintains the low level of CTNNB1 in cytoplasm through phosphorylation of CTNNB1 at serine-45 (Ser45), Ser33, Ser37 and threonine-41 by CSNK1A1 and GSK3β in the destruction complex[18,19]. Phosphorylated CTNNB1 is subsequently recognized and ubiquitinated by the beta-transducin repeat containing E3 ubiquitin protein ligase (BTRC). BTRC is a component of an E3 ubiquitin ligase. This process results in the proteasomal degradation of the phosphorylated CTNNB1[20]. In the absence of nuclear CTNNB1 translocated from the

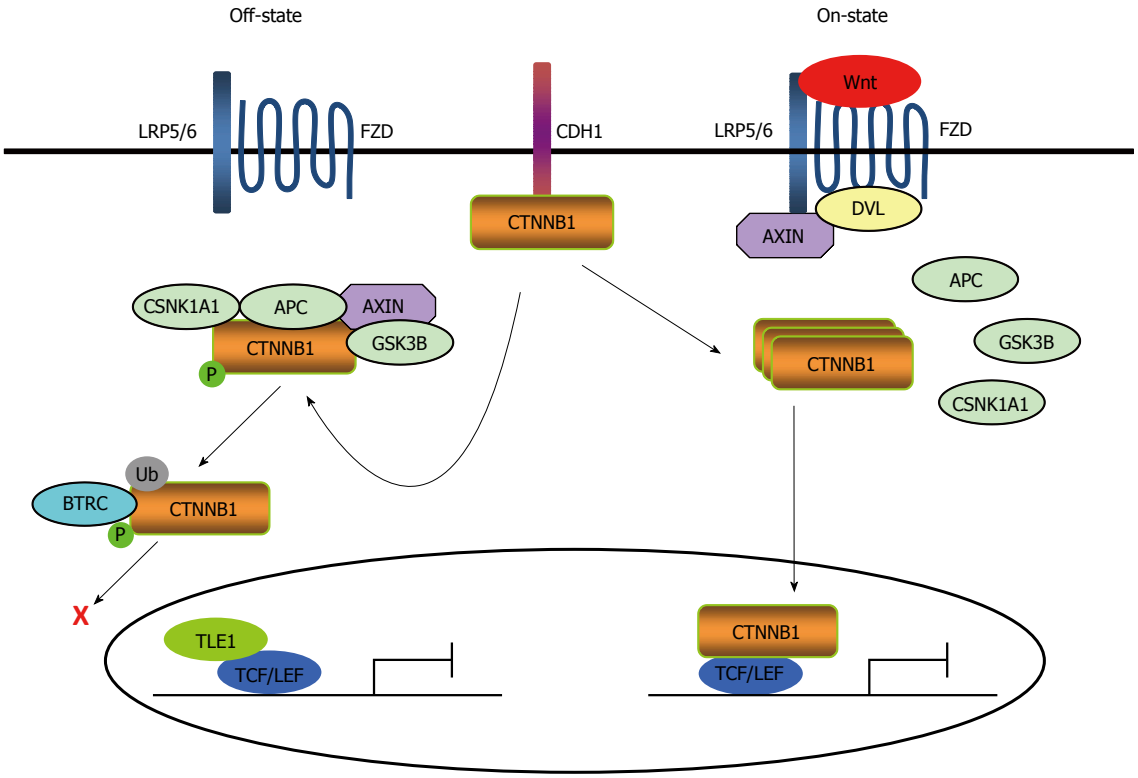

Figure 1 Canonical wingless/int-1signaling pathway. Three complexes are involved in the dynamic activating event: (1) the cell-surface receptor complex; (2) the destruction complex in the cytoplasm; and (3) the CTNNB1/TCF/LEF transcriptional complex in the nucleus. In a normal steady state, there are two pools for CTNNB1 in cells. One is known to interact with CDH1 at the cell-cell junction. The second is present in the destruction complex in cytoplasm, which is assembled by the scaffold proteins AXIN, APC, GSK3β, and CSNK1A1. CSNK1A1 and GSK3β phosphorylate CTNNB1 in the AXIN complex. Phosphorylated CTNNB1 is subsequently recognized and ubiquitinated by BTRC. In the absence of nuclear CTNNB1 translocated from the cytoplasm, TCF/LEF proteins bind to DNA and act as transcriptional repressors by binding to TLE1 proteins. These in turn interact with histone deacetylases whose activities lead to the transcriptional silence of chromatin. The binding of Wnts to FZDs, which form the cell-surface receptor complex, promotes the binding of scaffold proteins such as DVL to the FZD intracellular domains. This subsequently induces the aggregation and phosphorylation of LRP6 and the translocation of AXIN. Phosphorylated LRP6 also recruits AXIN to LRP6 on the plasma membrane. This allows AXIN to be inactivated, which then inhibits CTNNB1 phosphorylation. This in turn allows CTNNB1 to escape degradation, accumulate in the cytoplasm, and translocate to the nucleus. In the nucleus, CTNNB1 interacts primarily with members of the TCF/LEF family of transcription factors and triggers the activation of multiple intracellular signaling cascades. This results in the regulation of various cellular functions. CTNNB1: Catenin beta 1; TCF/LEF: T-cell-specific transcription factor/lymphoid enhancer binding factor; CDH1: Cell adhesion molecule cadherin 1; APC: Adenomatous polyposis coli; GSK3β: GSK3B, glycogen synthase kinase 3 beta; CSNK1A1: Casein kinase 1 alpha 1; FZD: Frizzled class receptor; BTRC: Beta-transducin repeat containing E3 ubiquitin protein ligase.

cytoplasm, T-cell-specific transcription factor (TCF)/lymphoid enhancer binding factor (LEF) proteins act as transcriptional repressors by binding to Groucho/transducin-like enhancers of split 1 (TLE1) proteins. The proteins interact with histone deacetylases, leading to the transcriptional silencing of chromatin[21-23] (Figure 1). In conclusion, three complexes are involved in the dynamic activating event: (1) the cell-surface receptor complex; (2) the destruction complex in the cytoplasm; and (3) the CTNNB1/TCF/LEF transcriptional complex in the nucleus.

Functionally, the Wnt signaling cascade can be activated through several pathways *via* stimulation of distinct Wnt receptors[24,25]. In vertebrates, ten members of the frizzled class receptor (FZD) family of proteins comprise a series of seven-pass transmembrane receptors that have been identified as Wnt receptors[26]. In addition to FZD proteins, single-pass transmembrane proteins, such as the low density lipoprotein receptor-related protein (LRP) 5 and LRP6, have been reported to function as Wnt receptors in the canonical Wnt pathway[27,28]. The binding of Wnts to FZDs which form the cell-surface receptor complex promotes the binding of scaffold proteins, such as disheveled (DVL) proteins, to the FZD intracellular domains. This, in turn, induces the aggregation and phosphorylation of LRP6 and the translocation of AXIN[29,30].

Phosphorylated LRP6 also recruits AXIN to LRP6 on the plasma membrane. This allows AXIN to be inactivated, which then inhibits CTNNB1 phosphorylation. As a result, CTNNB1 succeeds to escape degradation, accumulate in the cytoplasm, and translocate to the nucleus[31]. In the nucleus, CTNNB1 interacts primarily with members of the TCF/LEF family of transcription factors and triggers the activation of multiple intracellular signaling cascades. This results in the regulation of various cellular functions, including gene expression, cell growth and differentiation (Figure 1).

Non-canonical Wnt signaling

Non-canonical Wnt pathways are triggered by seve-

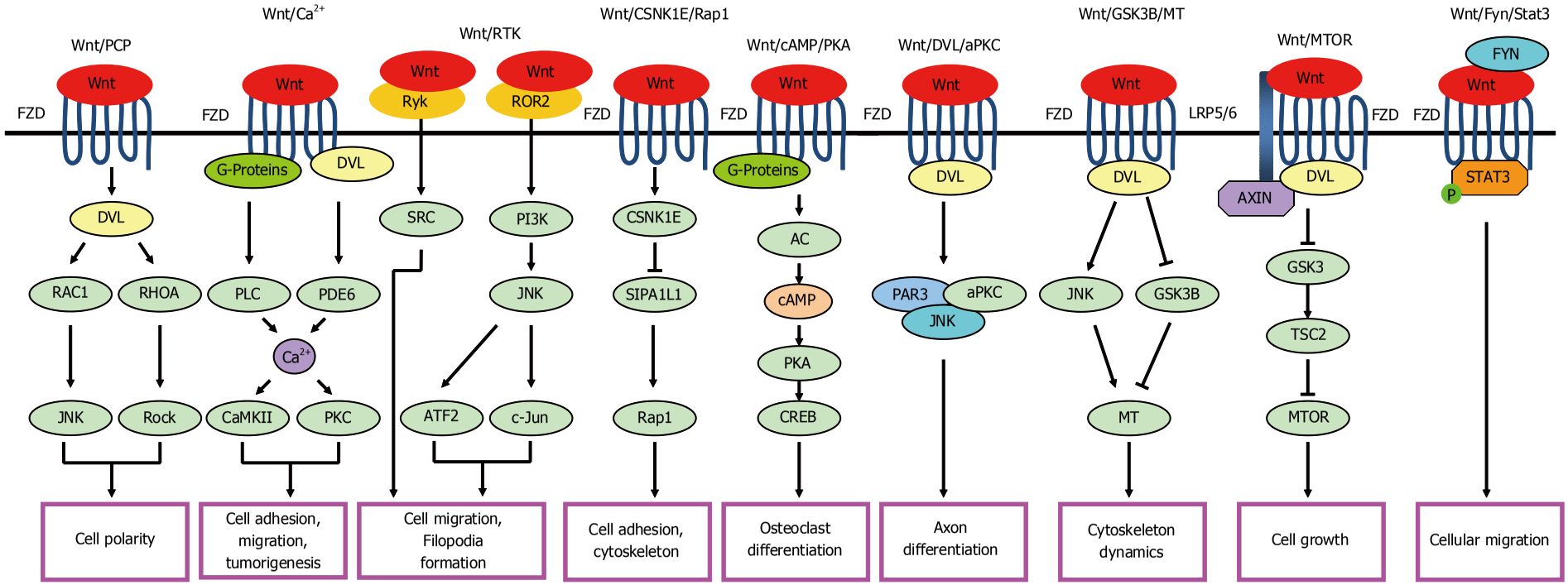

Figure 2 Non-canonical wingless/int-1 signaling pathway. Non-canonical Wnt pathways are mediated by several possible mechanisms which are independent of the CTNNB1-TCF/LEF transcriptional function, including: (1) Wnt/PCP pathway; (2) Wnt/Ca2+ pathway; (3) Wnt/RTK pathway; (4) Wnt/CSNK1E/Rap1 pathway; (5) Wnt/cAMP/PKA pathway; (6) Wnt/DVL/aPKC pathway; (7) Wnt/GSK3β/MT pathway; (8) Wnt/MTOR pathway; and (9) Wnt/FYN/STAT3 pathway. Lines ending with arrows or bars indicate activating or inhibitory effects respectively. Wnt: Wingless/int-1; CTNNB1: Catenin beta 1; TCF/LEF: T-cell-specific transcription factor/lymphoid enhancer binding factor; PCP: Planar cell polarity; RTK: Receptor tyrosine kinases; CSNK1E: Casein kinase I epsilon; cAMP: Cyclic adenosine monophosphate; PKA: Protein kinase A; DVL: Disheveled; aPKC: Atypical protein kinase C; GSK3β: GSK3B, glycogen synthase kinase 3 beta; MTOR: Mechanistic target of rapamycin; FYN: FYN proto-oncogene, Src family tyrosine kinase; STAT3: Signal transducer and activator of transcription 3.

ral possible mechanisms, which are all independent of CTNNB1-TCF/LEF transcriptional function (Figure 2). Among these non-canonical Wnt signaling pathways in vertebrates, the Wnt/planar cell polarity (PCP) pathway and the Wnt/Ca^{2+} pathway have been described in the most detail to date.

Wnt/PCP pathway: This pathway is often initiated by Wnt5a and Wnt11 through FZD and DVL. Next, small GTPases, such as ras-related C3 botulinum toxin substrate 2 (RAC1) and ras homolog family member A (RHOA), are activated by formation of the DVL-RAC1 complex and the DVL-dishevelled associated activator of morphogenesis 1-RHOA complex, respectively. The DVL-RAC1 complex then activates c-Jun N-terminal kinase (JNK). Finally, the triggered RHOA leads to Rho associated coiled-coil containing protein kinase (Rock) activation. This pathway regulates cell polarity in morphogenetic processes, including gastrulation and neural tube closure[32-34].

Wnt/Ca^{2+} pathway: In this pathway, Wnt5a/FZD2 activates phospholipase C *via* the heterotrimeric G proteins. This leads to the generation of dystroglycan 1 and inositol-trisphosphate 3, which increase the intracellular Ca^{2+} flux and levels. The Wnt/FZD complex also activates cyclic GMP-specific phosphodiesterase (PDE6), and

then increases intracellular Ca^{2+} concentration through the depletion of cellular cGMP and the inactivation of cGMP-dependent protein kinase. Ca^{2+} activates calcium/calmodulin-dependent protein kinase II and protein kinase C, which in turn inhibits the canonical Wnt pathway. This leads to a wide variety of effects, such as tissue separation during gastrulation in vertebrates and ventral patterning in Xenopus species, as well as cell adhesion, migration, neurodegeneration, inflammation, and tumorigenesis[35-37].

Wnt/Receptor tyrosine kinases pathway: The receptor tyrosine kinases (RTK) of the receptor-like tyrosine kinase (RYK) and RAR related orphan receptor A (RORA/ROR2) families function as extracellular Wnt-binding domains and are implicated in Wnt signaling[38].

Wnt/RYK pathway: RYK binds to Wnt-induced repulsion of axons and mediates cell migration in *Drosophila* and mice. SRC kinase may act downstream of RYK in flies, wherein it was originally identified as Derailed[36].

Wnt/ROR2 pathway: Wnt5a/ROR2 activates the phosphatidylinositol 3-kinase (PI3K)-cell division cycle 42 (CDC42)-mitogen-activated protein kinase kinase 7-JNK pathway, resulting in the activation of activating transcription factor 2 and c-Jun and the expression of PAPC[39]. ROR2 also binds to the actin-binding protein filamin A and promotes filopodia formation[40,41]. The Wnt5a/ROR2 pathway inhibits the canonical Wnt pathway[36].

Wnt/casein kinase I epsilon/TERF2 interacting protein (Rap1) pathway: Wnt8 activates casein kinase I epsilon (CSNK1E), which enhances the phosphorylation and degradation of signal-induced proliferation-associated 1 like 1 (SIPA1L1), a Rap1-specific GTPase-activating protein. Rap1 is thereby activated in a CTNNB1-independent manner. Rap1 regulates actin cytoskeleton and/or cell adhesion during vertebrate gastrulation[42].

Wnt/cyclic adenosine monophosphate/protein kinase A pathway: Wnt1/Wnt7a activates the G protein and adenylyl cyclase (AC) to increase cyclic adenosine monophosphate (cAMP) levels, which in turn activates protein kinase A (PKA) and the transcription factor cAMP responsive element binding protein 1 (CREB) and myogenic gene expression[36]. Wnt3a can also trigger the cAMP/PKA pathway[43], which could suppress osteoclast differentiation by PKA-mediated phosphorylation and inactivate the nuclear factor of activated T-cells 1 (NFATC1)[44].

Wnt/DVL/atypical protein kinase C pathway: Wnt/FZD signaling induces atypical protein kinase C (aPKC) stabilization and activation *via* interaction with DVL. This pathway can promote axon differentiation mediated by the pulmonary adenoma resistance (PAR) 3/PAR6/aPKC complex[45].

Wnt/GSK3β/microtubule pathway: Wnt/DVL increases microtubule (MT) stability through the concomitant inhibition of GSK3β and activation of JNK. This pathway is involved in the modulation of cytoskeleton dynamics[46].

Wnt/mechanistic target of rapamycin pathway: Wnt activates mechanistic target of rapamycin (MTOR)-mediated translational regulation in tumorigenesis *via* inhibiting GSK3-dependent phosphorylation of tuberous sclerosis 2 (TSC2). DVL, AXIN and APC are all involved in it. Activation of the Wnt/MTOR pathway promotes cell growth and tumorigenesis[47].

Wnt/FYN (FYN proto-oncogene, Src family tyrosine kinase)/signal transducer and activator of transcription 3 pathway: Wnt5/FZD2 can be triggered by FYN through its SH2 domain. The activated complex subsequently recruits and phosphorylates signal transducer and activator of transcription 3 (STAT3) on Tyr705 and finally contributes to the epithelial-mesenchymal transition (EMT) program, cellular migration, and tumor metastasis[48].

The non-canonical Wnt pathways have also been shown to play critical roles, such as in axon differentiation, cell adhesion, cell proliferation, migration and tumorigenesis, in multi-cellular animals.

GENETIC MECHANISMS OF WNT SIGNALING IN HCC

Increasing evidences have shown that the Wnt signaling pathway plays a vital role in HCC[49-51], especially the canonical Wnt pathway[52]. Additionally, two of the main non-canonical pathways (the Wnt/PCP pathway and the Wnt/Ca^{2+} pathway) are also involved in the development of HCC[15,53]. Interestingly, the canonical pathway is antagonized by non-canonical Wnt signaling in HCC[14,15]. Moreover, other signaling cascades have also been found to regulate the Wnt pathway through crosstalk[54-57].

Canonical Wnt signal in HCC

Twenty percent to 90% of HCC cases exhibit CTNNB1 activation[58], which promotes cell growth and invasive capability in c-Myc/transforming growth factor alpha transgenic mice[59]. Simultaneous mutation of CTNNB1 and HRAS leads to 100% incidence of HCC in mice[60]. However, the molecular mechanism of this process is less clear. As described above, three complexes are involved in the dynamic activation of the canonical Wnt signaling pathway. We discuss this below and

according to the regulation of the complexes, including the cell-surface receptor complex, the cytoplasmic destruction complex, and the nuclear CTNNB1/TCF/LEF transcriptional complex.

Dysregulation of the cell-surface receptor complex in HCC: Most of the Wnt ligands and their receptors have been reported to be highly expressed in HCC cell lines. Wnt3, Wnt9a and Wnt10b have displayed strong expression in most HCC cell lines, independent of differentiation status. Wnt2b, Wnt4, Wnt5a, Wnt5b and Wnt7b have been reported as overexpressed in poorly differentiated cell lines, while Wnt8b and Wnt9b have been reported as selectively overexpressed in well differentiated cell lines[14]. Almost all FZD receptors (except FZD9 and FZD10) and two co-receptors have also been reported as overexpressed in HCC cell lines[14]. Furthermore, LRP6 has also been found to be overexpressed in 38% of HCC[61].

It has been reported that HCV core protein correlates with increased Wnt1 and Wnt3a expression in HCC cell lines[62,63]. Interaction between Wnt3a and FZD7 could activate canonical Wnt signaling in different groups of HCC studies[64,65]. FZD7 overexpression has been shown to occur in early HCC and to contribute to enhanced tumor cell migration[65]. Overexpression of LRP6 has been shown to lead to hyperactivation of the canonical Wnt signaling pathway and to result in enhanced cell proliferation, cell migration, and invasion of human HCC[61,66].

Altered expressions of several secreted extracellular antagonists of Wnt ligands, such as secreted Frizzled-related proteins (SFRP), Wnt inhibitory factor-1 (WIF-1) and Dickkopf-related protein 3 (DKK-3), have been detected in HCC. Different SFRPs have been reported to bind with Wnt and thereby down-regulate their ability to activate FZD[67]. Numerous studies have shown that hypermethylation induces down-regulation of SFRPs (SFRP1 and SFRP5) and the subsequent activation of canonical Wnt signaling in HCC[68-71]. Down-regulation of WIF-1 and DKK-3 mediated by promoter methylation has also been reported to be a common event in HCC[72,73].

In addition, the scaffold protein DVL, which binds to the FZD intracellular domain to activate canonical Wnt signaling, has been shown to be up-regulated in a c-Myc/E2F transcription factor 1 transgenic mouse model of HCC[74]. The antagonisms of DVL, which negatively regulate the canonical Wnt signaling, including, dishevelled binding antagonist of beta catenin 2 (DACT2)[75], Prickle-1[76] and the human homologue of Dapper 1 (HDPR1)[77], are down-regulated in HCC.

Abrogation of the cytoplasmic destruction complex and CTNNB1 activation in HCC: Tumor formation is accelerated in HCC cells with active CTNNB1[78,79]. Nuclear accumulation of CTNNB1 is associated with proliferation in HCC cells, whereas CTNNB1 knockdown reduces migration and invasion of HCC cells[80]. However, the molecular mechanism for CTNNB1 activation in HCC still needs further investigation.

Researchers have reported that different degrees of mutations in CTNNB1 lead to the activation of CTNNB1. Reported mutations in exon 3 of CTNNB1 ranged from 2.8% to 44% in HCC cases[52,81-84]. The most frequently mutated site is Ser45, the principal site for phosphorylation mediated by CSNK1A1[85].

Since abnormal CTNNB1 redistribution has been reported in up to 90% of HCC cases[58], and the mutation rate of CTNNB1 in HCC is unmatched (2.8%-44%), it is implied that other mechanisms in addition to the CTNNB1 mutation are involved in the aberrant regulation of Wnt signaling in HCC. Mutations of the destruction complex members in HCC are also reported to contribute to hepatocarcinogenesis. AXIN1[52,84,86] and AXIN 2[86,87] mutations are observed in 5% to 54.2% and around 2.7%-37.5% of HCC cases, respectively. Conditional disruption of AXIN1 leads to the development of liver tumors in mice[88]. However, inactivating mutations of APC and GSK3β are quite rare in human HCC cases[86]. Nevertheless, deletion of APC showed significant connections to HCC through the activation of CTNNB1[89,90], while overexpression of wild-type APC in HCC cell lines reduces canonical Wnt signaling and results in growth suppression[91]. Elevated levels of inactive GSK3β are also observed in both human HCC tissues and mouse models of HCC harboring CTNNB1 accumulation[92-94]. Suppression of GSK3β activation by phosphorylation of Ser9 decreases CTNNB1 activity[92].

Actually, wild-type and mutated CTNNB1 transgenic mouse models indicate that abnormal CTNNB1 is not sufficient for carcinogenic transformation[95,96]. More factors are found in hepatocarcinogenesis mediated by Wnt signaling. Increasing evidences show that several etiologic factors which induce HCC might be involved in the aberrant regulation of canonical Wnt signaling, including HBV, HCV, and carcinogen exposure.

HBV-related HCC: A previous study has determined that mutations in AXIN1 were correlated with HBV-related HCC, whereas mutations in CTNNB1 were correlated with non HBV-related tumors[97]. This implies that mechanisms other than the mutation of CTNNB1 are involved in HBV-related HCC. However, a recent study has shown that genetic polymorphisms in CTNNB1 might affect tumor development and survival in HBV-related HCC[98]. The HBV x gene (HBx) up-regulates von Willebrand factor C and EGF domains (VWCE/URG11) and binds to APC to displace CTNNB1 from the destruction complex, which in turn activates CTNNB1[99]. Thereby, the canonical Wnt signaling is triggered[100].

HCV-related HCC: Inconsistent with the mechanism in HBV-related HCC, CTNNB1 mutation is shown to be

approximately twice as significant in HCV-related HCC compared with other causes[101]. Additionally, more studies have proven the tumor-associated role of Wnt signaling in HCV-related HCC[102]. It has been reported that HCV up-regulates microRNA-155 (miR-155), which promotes the nuclear accumulation of CTNNB1 and an accompanying increase in downstream targets[103]. NS5A protein and core protein of HCV may increase CTNNB1 by activating PI3K and increasing the phosphorylation of GSK3β at Ser9[63,104,105].

Carcinogen exposure-induced HCC: The increased accumulation of CTNNB1 has been shown in around 45% of aflatoxin B1 (AFB1)-associated HCC cases[106]. Further studies indicate that AFB1 exposure might activate the canonical Wnt signaling pathway by down-regulating miR-34[107]. However, there is also research showing a totally distinct role of AFB1 on CTNNB1. The results suggest that AFB1 down-regulates CTNNB1 in HCC[108]. Moreover, HCC is induced in transgenic mice whose liver tumors showed conditional expression of CTNNB1 at 6 mo after diethylnitrosamine (DEN) exposure. However, no tumor is formed in wild-type mice at 6 mo after DEN exposure, indicating that overexpression of CTNNB1 accelerates tumorigenesis and progression to HCC following DEN exposure[109].

Activation of the nuclear CTNNB1/TCF /LEF transcriptional complex in HCC

The human TCF/LEF family consists of four members: TCF-1, LEF-1, TCF-3, and TCF-4[51]. Increased LEF-1 in HCC tissues is associated with cyclin D1 overexpression in the nuclear compartment[110].

In our previous review, the role of aberrantly spliced TCF-4 variants in HCC was discussed[111]. Overexpression of TCF-4J in HCC cells up-regulates the expression level of hypoxia-inducible factor-alpha (HIF-2α) under hypoxia[112]. HIF-2α is capable of modulating TCF-4-mediated transcriptional activity by interacting with CTNNB1[113] and of up-regulating the expression of epidermal growth factor receptor (EGFR)[112]. HIF family proteins are involved in the development of HCC *via* promotion of angiogenesis[114]. EGFR promotes HCC cell proliferation and resistance to anti-cancer drugs[115]. In addition, a dominant-negative form of TCF-4 decreases the expression of c-Myc and cyclin D1 and suppresses the growth of BEL7402 cells[116]. Thirty-three percent of human HCC cases in which shorter survival periods are observed show c-Myc amplification[117]. Both N terminus of HCV NS5A and core protein increase TCF-4-dependent transcriptional activity and subsequently up-regulate the downstream targets, such as c-Myc and cyclin D1 in HCC[63,105].

Non-canonical Wnt signaling in HCC

Rare studies have demonstrated the role of non-canonical Wnt signaling in HCC. Several non-canonical Wnt signaling pathways have been proven to be involved in the regulation of hepatocarcinogenesis, such as the Wnt/PCP pathway[53] and the Wnt/Ca^{2+} pathway[15]. However, different factors induce distinct cell fates within the same pathways.

Cyclin-dependent kinase 14 (CDK14)[118], which is overexpressed in HCC tissues and confers cell invasive potential[119], can regulate cell cycle progression and cell proliferation by specifically interacting with members of cyclin proteins, such as cyclin D3 and cyclin Y[120,121]. Studies have demonstrated that CDK14 up-regulated DVL2 and Naked1 in non-canonical Wnt signaling in HCC by forming a direct complex with cyclin Y[53]. Exogenous overexpression of CDK14 and cyclin Y is also able to activate Rho GTPases (RHOA, RAC1, and CDC42) in HCC. The activated Rho GTPases result in the active formation of actin stress fibers[53], which lead to the modulation of cell motility[122].

Activation of non-canonical Wnt pathways, under some conditions, could suppress HCC. For instance, Wnt11 is reported to activate RHOA and Rock. Activated Rock subsequently inhibits RAC1 which contributes to decreased cell migration and motility in HCC[15].

In addition, the same Wnt ligand could also activate different non-canonical Wnt pathways in HCC. Exogenous overexpression of Wnt11 in HCC cells could also increase cytosolic free Ca^{2+}, and subsequently activate PKC, which translocates from the cytoplasm to the plasma membrane[15].

Regulation of Wnt signaling by crosstalk in HCC

Non-canonical Wnt pathway antagonizes the canonical pathway: It has been reported that the non-canonical Wnt pathway can inhibit canonical Wnt signaling in other cancers[123,124]. However, this phenomenon is rarely reported in HCC. Non-canonical Wnt ligand Wnt5a has been reported to inhibit TCF activation mediated by activated CTNNB1 in HCC cells[14]. Wnt11, which has been shown to inhibit HCC cell proliferation, antagonizes canonical Wnt signaling through phosphorylation of CTNNB1 and reduction of TCF-mediated transcriptional activity induced by activated PKC[15].

Other signaling pathways activate the Wnt signaling pathway: Accumulating evidences have demonstrated that activation of Wnt signaling can act in concert with other oncogenes, such as transforming growth factor beta (TGF-β)[54], hepatocyte growth factor (HGF)/c-Met pathway[55], HIF-1α/EMT pathway[125] and insulin/insulin-like growth factor-1 (IGF-1) pathway[57], to promote tumor progression (Figure 3).

Wnt pathway activation may be mediated by TGF-β[54,126,127]. Interactions between the TGF-β and CTNNB1 pathways are crucial for expression of CTNNB1 target genes in HCC[126]. The TGF-β effector Smad3 can promote the nuclear translocation of CTNNB1[128]. Recently, AXIN2 was reported to be

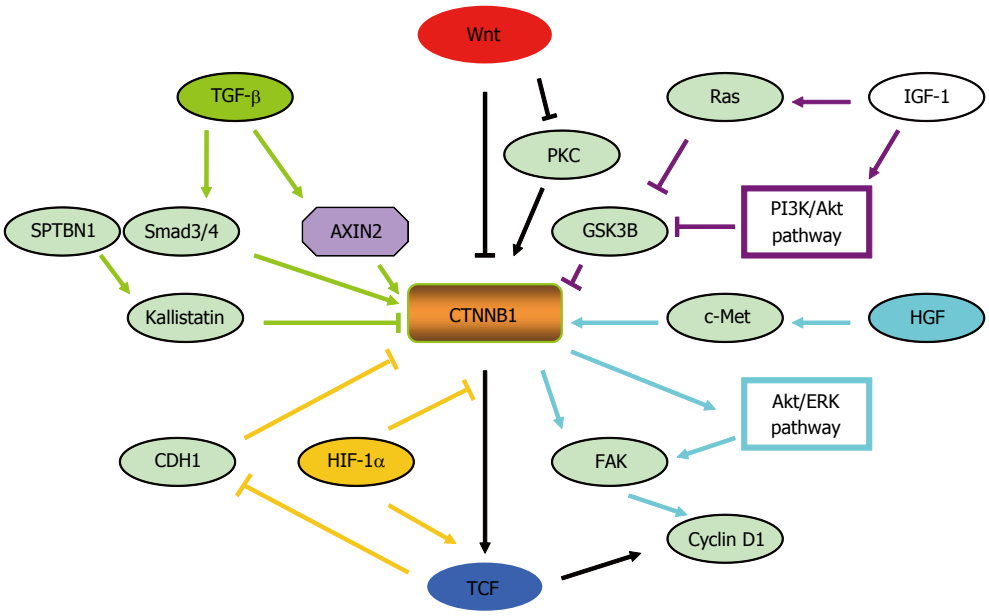

Figure 3 Regulation of wingless/int-1 signaling by crosstalk in hepatocellular carcinoma. The crosstalk between other signaling cascades and the Wnt signaling pathways involved in hepatocarcinogenesis are shown (see text). Lines ending with arrows or bars indicate activating or inhibitory effects respectively. The distinct line colors indicate the different pathways that crosstalk with Wnt signaling, including: Wnt signaling pathway (black), TGF-β pathway (green), HGF/c-Met pathway (blue), HIF-1α/EMT pathway (yellow), and IGF-1 pathway (purple). Wnt: Wingless/int-1; TGF-β: Transforming growth factor beta; HGF: Hepatocyte growth factor; HIF-1α: Hypoxia- inducible factor-1 alpha; EMT: Epithelial-mesenchymal transition; IGF-1: Insulin/insulin-like growth factor-1.

up-regulated by TGF-β treatment in HCC cell lines, resulting in the activation of Wnt signaling[129]. βII-spectrin (SPTBN1), an adapter protein for Smad3/Smad4 complex formation during TGF-β signal transduction, is down-regulated in HCC cells[130]. Loss of SPTBN1 promotes tumor formation and invasion of HCC cells through suppressing Wnt inhibitor Kallistatin and subsequently promoting CTNNB1 dephosphorylation and nuclear localization[130].

Crosstalk between the HGF/c-Met pathway and the Wnt pathway might also contribute to the progression of HCC. C-Met, a tyrosine kinase receptor of HGF, which can be associated with CTNNB1 at the inner surface of the hepatocyte membrane[131], is often co-activated with CTNNB1 in HCC[132]. Co-delivery of c-Met and constitutively active CTNNB1 into mouse livers rapidly induced primary hepatic tumors[132-134]. Monga *et al*[131] have shown that HGF treatment could induce the dissociation of CTNNB1 from c-Met and its subsequent translocation to the nucleus *via* tyrosine phosphorylation. Further studies have determined that CTNNB1 enhanced c-Met-stimulated focal adhesion kinase (FAK) activation and synergistically induced the activation of the AKT/extracellular receptor kinase (ERK)-Cyclin D1 signaling pathway in a FAK kinase-dependent manner[55]. FAK is also reported to be overexpressed in HCC[135] and required for CTNNB1-induced Cyclin D1 expression in a kinase-independent way[55].

EMT is a process of phenotype shifting of cells associated with embryogenesis, inflammation, and cancer metastasis[136]. HIF-1α is reported to mediate the hypoxia-induced EMT *via* up-regulation of transcription effectors such as TCF-3, which suppress CDH1 expression[137]. HIF-1α can compete with TCF-4 to bind with CTNNB1 and form the HIF-1α/CTNNB1 complex. Increased HIF-1α activity, in turn, leads to decreased canonical Wnt signaling activity, and consequently enhanced hypoxia-induced EMT in HCC[56].

Studies have demonstrated that the presence of insulin/IGF-1 could result in CTNNB1 stabilization through inhibition of GSK3β activity, which stimulates TCF/LEF-dependent transcription activation[57]. The activation of PI3K/Akt and Ras might mediate the inactivation of GSK3β[57].

CONCLUSION

Development of HCC is a multistage process precipitated by multiple specific molecular alterations. Several signaling pathways take part in this process, such as the PI3K/Akt pathway, the Wnt pathway, the TGF-β pathway, the HGF/c-Met pathway, and the IGF pathway. Among these, aberrant regulation of the Wnt signaling pathway appears to be an important event leading to inappropriate transcription of various oncogenic target genes. Most importantly, Wnt signaling might play vital roles in hepatocarcinogenesis through crosstalking with several different signaling cascades (Figure 3). However, the molecular mechanisms of the crosstalk in HCC context still demand further investigation.

Considering that targeting the Wnt signaling pathway might provide potential therapeutics in the treatment of HCC, extra studies are still needed.

Our recent study has shown that urolithin A, one of the intestinal metabolites of ellagic acid, exerts antiproliferative and antioxidant effects in HepG2 cells through the inhibition of canonical Wnt signaling[138]. In addition, several types of antagonisms, such as peptides, small synthetic compounds, and blocking antibodies, *etc*. could suppress tumor formation and metastasis by targeting different factors in the Wnt pathway. Some of them target the interaction between the Wnt ligand and the Fzd receptor; some target the destruction complex; the others could target the CTNNB1/TCF/LEF transcriptional complex[5,139-141]. Actually, some commercial medicines for other diseases have been found to modulate the Wnt signaling pathway. For instance, antipsychotic medications like dopamine D(2) receptor antagonist may treat symptoms of psychosis, at least in part, through modulation of the Wnt signaling pathway[142]. The non-steroidal anti-inflammatory drugs aspirin and indomethacin attenuate the canonical Wnt signaling pathway[143]. The cyclooxygenase-2 inhibitor celecoxib can inhibit CTNNB1-dependent transcription in colorectal cells[144] and suppress polyp formation in familial adenomatous polyposos patients[145]. However, these drugs may function through other signaling cascades either. Furthermore, there is still no inhibitor specific to the Wnt signaling pathway that have progressed to HCC clinical therapy. As a result, a better definition of the role of the Wnt pathway in the cascades network during hepatocarcinogenesis may reveal novel molecular targets which might be used for therapy of HCC.

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MINIREVIEWS

Split liver transplantation in adults

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Abstract

Split liver transplantation (SLT), while widely accepted in pediatrics, remains underutilized in adults. Advancements in surgical techniques and donor-recipient matching, however, have allowed expansion of SLT from utilization of the right trisegment graft to now include use of the hemiliver graft as well. Despite less favorable outcomes in the early experience, better outcomes have been reported by experienced centers and have further validated the feasibility of SLT. Importantly, more than two decades of experience have identified key requirements for successful SLT in adults. When these requirements are met, SLT can achieve outcomes equivalent to those achieved with other types of liver transplantation for adults. However, substantial challenges, such as surgical techniques, logistics, and ethics, persist as ongoing barriers to further expansion of this highly complex procedure. This review outlines the current state of SLT in adults, focusing on donor and recipient selection based on physiology, surgical techniques, surgical outcomes, and ethical issues.

Key words: Split liver transplantation; Adults; Graft survival; Graft size; Donor and recipient selection; Surgical technique; Ethical issues

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Core tip: Split liver transplantation (SLT) in adults is usually performed with the right trisegment graft or less frequently with the hemiliver graft. Both graft types require highly complex surgical techniques. Compared with the right trisegment graft, hemiliver SLT requires stricter donor and recipient selection to prevent graft dysfunction associated with size-mismatch. To achieve ideal graft-recipient paring, a clear understanding of surgical anatomy and recipient physiology is needed. With favorable circumstances, outcomes of adult SLT can be comparable to whole liver transplantation. The routine use of SLT, however, remains controversial due to various challenges, particularly under the current "sickest first" liver allocation policy.

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INTRODUCTION

Liver transplantation using partial grafts was born in the late 1980's as a rescue modality for a severe pediatric donor shortage. In 1984, Bismuth *et al*[1] described a new technique to decrease the size of an adult liver to fit a pediatric recipient. After successful experiences with this procedure, a new technique of "splitting" a whole liver graft was successfully introduced, allowing the simultaneous transplant of two recipients from one deceased donor liver[2-4]. Unlike reduced-size grafts, split liver transplantation (SLT) was initially characterized by higher morbidity and mortality[4,5]. Over time, however, technical advancements and better donor-recipient selection have led to more frequent use of SLT and better outcomes.

In SLT, deceased donor livers most commonly are split into a smaller left lateral segment (segment Ⅱ and Ⅲ) for children and a larger right trisegment (segment Ⅰ, Ⅳ-Ⅷ) for adults (Figure 1). This combination has contributed tremendously to the reduction of pediatric waiting list mortality[6]. Gains in knowledge have introduced the use of 2 hemiliver grafts, a left lobe (segment Ⅰ-Ⅳ) and a right lobe (segment Ⅴ-Ⅷ), for transplant in 2 adults or adult-sized recipients (Figure 1). Although hemiliver SLT theoretically doubles the number of liver grafts for adults, this technique has been underutilized due to technical, logistical, and ethical challenges[7]. Further advancement of SLT for adults requires a full understanding of the current state of SLT, focusing on the unique aspects of partial grafting from deceased donors. This review outlines existing practice in adult SLT, including donor and recipient matching, surgical techniques, and outcomes. Finally, ethical issues of adult SLT will be discussed, including how to justify SLT *vs* whole liver graft transplant and in what situations SLT provides the best benefits under the current liver allocation system guided by the Model for End-Stage Liver Disease (MELD) score.

DONOR AND RECIPIENT MATCHING

Donor selection

Careful donor selection and thorough consideration of split graft quality are essential in adult SLT. The upper donor age limit for SLT generally is considered to be between 40 and 50 years of age[8-10]. Prolonged ICU stay before organ recovery is unfavorable, but not a contraindication. Donor liver enzymes should be normal or mildly elevated[11,12]. If other risk factors are absent, split grafts with higher values of liver enzymes can be used[9,10]. While the impact of donor hypernatremia remains unknown, it can be unfavorable. The presence of obesity, history of heavy alcohol use, and low platelet counts upon donor admission could be a surrogate for hidden negative pathophysiology such as graft steatosis and fibrosis. The use of vasopressor to maintain donor hemodynamics can increase the risk of poor graft quality. Despite the lack of scientific evidence, these factors seem to be important to determine whether the liver is suitable for SLT.

During organ recovery, visual and manual evaluation by the donor team is of utmost importance. In the presence of abnormal visualization, a liver biopsy should be performed to rule out any pathology including macrosteatosis, inflammation, fibrosis, and cholestasis. When other donor and recipient factors are ideal, the presence of mild steatosis or inflammation is acceptable. Once the decision is made to proceed with splitting, coordination between donor and recipient teams is crucial in order to minimize cold ischemia time, which is the only modifiable donor factor.

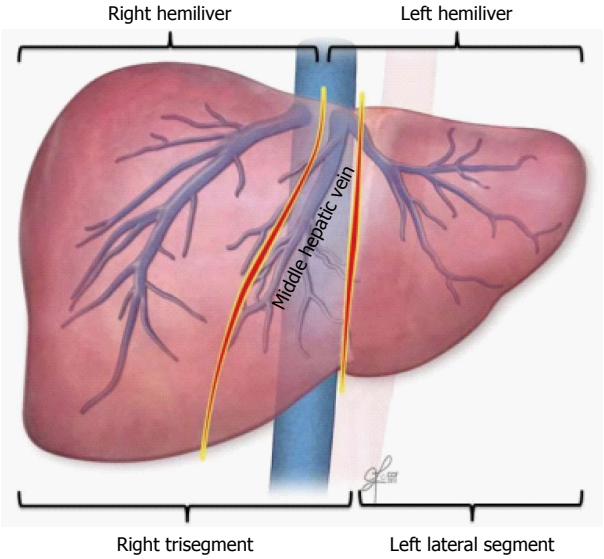

Figure 1 Graft types for split liver transplantation. The most common type is the left lateral segment graft (segment Ⅱ and Ⅲ) for children and the right trisegment graft (segment Ⅰ, Ⅳ-Ⅷ) for adults. In hemiliver splitting, the liver is split on the right side of the middle hepatic vein to yield the left hemiliver graft (segment Ⅰ-Ⅳ) and the right hemiliver graft (segment Ⅴ-Ⅷ) for 2 adults or adult-sized recipients.

Recipient selection

Once a donor liver is deemed to be splittable, choosing and matching an appropriate recipient is extremely important. Hemiliver SLT for adult recipients carries the potential risk of graft failure due to size mismatch, but with a right trisegment graft, graft size does not usually influence surgical outcomes. Recipient

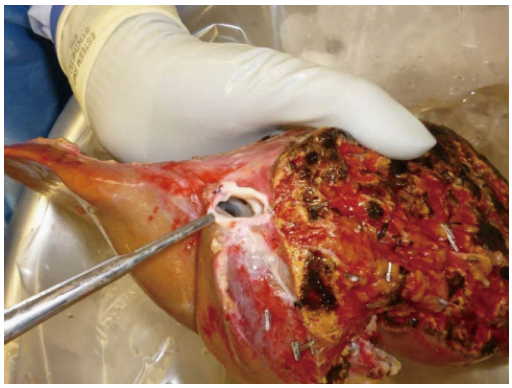

Figure 2 Single venous outflow of the left hemiliver graft. A large common channel of the left and middle hepatic veins is seen.

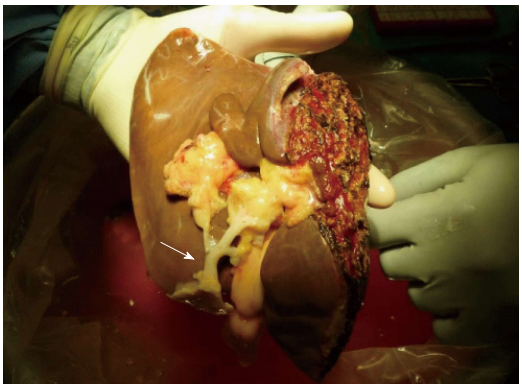

Figure 3 Left hemiliver graft with the celiac trunk (arrow). In this hemiliver split, the celiac trunk was retained with the left lobe graft and the remaining structures including the vena cava, main portal vein, and common hepatic duct were retained with the right lobe graft as described by Bismuth[3].

selection, therefore, can be more liberal with SLT utilizing a right trisegment graft than when hemiliver grafts are used[7]. Equally important, a right trisegment graft provides venous outflow similar to a whole liver graft and will generally tolerate portal hypertension in recipients[7]. On the other hand, recipient selection for hemiliver SLT requires more comprehensive assessment. Generally, teenagers or small adults with minimal portal hypertension are ideal recipients for hemiliver grafts. The use of hemiliver grafts for high-risk recipients, such as those with high MELD scores or severe portal hypertension, remains controversial[13]. Larger grafts should be used for recipients with severe portal hypertension in order to avoid small-for-size syndrome[10].

For living donor liver transplantation (LDLT), in order to meet a recipient's metabolic demand, the minimal graft size has been reported to be as small as a graft-to-recipient weight ratio (GRWR) of 0.6%-0.8%[14,15]. In contrast, the acceptable minimal graft size in adult SLT is unknown. Because split grafts have often experienced prolonged cold ischemia and brain death-related hemodynamic instability, recipients receiving split grafts appear to require a higher GRWR[16]. Lee *et al*[9] reported that a GRWR of 1.0% was the minimal requirement in hemiliver SLT to avoid early graft dysfunction. To achieve such graft-recipient matching, split grafts should be taken from larger donors and transplanted into smaller recipients[10].

Graft size

Graft size estimation is crucial in hemiliver SLT. Since liver imaging is rarely available in deceased donors, graft size estimation usually relies on standard calculation formulas using donor body surface area or body weight: whole liver volume (mL) = 1072.8 × body surface area (m^2) - 345.7 for Caucasians[17] and 706.2 × body surface area (m^2) + 2.4 for Asians[18]. More simply, whole liver weight can be estimated as 2% of donor body weight[19]. Lobe size can be determined based on standard lobar distribution, approximately 35% for the left lobe and 65% for the right lobe. It should be noted that since these estimations are not always accurate, graft weight can be underestimated to increase the risk of small-for-size related graft failure. Therefore, direct assessment by donor surgeons is quite important.

SURGICAL TECHNIQUES

Sharing patterns of split grafts

Lack of consensus regarding sharing patterns of major vessels and bile ducts between 2 split grafts, particularly when a liver is shared by 2 different centers, is one of the most important technical challenges facing SLT. The ideal and most favorable sharing pattern was originally described by Bismuth *et al*[3] in 1989. The principle concept of this sharing pattern is its avoidance of multiple small branches that would need to be reconstructed in recipients. Impeccable knowledge of surgical liver anatomy is crucial to understand why this sharing pattern is ideal in SLT. The left lobe frequently has a single branch of the portal vein, hepatic duct, and venous outflow that is a common channel of the left and middle hepatic veins (Figure 2), but multiple branches of small hepatic arteries often exist. On the other hand, the right lobe often has a single right hepatic artery, but multiple branches are commonly seen in the venous drainage, hepatic duct, and portal vein. According to the original sharing pattern by Bismuth, the left-sided graft retains the celiac trunk leaving a single right hepatic artery in the right-sided graft in order to avoid multiple small branches of hepatic artery in the left-sided graft (Figure 3). Then, the right-sided graft retains the remaining main branches, including the common hepatic duct, main portal vein, and vena cava[3]. Such a sharing pattern can lower the risk of surgical complications by avoiding multiple complex anastomoses. In current clinical practice, however, the primary transplant team often prefers to keep all main branches without consideration of actual donor anatomy or recipient needs, even leaving small multiple branches in the

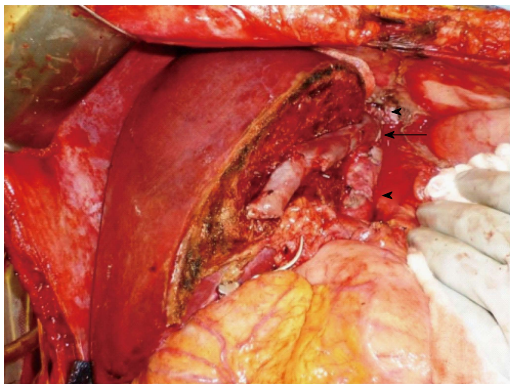

Figure 4 Right hemiliver graft with new middle hepatic vein. Implantation was performed using the conventional caval interposition technique. Two arrowheads indicate caval anastomoses. To prevent venous congestion in the anterior segment, an iliac vein graft was used to create new middle hepatic vein that is anastomosed to the orifice of the left and middle hepatic veins on the graft vena cava (arrow). From Hashimoto *et al*[10].

contralateral graft. While the primary team has the priority to keep main branches, the final decision should be made with flexibility based on donor anatomy and recipient need[10]. Such comprehensive sharing by 2 teams facilitates increased use of split grafts and improves recipient outcomes.

Donor surgery

SLT is a unique operation that requires establishing 2 complete sets of vascular inflow and outflow as well as biliary drainage from one liver graft. SLT organ recovery requires highly complex surgical techniques. Detailed techniques of *in situ* splitting are described previously[20]. The first and most important step for successful SLT is the capability of donor team to make a timely and reliable decision about whether to proceed with splitting. In order to achieve this, the donor team needs to fully understand the recipient situation, including body size, medical urgency, severity of portal hypertension, and surgical anatomy. With visualization of the donor liver, careful assessment of suitability for SLT should be conducted in terms of size, quality, and anatomy. Intraoperative cholangiogram is mandatory to determine splittability. Second, donor operation time should be minimized because of frequent hemodynamic instability in brain dead donors and to avoid compromising graft quality of other organs to be recovered. Hepatic hilar dissection also should be minimized, except for anatomical assessment, because this step can be performed safely on the back table. Liver hanging maneuver is effective for *in situ* parenchymal transection[20,21]. It is important to have a low threshold to cross clamp in case the donor becomes unstable during *in situ* splitting, necessitating a switch to the *ex vivo* technique. Finally, complex back table procedures include the division of vessels and bile duct and venous reconstruction to facilitate venous drainage of the anterior segment in the right lobe graft[20].

Recipient surgery

Excellent venous outflow is essential for successful SLT. Since the right trisegment graft usually retains the entire vena cava, caval anastomosis can be done with either the piggyback or the standard technique, as is done in whole liver transplantation. Such anatomical advantage promises excellent venous outflow. In SLT using the left hemiliver, our standard technique at Cleveland Clinic uses the common channel of the left and middle hepatic veins anastomosed to the recipient venous cuff created with all 3 hepatic veins as the piggyback technique in whole liver transplantation (Hashimoto, unpublished data). This technique promises excellent venous outflow. When the vena cava is retained with the right hemiliver graft, excellent venous outflow can be achieved with a new middle hepatic vein draining into the donor vena cava (Figure 4). When the vena cava is not retained with the right hemiliver graft, a complex venous reconstruction is necessary, as is done with LDLT. Portal inflow should be modified in split grafts of marginal size[22]. Splenic artery ligation, splenectomy, and hemi-portocaval shunt are well known techniques for portal inflow modification. Of these, the use of hemi-portocaval shunt is controversial because of increased risk of portal steal phenomenon[23]. In biliary reconstruction, unnecessary tissue dissection disrupts blood supply to the recipient bile duct and increases the risk of biliary ischemia, bile leak and stricture. Thus, the minimal dissection technique utilized in LDLT should be used for SLT to optimize blood supply to the recipient bile duct, particularly when choledococholedocostomy is performed[24].

OUTCOMES

Right trisegment grafts

SLT using the right trisegment graft initially had an increased risk of morbidity and mortality in adult recipients[4,5]. While surgical outcomes have improved with experience, outcomes for right trisegment graft transplantation are still controversial[25]. Due to the procedure's technical complexity, the incidence of biliary and vascular complications can be as high as 40% and 25%, respectively. However, when multiple risk factors are avoided (short ischemia time, non-urgent recipient status, young donor age, *etc*.), the right trisegment graft can achieve excellent outcomes and is no longer considered to be marginal by experienced centers[26,27].

Hemiliver grafts

Data of hemiliver SLT for 2 adults are limited. Aseni *et al*[28] reported a recent Italian multicenter experience of hemiliver SLT, showing inferior 5-year survival compared to whole liver transplantation (63% *vs* 83%). However, under certain circumstances, long-term survival after hemiliver SLT is equivalent to

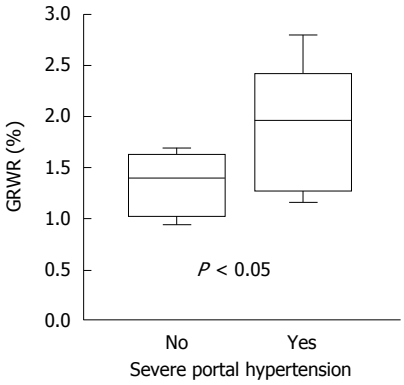

Figure 5 Distributions of graft-to-recipient weight ratio in recipients who received hemiliver grafts at Cleveland Clinic. With ideal graft-recipient matching, the majority of recipients achieved a graft-to-recipient weight ratio (GRWR) > 1.0%. More importantly, hemiliver grafts with low GRWR were avoided in recipients with severe portal hypertension in order to prevent small-for-size related graft failure. A line within the box indicating the mean and the lower and higher boundaries of the box indicating the 25th and 75th percentile, respectively. Whiskers below and above the box indicate the 10th and 90th percentiles. From Hashimoto *et al*[10].

whole liver transplantation or LDLT[9,10,12,29]. Importantly, the impact of graft size on survival seems to be more prominent in hemiliver SLT compared to the right trisegment graft. Accordingly, appropriate graft-recipient selection is critical to avoid small-for-size grafting and to promote optimal outcomes. As mentioned in *Recipient selection*, when a GRWR is greater than 1.0%, hemiliver graft survival appears to be favorable[9]. Our experience at Cleveland Clinic also demonstrates that avoiding smaller grafts for recipients with severe portal hypertension facilitates desirable outcomes (Figure 5)[10]. This strategy increases safety and effectiveness of hemiliver grafts and could result in wider application of hemiliver SLT.

The small-for-size grafts that can result from SLT, particularly hemiliver grafts, often receive excessive portal flow, which causes hepatic arterial spasm *via* hepatic arterial buffer response[30,31]. Importantly, this may increase the risk of hepatic artery thrombosis[31]. Such arterial spasm can cause poor blood supply to the graft biliary system, resulting in an increased risk of biliary complications[32]. Another important surgical risk is early graft failure due to graft-recipient size mismatch. When a small-for-size graft is used for a recipient with severe portal hypertension, modification of the portal inflow may be necessary to prevent graft failure. If this occurs, early retransplantation should be considered before the onset of renal failure or sepsis.

ETHICAL ISSUES

Split graft vs whole liver graft

Creating two extended criteria split grafts from a standard criteria whole liver raises a variety of ethical issues[33,34]. Since partial grafting per se is a risk factor for graft failure[35], one ethical issue is whether it is best to proceed with SLT or wait for a smaller whole liver graft. To justify the use of split grafts, SLT needs to show similar or better outcomes compared to whole liver transplantation, as LDLT has been able to demonstrate[36]. Unfortunately, SLT is not yet considered the standard of care for adult recipients, but it does potentially give recipients greater opportunity for a life-saving transplant. Given unanswered ethical questions, however, recipients should have the unequivocal right to refuse a split graft with complete and accurate national and center-specific information. Thorough discussion of the risks and benefits of SLT with transplant candidates should take place at the time of evaluation, listing, and organ offer[37].

Split liver transplantation in adults under MELD allocation

The use of split grafts for high MELD recipients is controversial[10,13]. Under the philosophy of the "sickest first" liver allocation, splittable donors are often allocated to those with a high MELD score who are generally unsuitable for SLT. When a donor liver is splittable, the best reason to proceed with SLT is when a primary recipient is too small to receive a large whole donor liver. Since small adult candidates are often bypassed on the waiting list when a large donor becomes available, SLT can overcome the large-for-size mismatch and increase opportunity for transplantation for these candidates. For small recipients, split grafts can provide enough liver volume to tolerate portal hyperperfusion, which is considered to be one of the major factors resulting in small-for-size related graft failure. According to our experience, after the primary recipient is transplanted, the leftover split graft can be used safely and effectively for the secondary recipient with similar outcomes[10]. While this graft-recipient combination helps achieve excellent survival after SLT, such ideal matching rarely happens under the MELD allocation. Even with ideal matching, various challenges and higher complication rates result in the underutilization of split grafts, particularly when hemiliver SLT is indicated.

CONCLUSION

SLT is an important technique to increase the availability of livers for adults in need of life-saving liver transplantation. As experience has grown worldwide, resulting in technical advancements and better donor-recipient matching, this highly complex surgical technique has become more feasible and has achieved excellent outcomes. However, the routine application of adult SLT will only be possible when certain challenges are addressed and resolved. While ideal donor-recipient matching is hindered under the current "sickest first" liver allocation, patients can still benefit from SLT under certain circumstances. Continued experience and advancement of SLT will better define the role of SLT in addressing the current severe donor

shortage and reducing wait list mortality in adults.

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MINIREVIEWS

Hepatobiliary and pancreatic ascariasis

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Abstract

Hepatobiliary and pancreatic ascariasis (HPA) was described as a clinical entity from Kashmir, India in 1985. HPA is caused by invasion and migration of nematode, *Ascaris lumbricoides*, in to the biliary tract and pancreatic duct. Patients present with biliary colic, cholangitis, cholecystitis, hepatic abscesses and acute pancreatitis. Ascarides traverse the ducts repeatedly, get trapped and die, leading to formation of hepatolithiasis. HPA is ubiquitous in endemic regions and in Kashmir, one such region, HPA is the etiological factor for 36.7%, 23%, 14.5% and 12.5% of all biliary diseases, acute pancreatitis, liver abscesses and biliary lithiasis respectively. Ultrasonography is an excellent diagnostic tool in visualizing worms in gut lumen and ductal system. The rational treatment for HPA is to give appropriate treatment for clinical syndromes along with effective anthelmintic therapy. Endotherapy in HPA is indicated if patients continue to have symptoms on medical therapy or when worms do not move out of ductal lumen by 3 wk or die within the ducts. The worms can be removed from the ductal system in most of the patients and such patients get regression of symptoms of hepatobiliary and pancreatic disease.

Key words: *Ascaris lumbricoides*; Cholecystitis; Biliary calculi; Cholangitis; Pancreatitis; Recurrent pyogenic cholangitis

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Core tip: *Ascaris lumbricoides* infects more than 1.4 billion people throughout the world. The impact of diseases caused by the parasite had been underestimated. Hepatobiliary and pancreatic ascariasis (HPA) as a clinical entity came in to limelight of late with developments in biliary imaging. Now the disease is recognized as major health problem in endemic regions of the world. However, clinicians all over the world need to be aware of HPA as the disease can be seen in nonendemic areas in migrant population. This article shall focus on the impact of HPA in healthcare in endemic zones and highlight the diagnosis and management options.

Khuroo MS, Rather AA, Khuroo NS, Khuroo MS. Hepatobiliary and pancreatic ascariasis. *World J Gastroenterol* 2016; 22(33): 7507-7517 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i33/7507.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i33.7507

INTRODUCTION

Hepatobiliary and pancreatic ascariasis (HPA) is caused by entry of the nematode, *Ascaris lumbricoides* (*A. lumbricoides*), from the duodenum in to the biliary and pancreatic ductal lumen[1]. Patient present with wide spectrum of symptoms of biliary and pancreatic disease[2]. On repeated invasion, worms often cannot come out of the ductal lumen and die. Subsequent to worm death, sludge and stones form within the bile and hepatic ducts and evolves in to clinical entity of recurrent pyogenic cholangitis[3,4].

HISTORICAL BACKGROUND

HPA as a clinical disease was described from Kashmir, India in 1985[1]. Ascariasis is common infection in Kashmir, India. Around 70% population especially children are infected with this helminth[5]. Surgeons of this region had encountered worms in the bile ducts rarely in their entire surgical practice and impact of this disease in the community had never been highlighted. The organism is highly motile and can move in and out of ducts over short periods and was not expected to be present in the ducts at surgery. With advancement in biliary endoscopy, we investigated the epidemiology of hepatobiliary and pancreatic disease caused by ascariasis in Kashmir, India. Over a 6-mo period, endoscopic retrograde cholangiopancreatograms (ERCPs) were performed in 160 patients presenting with upper abdominal pain to the emergency room of a tertiary care hospital[1]. Thus, ascariasis was nearly as common as gall stones in causing biliary and pancreatic disease. The clinical features, radiological findings and treatment outcome of these patients was evaluated[1]. Originally we named this disease as "Biliary ascariasis". Over the years, we appreciated that ascariasis, in addition to biliary disease causes hepatic and pancreatic disease in a substantial percentage of patients[6] and to include these components of the disease in definition, named it as HPA[7]. We appreciated that ERCP had limitation as a diagnostic tool for HPA, as it was invasive and not available to primary care physicians in the community[8]. To circumvent this, we established ultrasound as an excellent diagnostic modality[9,10]. We defined HPA as an important healthcare concern in this community and over a period of 9 years (1983 to 1991), identified 500 patients with HPA[2] and 263 patients with hepatolithiasis[3] (an aftermath of biliary ascariasis in this community) from a single center from Kashmir, India. We reported on the natural course of HPA[11] and established the algorithm for management of this entity including role of biliary endotherapy[7,12-15].

ASCARIS LUMBRICOIDES

Morphology

Ascaris lumbricoides inhabits gastrointestinal tract of humans[16]. The adult worm life span is around 6 to 18 mo. The adult worm is long, elongated, cylindrical organism with sexual dimorphism. The dimensions of male ascaris are around 15-30 cm × 2-4 mm and female dimensions are 20-40 cm × 3-6 mm. The worm has outer chitinous layer forming the integument of the organism. They reveal strong motor activity through single layer of longitudinal muscle. The worm is devoid of circular muscle fiber layer. The body cavity contains all the viscera including alimentary canal, excretory system, nervous system and the reproductive system. Alimentary tract is a longitudinal canal and consists of mouth, pharyngeal cavity, esophagus, midgut, rectum and cloaca. Excretory system extends all along body as 2 linear streaks. The male and female genital organs are well developed in both sexes. The female releases an estimated 240000 eggs per worm per day. The egg may be fertilized or unfertilized. The fertilized egg (size: 30-40 × 40-60 μm) is ovoid, mamillated, and golden brown and shows evidence of embryonation. The unfertilized egg is elongated (88 to 95 × 44 μm) with thin shell, irregular outer surface and poorly differentiated retractable granules inside[17,18].

Life cycle

A. lumbricoides is a geohelminth and requires moist, shady soil for embryonation of the eggs. Normal habitat for the adult is the jejunum. Four stages in the life cycle include: embryonation, ingestion, larva migration and maturation (Figure 1). Ascaris eggs pass out with the feces. The fertilized eggs embryonate in around 2 wk' time period and molt twice to transform to infective larvae. Eggs remain viable and infective in soil for 10 years. Infective larvae are ingested and start human infection. Eggs are dissolved in stomach by gastric juice and rhabdoid larva of 200 to 300 um are released. The larvae reach the caecum and

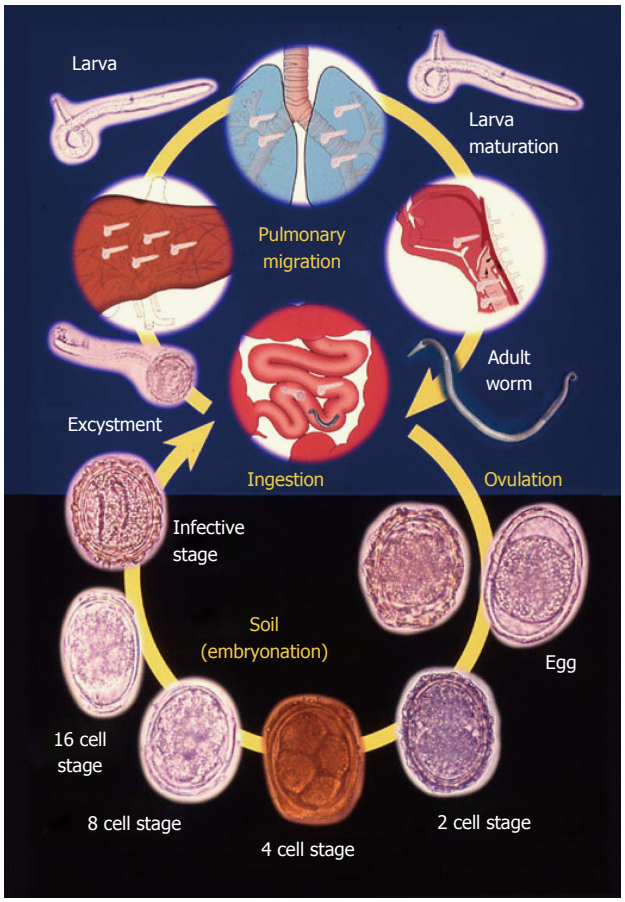

Figure 1 Life cycle of *Ascaris lumbricoides*. Adapted from khuroo *et al*[5].

penetrate across mucosa in to portal vein radicle and reach liver. Larvae again molt twice and considerably enlarge in size. They cross the hepatic sinusoids in to hepatic veins and right heart and reach lungs. Larvae penetrate in to alveoli and traverse along tracheobronchial mucosa to reach larynx, where they are swallowed. Larvae attain sexual maturity in upper small bowel and transform in to adult male and female ascarides. The time taken from ingestion to maturation is around 4 mo[5,16].

Global prevalence

Ascariasis is prevalent worldwide with an overall prevalence of 25%. An estimated 1.4 billion people are infected. Ascariasis is ubiquitous in the Indian subcontinent, China, African continent and Latin America. The prevalence in Japan has dropped over time and now is around 0.04% only. The infection is uncommon in large cities in Europe; however, some rural area have high prevalence. In United States, around 4 million people are infected and ascariasis is the third common helminth infection. Most of those infected are immigrant from developing countries[5,7].

Clinical disease

Majority of ascaris infections are asymptomatic[16]. Clinical disease is restricted to small percentage of individuals with heavy worm-load (Table 1). Estimated 1.2 to 2 million cases of clinical disease occur per year with around 20000 deaths. Ascaris during pulmonary migration and maturation causes the syndrome of Ascaris pneumonia[19,20]. It occurs 4 to 16 d after ingestion of the infective larvae and lasts for 2 to 3 wk. Patients present with seasonal frequent spasmodic cough and wheezing, shortness of breath, and retrosternal distress. Children may present with status asthmaticus, needing intensive care. Diagnosis is established by peripheral eosinophilia and presence of filariform larvae in sputum or gastric aspirate (Figure 2). Ascaris-induced intestinal obstruction is common occurrence in children in endemic areas[21-24]. It is caused by aggregated worm mass blocking the bowel lumen. Massive worm aggregates may cause bowel infarction and gangrene, needing bowel resection (Figure 3). Peritoneal ascariasis occurs due migration of worms in to the peritoneum. In endemic areas worms may enter appendix lumen and cause appendicular

Table 1 Clinical syndromes related to ascariasis in endemic areas

Disease classification	Pathogenesis	Clinical syndromes
Ascaris pneumonia	Larval migration in lungs	Self-limiting pneumonia Status asthmaticus needing ICU admission
Intestinal ascariasis	Ascarides aggregation in small bowel lumen	Intestinal obstruction, bowel infarction and gangrene
Appendicular ascariasis	Ascaris blocking appendix orifice	Appendicular colic, appendicitis, appendicular gangrene
Peritoneal ascariasis	Ascaride-related gut perforation	Peritonitis, septic shock
Gastric ascariasis	Ascarides in stomach and esophagus	Pyloric obstruction (ascaris in antrum and blocking pylorus) Nocturnal chocking (ascaris traversing in to gullet at night) Unique retrosternal itching (ascarides in fundus and lower esophagus)
Hepatobiliary and pancreatic ascariasis	Duodenal ascariasis invading ampullary orifice	Biliary colic (duodenal ascariasis) Acute cholangitis (hepatic ascariasis; massive worm load can cause septic cholangitis and shock) Acalculous cholecystitis (choledochal or gall bladder ascariasis; ascarides in gall bladder may cause gall bladder gangrene) Hepatic abscess (hepatic ascariasis) Acute pancreatitis (duodenal ascariasis or pancreatic ascariasis, ascaride in pancreatic duct can cause necrotizing pancreatitis) Hepatolithiasis (dead ascarides in hepatic ducts forming nidus of sludge/stones)
Stunting of growth, cognitive dysfunction and malnutrition	High ascaride load in children	-

Figure 2 Sputum examination of a child with ascaris pneumonia showing filariform larva.

Figure 3 Massive aggregate of ascarides within jejunum causing obstruction, infarction and gangrene of bowel necessitating bowel resection. Adapted from Khuroo[6].

colic, acute appendicitis and may lead to gangrene of appendix[25]. Syndrome of gastric ascariasis occurs when worms migrate to stomach and esophagus[26]. Gastric ascariasis causes unique dyspeptic symptoms which include worm-related pyloric obstruction, recurrent nocturnal chocking and retrosternal irritation. HPA is a common and well-described entity caused by ascariasis[5]. Children in endemic area with heavy worm-load present with stunting of linear growth and defects in cognitive functions. Children may also show signs of malnutrition as a result of stealing of nutrients by the worms[5].

HEPATOBILIARY AND PANCREATIC ASCARIASIS

Prevalence

HPA is a disease prevalent in endemic areas of world. Large series of patients have been published from several states of India including Kashmir[2], Kolkata[27] and Assam[28] and several other endemic countries namely Saudi Arabia[22,29], Syria[30], Philippines[31] and South Africa[24,32-35]. However, HPA has not been

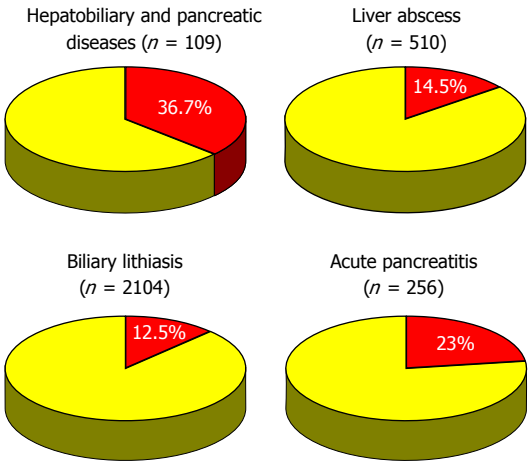

Figure 4 Impact of hepatobiliary and pancreatic ascariasis as an etiologic factor in biliary disease, hepatic abscesses, biliary lithiasis and acute pancreatitis. Numbers in parenthesis shows number of patients with disease and percentages are those caused by ascariasis. Adapted from Ref. [1,3,6,37].

reported from several regions of India. This is related to density of worm load in the community. HPA is restricted to population with high endemicity with high worm load in the population[5]. Prevalence of HPA was estimated by ultrasound in urban population in Kashmir. Five (0.45%) of the 1104 subjects evaluated had evidence of hepatobiliary and pancreatic ascariasis[36]. The impact of HPA on healthcare in Kashmir, India is phenomenal. *A. lumbricoides* is the etiological factor for 36.7% of patients with hepatobiliary diseases[1], 23% of patients with acute pancreatitis[6], 14.5% patients with liver abscesses[37] and 12.5% patients with biliary lithiasis[3] (Figure 4).

Pathogenesis

Ascaris natural habitat is jejunum[16]. HPA is initiated by proximal movement of the organisms in to duodenum (duodenal ascariasis). Heavy worm-load is the main factor for forward march of the ascarides (Figure 5)[5]. They have a propensity to explore the orifices and in duodenum, the organism repeatedly enters in to and out of the orifice of ampulla of Vater. The adult worm blocks the ampullary orifice and obstructs both the bile and the pancreatic duct. In addition, the writhing movements of the worm excites marked sphincter spasm and dysmotility[38]. Patient with duodenal ascariasis present with severe biliary colic[2] (Figure 6). Some of these patients may present with acute pancreatitis[6]. Ascarides often enter the bile duct and traverse up along the bile duct lumen (choledochal ascariasis). Ascarides placed in the bile duct lumen enter and obstruct the cystic duct orifice causing obstructive cholecystitis (Figure 7). The ascarides often move in to intrahepatic ducts (hepatic ascariasis). Left ductal system is more often reached than right ductal system. While in hepatic ducts, worms lead to various grades of acute cholangitis. A proportion of such patients present with suppurative cholangitis,

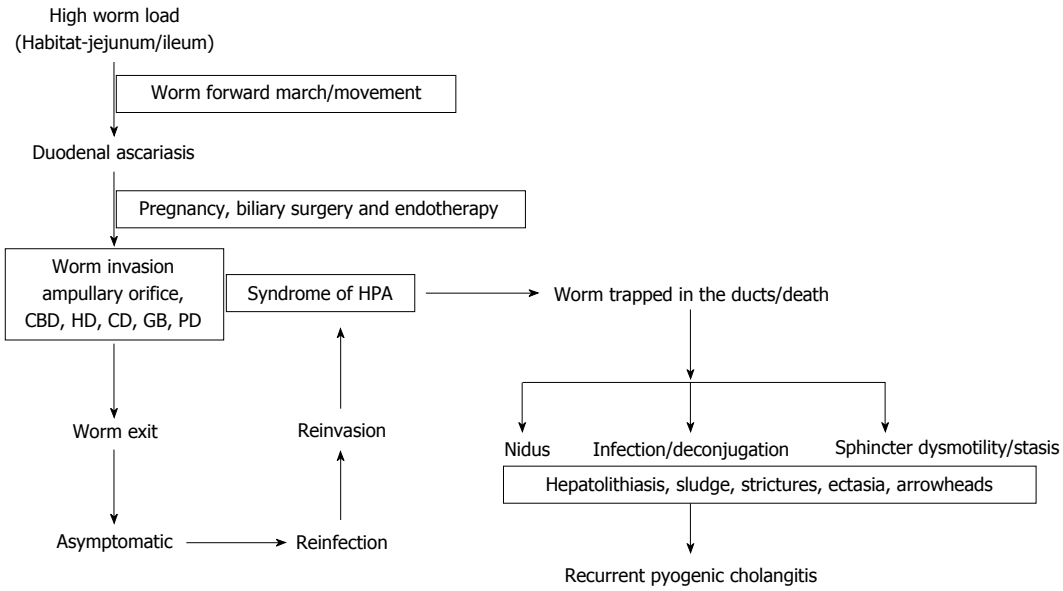

Figure 5 Pathogenesis and natural course of hepatobiliary and pancreatic ascariasis in high endemic regions.

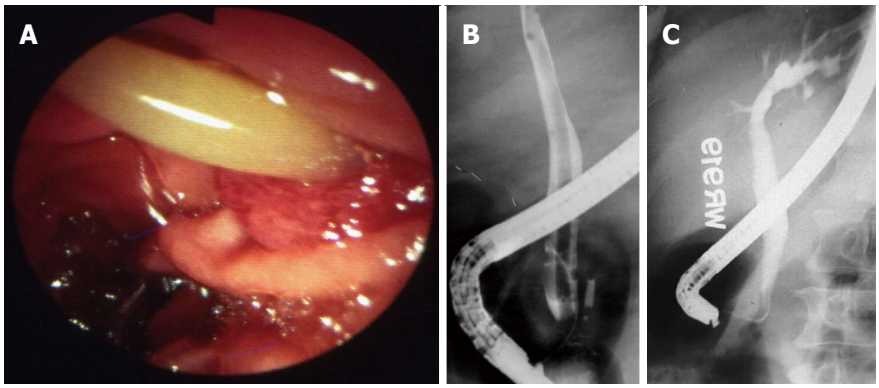

Figure 6 Duodenal ascariasis presenting as biliary colic. A: Duodenoscopy showing adult ascaride in the ampullary orifice; B: Endoscopic retrograde cholangiogram showing long linear filling defect in the common bile duct; C: Cholangiogram after extraction of worms from bile duct. Patient had immediate relief of biliary colic. Adapted from Khuroo[5].

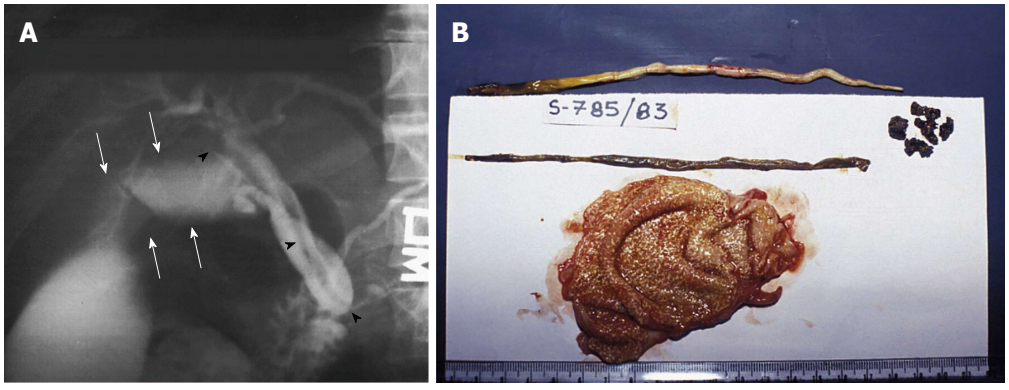

Figure 7 Choledochus ascariasis blocking orifice of cystic duct, causing acute obstructive cholecystitis, needing emergency cholecystectomy. A: Endoscopic retrograde cholangiogram showing linear filling defect in common bile duct (black arrows). Cystic duct and gall bladder is grossly dilated (white arrows); B: Gall bladder showing inflamed gall bladder with 2 adult ascarides and few stones around macerated dead worm. Adapted from Khuroo[5].

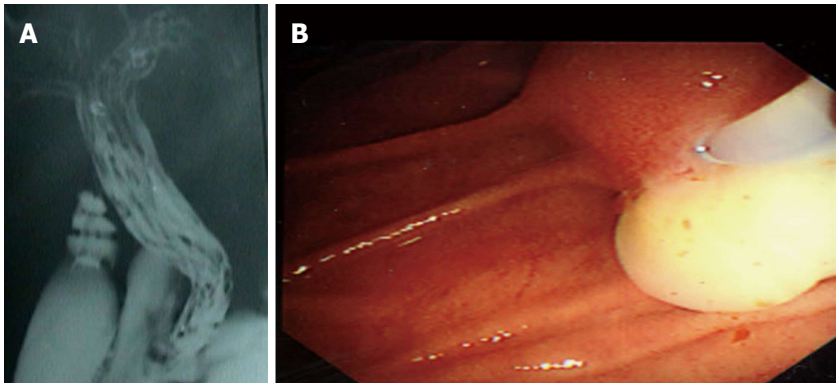

Figure 8 Hepatic ascariasis presenting as suppurative cholangitis. A: Endoscopic retrograde cholangiogram showing palisading of ascarides in common bile ducts and hepatic ducts; B: Duodenoscopic view showing pus exuding from ampullary orifice.

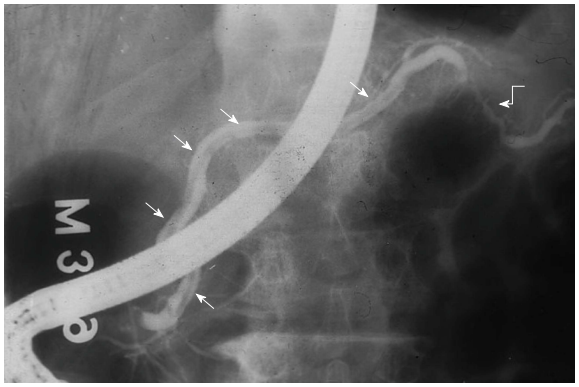

Figure 9 Pancreatic ascariasis presenting as necrotizing pancreatitis. A linear filling defect is seen all along the pancreatic duct (straight arrows). A stricture is seen in the tail region (curved arrow) reminiscent of pancreatic necrosis. Adapted from Khuroo *et al*[2].

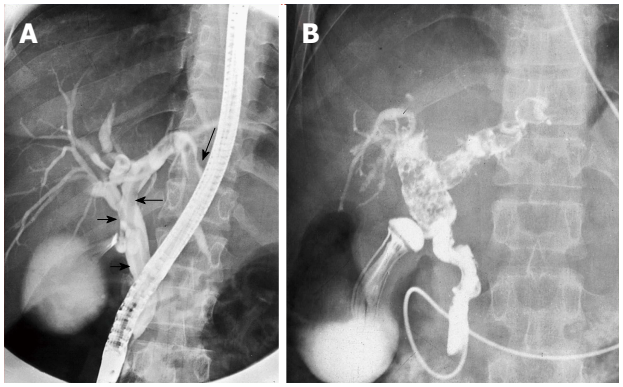

Figure 10 Recurrent pyogenic cholangitis aftermath of biliary ascariasis. A: Endoscopic retrograde cholangiogram obtained 5 years prior to present admission revealing two long linear smooth filling defect in the common and left hepatic duct (arrows); B: Cholangiogram performed during present admission. Cholangiogram revealed biliary dilatation with multiple filling defects and cholangitic changes in the common hepatic, right and left hepatic duct. Naso-biliary tube is in place to treat pyogenic cholangitis. Adapted from Khuroo *et al*[4].

septicemia and septic shock and need intensive care management and urgent endotherapy to decompress biliary tract[2] (Figure 8). Less often ascarides enter the gall bladder (gall bladder ascariasis) and such patients present with biliary pain and cholecystitis[10]. Rarely ascarides may enter pancreatic duct (pancreatic ascariasis) (Figure 9). Patients with pancreatic ascariasis often present with severe necrotizing pancreatitis, which may be fatal[6,39,40].

Ascarides make repeated traverses in to and out of the ductal lumen, as long as they are alive. Ascaris mobility within the ducts is maintained usually till 10 d on serial ultrasound examinations and have a chance to move out of ducts. Often, the ascarides get trapped inside the bile ducts, die and become the nidus and source of biliary sludge and brown pigment biliary calculi (Figure 10)[4,41-43]. Gallbladder is often spared of the stone formation. The stones in the hepatic ducts are related to number of reasons. Dead worm fragments and ascaris ova form a nidus and ideal sites for stone formation. The enteric organisms usually *Escherichia coli* which enter the bile ducts have high beta glucoronidase activity which deconjugate bile pigments. Ascarides in the bile ducts lead to impaired drainage, stasis and formation of biliary sludge and stones. In addition, ascaris invasion causes papillary edema and sphinter of Oddi motor dysfunction which in turn leads to impaired biliary drainage[38]. Malnutrition with low protein intake lowers glucaro 1, 4 lactone, a natural inhibitor of glucoronidase, levels in bile in such patients and accelerates stone formation[4,41].

Clinical profile

HPA is a disease of adults (mean age 35 years with age range 4 to 70 years) with female predominance (female: male ratio 3:1)[1,2]. Ascariasis is more often prevalent in children, however, HPA is seen less often in children[13]. This may be due to smaller size of the ampullary orifice. HPA is commonly seen in pregnant women, possibly due to hormone-induced relaxation and dilatation of ampullary orifice, making it easier for ascarides to enter the ducts. On the same analogy, worms in pregnant women reach gall bladder more often than in non-pregnant population[44-46]. Biliary surgery/interventions namely cholecystectomy, choledocholithotomy, sphincteroplasty, and endoscopic sphincterotomy performed for gallstone disease predisposes patient to ductal invasion

by ascarides[11,31,47]. These procedures cause widened ampullary orifice and lead to easier passage of worms into the bile ducts.

HPA can cause six distinct clinical presentations including[2] (Table 1): (1) biliary colic: Biliary colic presents as sudden severe pain right hypochondrium associated with nausea and vomiting. Vomitus often contains adult live worm and points to occurrence of migration of worms in to duodenum and stomach. The pain may be recurrent in nature or prolonged demanding large doses of narcotic analgesics. Such patients do not develop fever and jaundice to suggest cholangitis. In most patients, ascarides are located in the duodenum and enter in and out ampulla of Vater (duodenal ascariasis) (Figure 6); (2) acute cholangitis: The patients present with right hypochondrium pain, chills and fever, icterus, hepatomegaly, high white cell count and elevated liver tests including bilirubin, transaminases and alkaline phosphatase[48]. A subset of such patients develop suppurative cholangitis and develop systemic sepsis with low blood pressure and acidosis. ERCP shows multiple ascarides in the intrahepatic ducts (Hepatic ascariasis). At duodenoscopy, pus is seen coming out of the ampullary orifice and pus can be aspirated out of the ductal lumen (Figure 8); (3) acalculous cholecystitis: The patients present with pain of in the right upper quadrant and chills and fever. The pain is referred to right shoulder and scapular region. Fever is low grade in nature. Abdominal examination reveals tender right hypochondrium with a tender mass and rebound tenderness and guarding. Ultrasound reveals distended, thick-walled gallbladder, filled with sludge[10,49]. In most patient's ascarides are placed in the bile ducts (Choledochal ascariasis) and block cystic duct orifice, leading to cystic duct dilatation and gallbladder distension (Figure 7). Occasionally worms may traverse the cystic duct lumen and manage to enter the gallbladder (Gallbladder ascariasis). Most of these patients respond to treatment without complications. However, some patients may present with gangrenous cholecystitis needing urgent cholecystectomy; (4) hepatic abscess: Patients with hepatic abscesses develop pain right hypochondrium, high fever, point tenderness in one intercostal space and edema of the right lateral chest wall[37]. Ascarides are placed in the intrahepatic ducts in most of such patients. Pus from the abscess often reveals ova of ascaris and /or fragments of dead ascarides; (5) acute pancreatitis: The patients present with epigastric pain referred to the back, vomiting, and raised serum amylase and alkaline phosphatase. Pancreatitis may be obstructive in nature by ascarides in the ampullary orifice or may be severe necrotizing type due worms entering the pancreatic duct. Ninety percent of patients had mild edematous pancreatitis and 10% develop necrotizing pancreatitis[39,50,51]. A subset of patients with worms in the pancreas develop hemorrhagic pancreatitis, which may be fatal (Figure 9)[40]; and (6) recurrent pyogenic cholangitis: Recurrent pyogenic cholangitis is manifested by biliary tract infection and formation of sludge and stones in the hepatic ducts[3]. In addition, intrahepatic bile ducts develop strictures, excessive branching and arrowhead formation. There are secondary changes in the hepatic parenchyma which include formation of microabscesses, atrophy of liver secondary to chronic biliary obstruction and biliary cirrhosis with progressive end stage liver disease. Recurrent pyogenic cholangitis in Kashmir is an aftermath of repeated worm invasion of bile and hepatic ducts[4,11]. Several pathogenic mechanisms play part in formation of brown pigment stones[38]. Patients develop attacks of pain right upper quadrant with shaking chills and high grade fever and jaundice. Patients may develop septicemia with septic shock. Disease is usually progressive and if untreated, ends up in advanced liver disease.

Natural course

Long-term follow-up of 500 patients with HPA revealed that majority of the patients (54.8%) have duodenal ascariasis and in such patients, ascarides only invade the ampulla of Vater leaving the ducts free of organisms[2]. Ascarides persist within the ductal lumen in a small percentage (2.4%) of patients, while in remaining patients (42.2%) worms move out of ducts within 10 d' follow-up. Ductal reinvasion was observed in 15.4% of patients. Over a 2-year period, seven patients (1.4%) had developed intrahepatic brown pigment stones. None of patients had common bile duct, gallbladder or pancreatic duct calculi. Histopathological analysis of stones revealed segments of adult ascaris forming nidus of the stones.

Diagnosis

Laboratory tests ae not useful in the diagnosis of HPA. However, estimations of blood counts, liver and kidney function tests and serum amylase do help in evaluating the pattern and severity of hepatobiliary and pancreatic disease. Identification of ascaris eggs in the stool has little diagnostic value in endemic areas, as prevalence of ascariasis in such regions may vary from 30% to 90%.

Diagnosis of HPA can be made by ultrasonography, duodenoscopy and ERCP[8-10]. Of late, MRI and MRCP can help in diagnosis of HPA and may replace ERCP if therapeutic procedure is not envisaged (Figure 11)[52-54].

Ultrasound biliary imaging is useful to visualize ascarides in stomach, the duodenal lumen, biliary tree, pancreatic ducts and gall bladder (Figure 12) (Table 2)[55]. Ultrasound also depicts changes secondary to worm invasion including cholecystitis, hepatic abscesses, and pancreatitis. Ascarides in stomach and duodenum are well visualized in water filled lumen as actively motile linear or curved structures. The alimentary canal of the parasite is shown as anechoic tubular structure. In one study from Kashmir, worms were visualized in all the 22 patients with multiple

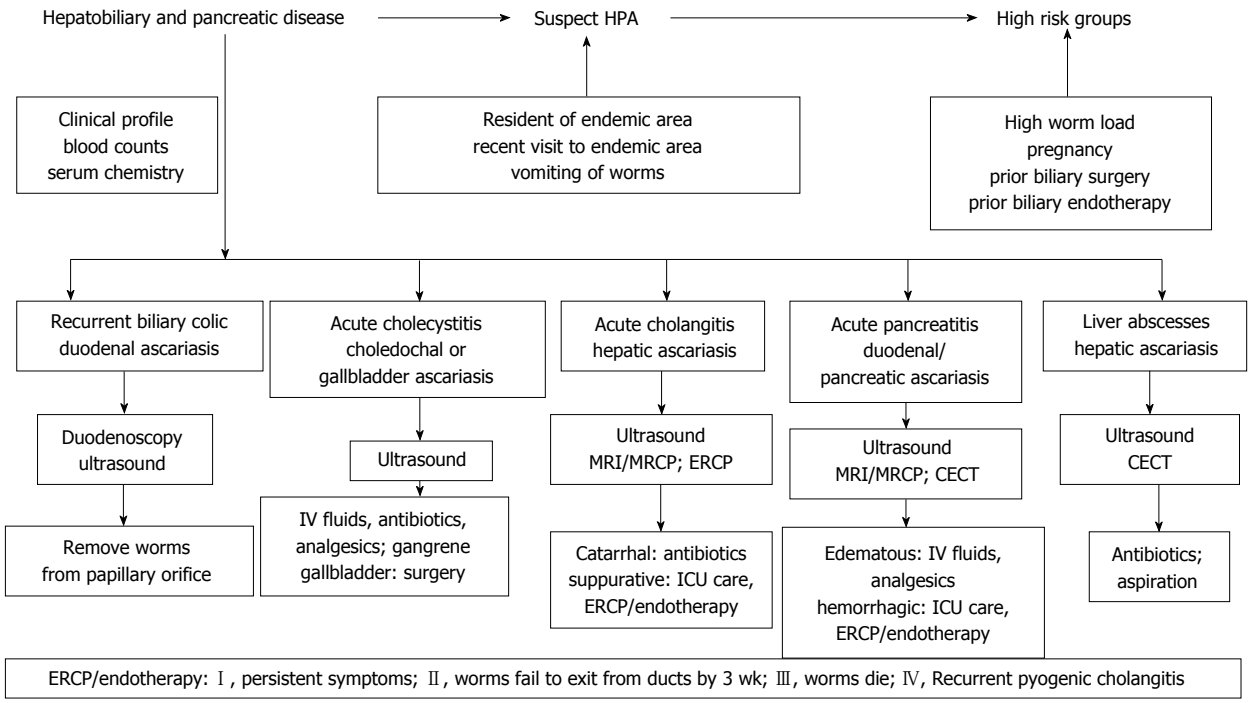

Figure 11 Flow chart showing diagnostic and management algorithm.

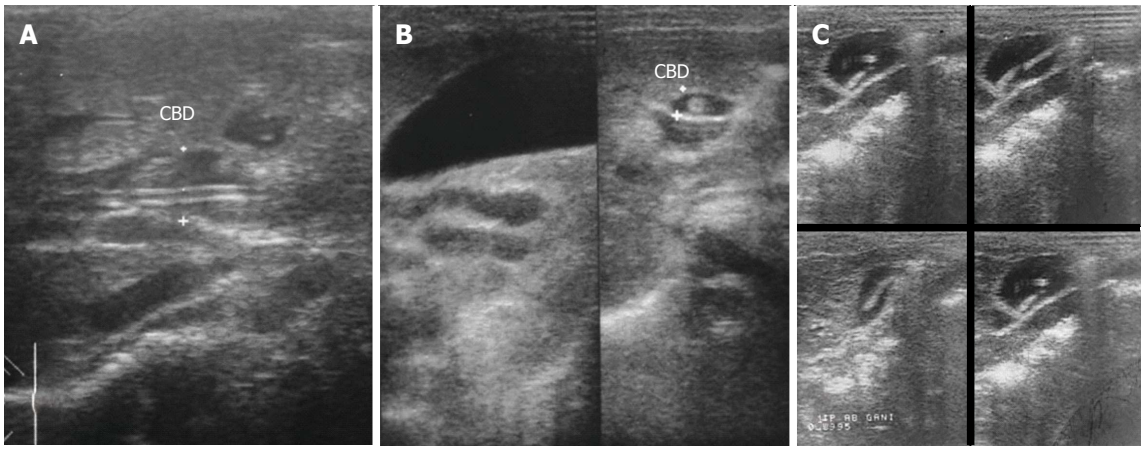

Figure 12 Ultrasonography images in hepatobiliary and pancreatic ascariasis. A: Biliary ultrasound depicting four-line sign; B: Ultrasound showing tube like structure within common bile duct with distended gall bladder; C: Ascarides in gall bladder showing active movements as seen in serial images. Adapted from Khuroo[5].

Table 2 Ultrasonography findings of hepatobiliary and pancreatic ascariasis

Site of ascarides	Ultrasonography findings
Stomach and duodenum	Long linear or curved echogenic strips without acoustic shadowing within water filled duodenum and stomach. The structures show active motility
Hepatobiliary ascariasis	Single or multiple long linear or curved echogenic structures without acoustic shadowing
	Thick long linear or curve non-shadowing echogenic strip containing a central longitudinal anechoic tube (four-line sign), representing the digestive tract of the worm
	Characteristic writhing movement of the echogenic strips within the ducts
	Gall bladder and cystic duct distension; gall bladder wall edema; sludge within gall bladder
	Multiple liver abscesses
Gall bladder ascariasis	Long coiled echogenic structure within gall bladder lumen
	Tubular echogenic structures within gall bladder lumen
	Echogenic structures exhibiting rapid movements
	Distended gall bladder, gall bladder wall edema
Pancreatic ascariasis	Long linear nonshadowing echogenic strips within a dilated pancreatic duct
	Edematous pancreas

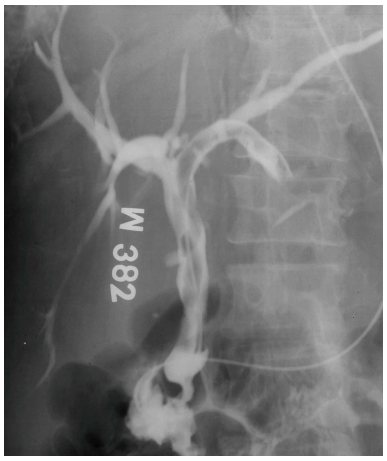

Figure 13 Hepatic ascariasis presenting as suppurative cholangitis managed with nasobiliary tube drainage. Note ascaride placed in common bile duct and left hepatic duct. Nasobiliary tube is in place to decompress and drain the duct.

duodenal and gastric ascariasis[26]. However, ultrasound has limitation in detecting single worm in the duodenal lumen and invading the ampullary orifice[2]. Ascarides in the bile duct lumen show characteristic sonographic appearances[56-58]. The bile ducts are often dilated and gallbladder may be distended with wall edema and sludge within the lumen. The worm is seen as thick, long, linear or curved, non-shadowing echogenic structure/'s (four-line sign) devoid of acoustic shadowing, often with anechoic tubular structure, which represents the alimentary canal of the worm[59]. Ascarides in the bile ducts show characteristic writhing movement and these can be well appreciated on real time ultrasound examination. Ascarides in the bile ducts need to be differentiated from bile duct stones which are visualized as high level echogenic structures throwing acoustic after shadowing. The stones lack characteristic writhing mobility in the ducts, however, may change position within bile ducts with change in the body posture. When compared with ERCP, ultrasound identified ascarides in the bile ducts in 24 (92.3%) of the 26 patients of choledochal ascariasis. Sonography detected ascarides within the bile duct in all 20 patients with 2 or more than 2 worms in the duct and in 4 of the 6 patients with single worm in the duct. In 2 patients with single worm in the bile duct ultrasound findings were reported as normal. Ultrasound was false positive in another 2 patients with biliary obstruction and sludge in the bile ducts[9]. Ultrasonography was accurate in evaluating exit of worms from the ducts in all patients who showed sonographic appearances of HPA. The ascarides in the gallbladder are seen on ultrasound as long, curved and coiled up echogenic structures and their fast, dancing movements are well seen on real time ultrasound examination[60]. Gallbladder is markedly distended with wall edema and sludge within the lumen. Ultrasonography is accurate to detect ascarides in the gallbladder. We visualized ascarides in all 13 such patients within gallbladder lumen. In contrast ERCP had limitation in detecting worms in the gallbladder[10]. Serial ultrasound examination accurately determined the exit of worms from gallbladder lumen. Thus real time ultrasound has advantages over ERCP in this condition, may it be diagnosis or follow up. Pancreatic ascariasis reveals edematous pancreas and ascardies may be visualized within pancreatic ductal lumen. However, ultrasound has limitation in diagnosis of pancreatic ascariasis and picked up worms in the pancreatic duct in only 2 of the seven patients with pancreatic ascariasis. In contrast ERCP has distinct advantage as ascarides can be accurately picked up in the duodenal lumen as well as in the pancreatic ducts[7].

On ERCP, the worms in the dilated bile or pancreatic ducts cause filling defects which have smooth, long and linear characteristics. In addition to above, ascarides may cause a variety of defects namely curved or forming loops[8]. Duodenoscopy often reveals ascarides in the duodenum invading the ampullary orifice. Till late, ERCP has been the investigation of choice for diagnosis of HPA. However, it has several limitations. The procedure is invasive, not available in peripheral health centers in developing countries and difficult to perform on multiple occasions to evaluate worm exit form ducts. Thus ultrasonography should be the initial investigation in diagnosis and follow up of patients with of HPA. ERCP should be employed in selected cases of HPA. ERCP is often needed in patients with duodenal ascariasis, as a single worm in the duodenum and entering in and out of ampullary orifice and causing severe biliary colic may not be visible on ultrasonography. Once the worm is visualized on duodenoscopy, the worm can be extracted to give immediate relief of pain and it is not necessary to fill the ductal system. ERCP is also needed in cases where ultrasound fails to show characteristic worm appearances or is normal in cases with high clinical suspicion. In addition, ERCP has distinct advantages in diagnosis of pancreatic ascariasis as ultrasound has low diagnostic pick up in such patients.

Treatment

The treatment for HPA is to treat various clinical syndromes by appropriate means and administration of anthelmintic drugs. The ascarides once paralyzed are usually expelled out by peristaltic activity of the gut[2,15]. A number of anthelmintic drugs have been developed to effectively manage ascariasis. Anthelmintic drugs which are very effective include pyrantel pamoate, mebendazole, albendazole and ivermectin[5]. Administering anthelmintic directly in to the bile ducts is not advised as it can impede movement of live worms out of the ducts[12,13]. Endotherapy should be performed in case patient's symptoms do not subside on intensive medical treatment and/or ascrides fail to move out of the ductal lumen up to 3 wk of follow up. Patients with suppurative cholangitis often need emergency ERCP and emergency nasobiliary drainage as a first line procedure (Figure 13). Endoscopic removal of live and dead

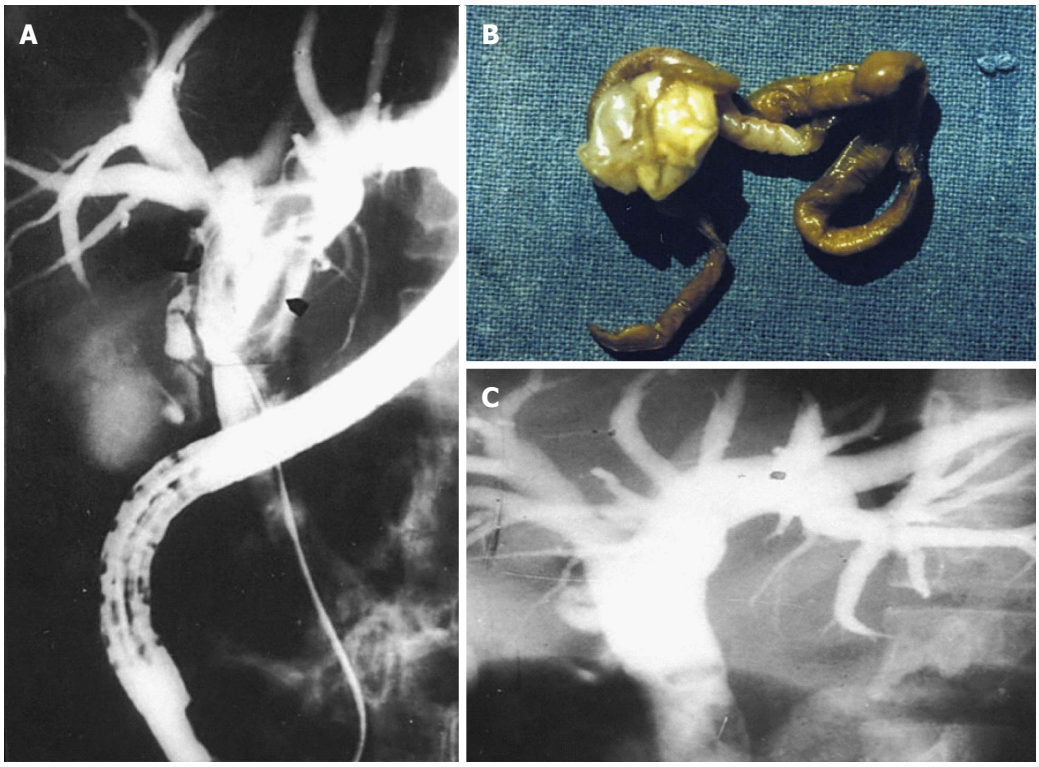

Figure 14 Endotherapy in hepatobiliary ascariasis. A: Cholangiogram showing dilated ducts multiple linear filling defects; B: Dead ascaride extracted from biliary tract; C: Cholangiogram obtained after extraction of dead ascaris. Ducts are free of filling defects. Adapted from Khuroo *et al*[15].

ascarides from ampullary orifice or ductal system is needed in case of severe symptomatic disease or when ascarides are dead within the ductal system (Figure 14). The worms can be removed from the ductal system in most of the patients and such patients get regression of symptoms of hepatobiliary and pancreatic disease.

Control

Control of ascariasis is possible by improving sanitation combined with health education and personal hygiene. Drugs namely pyrantel pamoate, mebendazole, albendazole and ivermectin are safe and effective and can be used for mass chemotherapy. However, several factors jeopardize the fight against this pathogen including cultural taboos, poor resources and financial problems, and huge biotic potential of the pathogen.

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ORIGINAL ARTICLE

Basic Study

Haemoxygenase modulates cytokine induced neutrophil chemoattractant in hepatic ischemia reperfusion injury

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Author contributions: Tapuria N performed the majority of experiments and wrote the manuscript; Junnarkar S helped with experiments and sample collection; Abu-amara M helped with analysis; Fuller B performed literature review; Seifalian AM coordinated the research; and Davidson BR supervised the research.

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Abstract

AIM

To investigate the hepatic microcirculatory changes due to Haemoxygenase (HO), effect of HO inhibition on remote ischemic preconditioning (RIPC) and modulation of CINC.

METHODS

Eight groups of animals were studied - Sham, ischemia reperfusion injury (IRI) the animals were subjected to 45 min of hepatic ischemia followed by three hours of reperfusion, RIPC (remote ischemic preconditioning) + IRI group, remote ischemic preconditioning in sham (RIPC + Sham), PDTC + IR (Pyridodithiocarbamate, HO donor), ZnPP + RIPC + IRI (Zinc protoporphyrin prior to preconditioning), IR-24 (45 min of ischemia followed by 24 h of reperfusion), RIPC+IR-24 (preconditioning prior to. After 3 and 24 h of reperfusion the animals were killed by exsanguination and samples were taken.

RESULTS

Velocity of flow (160.83 ± 12.24 μm/s), sinusoidal flow (8.42 ± 1.19) and sinusoidal perfusion index (42.12 ± 7.28) in hepatic IR were lower ($P < 0.05$) in comparison to RIPC and PDTC (HO inducer). RIPC increased velocity of flow (328.04 ± 19.13 μm/s), sinusoidal flow (17.75 ± 2.59) and the sinusoidal perfusion index (67.28 ± 1.82) ($P < 0.05$). PDTC (HO induction) reproduced the

effects of RIPC in hepatic IR. PDTC restored RBC velocity (300.88 ± 22.109 μm/s), sinusoidal flow (17.66 ± 3.71) and sinusoidal perfusion (82.33 ± 3.5) to near sham levels. ZnPP (HO inhibition) reduced velocity of flow of RBC in the RIPC group (170.74 ± 13.43 μm/s and sinusoidal flow in the RIPC group (9.46 ± 1.34). ZnPP in RIPC (60.29 ± 1.82) showed a fall in perfusion only at 180 min of reperfusion. Neutrophil adhesion in IR injury is seen in both postsinusoidal venules (769.05 ± 87.48) and sinusoids (97.4 ± 7.49). Neutrophil adhesion in RIPC + IR injury is reduced in both postsinusoidal venules (219.66 ± 93.79) and sinusoids (25.69 ± 9.08) ($P < 0.05$). PDTC reduced neutrophil adhesion in both postsinusoidal venules (89.58 ± 58.32) and sinusoids (17.98 ± 11.01) ($P < 0.05$) reproducing the effects of RIPC. ZnPP (HO inhibition) increased venular (589.04 ± 144.36) and sinusoidal neutrophil adhesion in preconditioned animals (121.39 ± 30.65) ($P < 0.05$). IR after 24 h of reperfusion increased venular and sinusoidal neutrophil adhesion in comparison to the early phase and was significantly reduced by RIPC. Hepatocellular cell death in IRI (80.83 ± 13.03), RIPC + IR (17.35 ± 2.47), and PTDC+IR (11.66 ± 1.17) ZnPP + RIPC + IR (41.33 ± 3.07) reduced hepatocellular death. ZnPP significantly increased hepatocellular death ($P < 0.05$ PTDC/RIPC *vs* ZnPP and IR). The CINC cytokine levels in sham (101.32 ± 6.42). RIPC + sham (412.18 ± 65.24) as compared to sham ($P < 0.05$). Hepatic IR (644.08 ± 181.24) ($P < 0.05$). RIPC CINC -1 levels in the early phase (401.62 ± 78.56). And PDTC (HO inducer) CINC-1 levels in hepatic IR (413.36 ± 63.06) were significantly lower. HO inhibition in preconditioned animals with Zinc protoporphyrin increased serum CINC levels (521.81 ± 74.9) ($P < 0.05$). The serum CINC levels were high in the late phase of hepatic IR (15306 ± 1222.04). RIPC reduced CINC levels in the late phase of IR (467.46 ± 26.06), $P < 0.05$.

CONCLUSION

RIPC protects hepatic microcirculation by induction of HO and modulation of CINC in hepatic IR.

Key words: Reperfusion injury; CINC; Microcirculation; Ischaemic preconditioning; Remote preconditioning; Haemoxygenase

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Core tip: This study is novel in demonstrating the *in vivo* microcirculatory changes due to haemoxygenase (HO) induced by Remote ischaemic preconditioning by brief hind limb ischemia (RIPC) in hepatic ischemia reperfusion (IR) injury. HO also decreased CINC levels (cytokine secreted from kupffer cells in hepatic IR) which is significant in reducing neutrophil recruitment and IR injury. HO inhibition abolished the protective effect of RIPC on hepatic microcirculation and was associated with significantly elevated CINC levels, serum transaminases and hepatocellular death. These findings are novel and have not been demonstrated in previous studies. RIPC may have a potential role in donor preconditioning.

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INTRODUCTION

Ischemia reperfusion injury (IRI) in liver transplantation remains a concern since the incidence of primary nonfunction is 5%-10% in liver transplantation and the problem is aggravated in fatty livers. Recent animal experiments by our group have demonstrated that remote ischemic preconditioning (RIPC) by brief periods of limb ischemia and reperfusion significantly improved liver function, microvascular flow and modulation of hepatic microcirculation[1,2]. The role of haemoxygenase (HO)-1 in IR and RIPC has been investigated previously.

Evidence for HO-1 in IR

Observations in animal models of ischemia reperfusion injury of the liver and kidney suggest that there is an increase in microsomal haem content accompanied by a decrease in cytochrome-p450 content[3]. Haem is a source of reactive oxygen species *i.e.,* free oxygen radicals which cause disruption of mitochondrial membranes associated with oxidative stress. The free radicals also inflict endothelial injury and swelling. This leads to reduced flow of red blood cells and sludging. Following reperfusion excessive oxygen results in free radical generation under the influence of the xanthine oxidase system in addition to haem. HO the rate limiting enzyme in the degradation of haem catalyzes the oxidative degradation of haem. There are three isoforms of haemoxygenase, HO-1 (inducible HO) also known as heat shock protein, HO-2 (constitutive HO found mainly in brain and testis) and HO-3 which is related to HO-2 but not well characterised. HO-1 is responsible for degradation of haem in senescent RBCs. Degradation of haem and formation of CO results in consumption of free radicals. Thus HO-1 enhances scavenging of free radicals and could potentially reduce hepatic IR injury by promoting haem degradation[4]. Based on this hypothesis experiments were conducted by Katori *et al*[4] and they found that the HO system was beneficial in reducing hepatic IR in animal liver transplant models. Kato showed that pre-treatment of donor rat livers with cobalt protoporphyrin (COPP) reduced hepatic IR after cold preservation and reperfusion in *ex vivo* models with significantly increased bile flow and portal flow as

compared to non-treated livers[5]. In liver transplant models pre-treatment with COPP enhanced rat survival and decreased histological severity of IR injury in the liver as compared to nontreated rats. The beneficial effects of haemoxygenase have been demonstrated in genetically fat zucker rats[3] with significantly decreased hepatic IR injury in steatotic livers. Thus HO has a key role in the modulation of free radical generation and protection against IRI.

HO-1 has been shown to exert four major beneficial effects: (1) antioxidant function; (2) antiapoptosis; (3) anti-inflammatory function; and (4) maintenance of microcirculation. The functions of HO-1 and CO seem to be related to their ability to modulate immune function[6]. CO has been shown to exert anti-inflammatory actions, regulate cGMP activity through activation of guanylate cyclise which is known to regulate endothelial-dependent vasodilatation and inhibit platelet aggregation[7]. CO inhibits apoptosis by activating MAPK[8]. Endogenously generated CO rather than NO generated from iNOS has been shown to preserve sinusoidal perfusion and to limit hepatic dysfunction in a model of haemorrhagic shock in rats[9]. HO activity may be linked to iNOS (haem protein)[10]. INOS contains haem molecules. CO generated by HO-1 binds to haem ligands in iNOS and prevents binding of NO donor substrates thus producing an inhibitory effect on iNOS[11].

Further studies by McCarter *et al*[12] have shown that in ischemia reperfusion of the limbs there is remote organ injury and the expression of haemoxygenase in remote organs after 3-4 h of limb reperfusion was associated with remote organ protection[12]. Wunder *et al*[13] showed an increase in neutrophil adhesion in postsinusoidal venules and sinusoids following limb ischemia reperfusion injury in a rat model and the administration of chromium mesoporphyrin (HO-blocker) significantly enhanced the number of adherent neutrophils whereas administration of haemin(HO-inducer) significantly reduced the number of adherent neutrophils suggesting the role of HO-1 in remote organ protection[13,14].

RIPC and HO-1

Recently Lai *et al*[15] in a rat model of partial hepatic IR injury showed that remote ischaemic limb preconditioning confers cytoprotection and protection of liver function against IRI due to HO-1 expression. In a rat model of partial hepatic IR, Lai *et al*[15] preconditioned the liver by four brief cycles of prior hind limb ischemia (10 min) followed by 10 min of reperfusion followed by hepatic ischemia and 240 min of reperfusion. Lai *et al*[15] demonstrated that RIPC decreased parenchymal injury, increased HO-1 expression in the liver and HO-1 inhibition by zinc protoporphyrin abolished the protective effects of RIPC. They also showed increased HO-1 expression in Kupffer cells in preconditioned livers.

What is CINC-1?

Functionally, CINC-1 is described as a major neutrophil chemoattractant and activator. CINC-1, induced by IL-1, TNF- and bacterial products, promotes both neutrophil rolling and adhesion, likely through the upregulation of surface integrins. It is also induced early in macrophages and declines more quickly in expression[16]. Kupffer cells, which comprise the largest fixed macrophage population in the liver are the prime source of CINC.

What is the role of CINC-1 in hepatic IR?

Hisama *et al*[16] demonstrated that serum CINC levels peaked 6 hours after reperfusion in animal models leading to activation and recruitment of neutrophils and CINC inhibition was associated with decrease in neutrophil recruitment in IR.

Aim of this study

Lai *et al*[15] demonstrated increased HO-1 expression in kupffer cells in preconditioned livers. However they did not show the changes in microcirculation in hepatic IR or the effect of RIPC induced HO expression on Hepatic IR.

There has been no study which has demonstrated the effect of RIPC on CINC production. As Kupffer cells are known to produce CINC (Cytokine induced chemoattractant responsible for neutrophil recruitment) we investigated potential modulation of CINC production by RIPC induced HO.

MATERIALS AND METHODS

Animals and surgical procedures

All experiments were conducted under project license from home office United Kingdom in accordance with the animals' scientific act 1986. Male Sprague - Dawley rats, weighing 250-300 gms were used. Animals were kept in a temperature controlled environment with a 12 h light-dark cycle and allowed tap water as well as standard rat chew pellets libitium. Animals were anaesthetized with 4 L/min of isoflurane (Baxter, Norfolk, United Kingdom) and maintained with 1-1.5 L/min of O_2 and 0.5%-1.0% Isoflurane. They were allowed to breathe spontaneously through a concentric mask connected to an oxygen regulator and monitored with a pulse oximeter (Ohmeda biox 3740 pulse oximeter, Ohmeda, Louisville, United States).

Polyethylene catheters (0.76-mm inner diameter, Portex, Kent, United Kingdom) were inserted into the right carotid artery for monitoring of mean arterial blood pressure and the left jugular vein (0.40-mm inner diameter, Portex, Kent, United Kingdom) for administering normal saline (1 mL/100 gm/h body weight to compensate for intraoperative fluid loss. The animals were placed in supine position on a heating mat (Harvard apparatus Ltd., Kent, United Kingdom) to maintain their temperature.

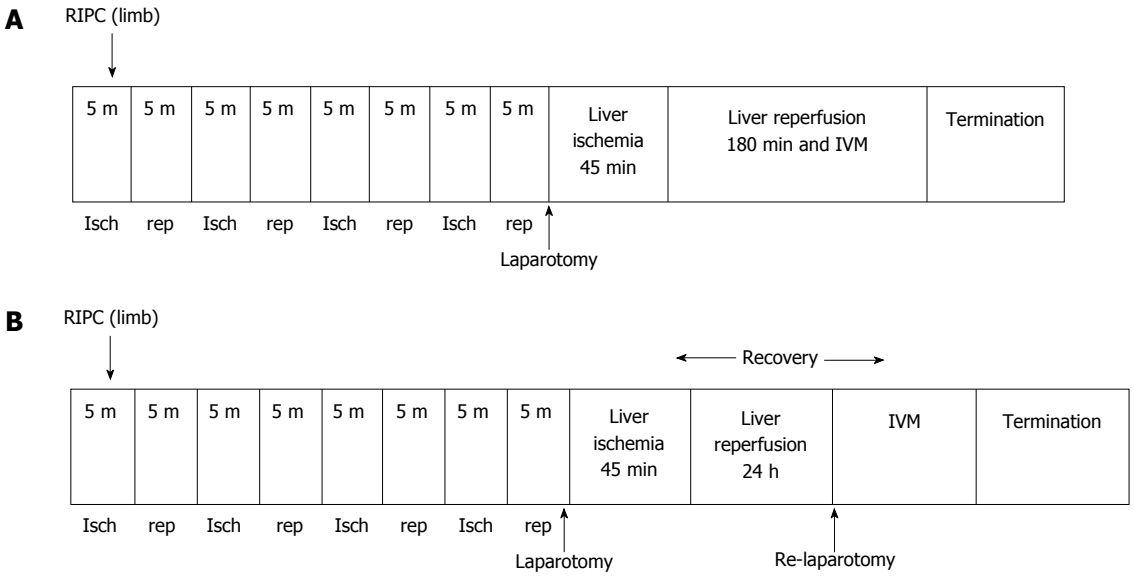

Figure 1 Experimental protocol. A: Early phase of hepatic IR; B: Late phase of hepatic IR. IR: Ischemia reperfusion.

Experimental design

Hepatic IR models for early (Figure 1A) and late phase of IR (Figure 1B). Laparotomy was performed through a midline incision. The hepatic ligaments were cut and the liver was mobilised for exposure. A standard model of lobar hepatic ischemia of the left lateral and median lobes of (70% of liver)[17] was used. Ischemia was induced by clamping the corresponding vascular pedicle with an atraumatic microvascular clamp. Hepatic ischemia was induced for a period of 45 min followed by 3 h reperfusion to study the early phase of hepatic IR and 24 h of reperfusion to study the late phase of hepatic IR. Experiments were terminated by exsanguination of animals at the end of the reperfusion period. All animals had a bolus of heparin (20 U/kg, intravenously) prior to clamping to prevent potential thrombus formation in the hepatic artery. Global ischemia was not induced since the aim of this study was to investigate the effects of warm IR. Lobar ischemia in this model prevented splanchnic congestion and portal hypertension.

Limb preconditioning

A tourniquet was applied around the thigh in one of the hind limbs. Limb perfusion was monitored by a laser Doppler (Moor instruments, Surrey, United Kingdom) and the tourniquet was tightened until no flow was detected. The procedure involved 5 min of ischemia followed by 5 min of reperfusion. This was repeated four times[18].

Recovery after surgery

Animal behaviour was assessed every hour for 4 h and then at 22 and 24 h. Signs of poor clinical condition were lethargy, ruffled fur and guarding upon abdominal palpation, lack of grooming and decreased food intake. Animals which appeared to do poorly were killed before the 24 h reperfusion end point

Re-laparotomy, monitoring and intravital microscopy

The animals were re-anaesthetized after 24 h of reperfusion, homeostasis and monitoring was undertaken as described in the section on animal procedures. The abdominal sutures were carefully opened up and the liver was examined under the intravital microscope.

Animal care

All animals were looked after according to home office United Kingdom guidelines ensuring that they did not suffer pain or distress during these experiments. They were anaesthetised with isoflurane and exsanguinated for collection of tissues and bloods.

Experimental groups (*n* = 6 in each group): Eight groups of animals were studied: (1) Group one (Sham) in which animals were subjected to laparotomy only and underwent an identical experimental protocol without clamping; (2) Group two (IRI) the animals were subjected to 45 min of ischemia followed by three hours of reperfusion; (3) Group three were preconditioned prior to IRI (RIPC + IRI) group. Protocols described above for preconditioning and inducing ischemia were used; (4) Group four sham animals were preconditioned (RIPC + Sham); (5) Group five animals were given Pyrrolidine dithiocarbamate (PDTC) 100 mg /kg 30 min prior to IR (PDTC + IR); (6) Group six animals were given Zinc protoporphyrin (ZNPP) 1.5 mg/kg (HO-1 blocker) by intraperitoneal route 1 h prior to RIPC and subsequent IRI (ZnPP + RIPC + IRI); (7) Group seven animals were subjected to ischemia for 45 min followed by reperfusion for 24 h and then subjected to intravital microscopy (IR-24); and (8) Group eight animals were

Table 1 Scoring used

Numerical assessment	0	1	2	3	4
Sinusoidal congestion	None	Minimal	Mild	Moderate	Severe
Vacuolation	None	Minimal	Mild	Moderate	Severe
Necrosis	None	Single cell	30%	60%	> 60%

preconditioned followed by ischemia for 45 min and reperfusion for 24 h (RIPC + IR-24).

Intravital videofluorescence microscopy

The liver was placed upon a glass mount and covered with a cover slip along with continuous normal saline irrigation. A drop of saline was placed on the cover slip to enable immersion of the microscope lens tip. A Nikon (Tokyo, Japan) microscope (Nikon epi-illumination system with filter block set suitable for Texas Red, FITC and DAPI dyes) coupled to a CCD camera (JVC TK-C1360B (Osaka, Japan) colour video camera) was used. Magnification provided was 10 × and 40 ×. The microscopy images were transferred by camera to a video monitor and recorded for offline analysis. Frame by frame analysis of the recorded images for quantitative analysis was performed. Microcirculation was evaluated by measuring acinar perfusion in ten randomly chosen acini and leukocyte endothelial interaction in ten postsinusoidal venules. LUCIA (lab universal computer image analysis, Nikon, Tokyo, Japan) software was used to analyse the images.

RBC velocity (V): (1) 0.5 mL of FITC labelled red cells suspended in glucose saline buffer solution (20 mgFITC/mL of RBC) were given intravenously. Ten randomly chosen nonoverlapping rappaport acini were assessed[19]; and (2) The RBC velocity was assessed by measuring the length (L) of RBC movement (microns) in each sinusoid in subsequent frames. Twenty-five frames were captured per second. Hence the formula = L × 25/number of frames moved was used for calculating velocity.

Sinusoidal perfusion and perfusion index

The sinusoidal perfusion index was evaluated as ratio of perfused hepatic sinusoids Continuous perfusion (Scp) + intermittent perfusion (Sip) to the total visible sinusoids which includes non-perfused sinusoids (Snp). Perfusion index = (Scp + Sip/Scp + Sip + Snp).

Sinusoidal diameter (D): This was measured by assessing the length across the sinusoids and expressed in microns.

Sinusoidal blood flow: This was calculated using the formula- Velocity (V) ×22/7× (D/2)2.

Neutrophil adhesion: Rhodamine 6G (0.3 mg/kg)[20] was given intravenously for staining of neutrophils[13,14,21]. The numbers of stationary leuokocytes for a period of 30 s under green filter light were expressed as leukocytes/mm^2. The area of the vessels was calculated using the product of diameter and length assuming cylindrical geometry (3.14 × D × L)[22,23].

Hepatocellular death

Propidium iodide[12] (0.05 mg/kg i.v.) was injected intravenously to stain nuclei of dead hepatocytes which was expressed as number of dead cells/HPF[13,14,21].

Tissue and blood collection

Animals were killed by exsanguination and blood was collected in BD vacutainer tube SST^{m}II advance 5.0 mL tubes for Serum, BD vacutainer tube LH 102 I.U > (6 mL) for citrated plasma and BD vacutainer K 2E 7.2 mg (4 mL) for EDTA plasma and centrifuged at 3000 rpm for 10 min to sediment the RBC. Serum and plasma samples were frozen in liquid nitrogen and subsequently stored in -80 degees Celsius. Liver tissue was snap frozen and stored at -80degrees Celsius. Tissues were also fixed in 10% formalin and embedded in paraffin for histology.

Histology

Sections were cut at 5μ and stained with haematoxylin and eosin for histological analysis. Modified Suzuki criteria were used to describe the histological changes in the early phase of hepatic IR. The scoring used is as shown (Table 1).

Immunohistochemistry for HO-1

The paraffin fixed slides were dewaxed in several changes of xylene and then rehydated through absolute alcohol, 90%alcohol, 70%alcohol to distilled water or deionised water (1-2 min in each solvent). Sections of 5μ were used. Mercury pigment was removed with iodine. In order to inactivate endogenous peroxidase and retrieve antigen the slides were immersed for 30 min in a solution of 0.3% H_2O_2 (4 mL of H_2O_2 per 100 mL) in methanol and were rinsed in phosphate buffer solution (PBS). This was followed by blocking of non-specific background with 100 mL of normal rabbit serum (diluted 1:30 with PBS) for 30 min. Primary rabbit polyclonal antibody was diluted in PBS containing 0.05% bovine serum albumin and 0.01% sodium azide at room temperature for 2 h. The slides were rinsed with PBS three times for 5 min each and then incubated for 30 min with 50-100 mL of 1:200 diluted biotinylated antibody solution(anti rabbit IgG). This was followed by three rinses with PBS for 5 min each. They were then incubated with secondary antibody for 30 min at room temperature.

To develop the reaction the sections were incubated with vectastain ABC reagent (Santa cruz technology) using peroxidise as substrate and 3, 3' diaminobenzidine tetrahydrochloride as chromogen. Sections were then counterstained with hematoxylin.

Scoring system used for grading immunohistochemistry changes

The scoring system used for HO-1 expression is based on the system used for assessing oestrogen receptor status of invasive ductal carcinoma cells of the breast in paraffin embedded section[23-25]. The cells which are positive for HO-1 marker are graded into 4 grades depending on the intensity of staining: 0 = No staining; 1 = weak staining; 2 = moderate staining; 3 = strong staining.

This was multiplied with the percentage of cells positive. The percentage was derived by counting the number of positive cells in a total of 100 cells in a high power field. Hence the minimum score possible was 0 and the maximum 100.

Western blot for HO-1

Haemoxygenase protein was identified using Western blotting. Frozen samples of Liver tissue (100-200 mg) were homogenised in TOXEX buffer (20 mmol/L HEPES [pH 7.9], 0.35 mol/L NaCl, 20% glycerole, 1% Nonidet P-40, 1 mmol/L $MgCl_2$, 0.5 mmol/L ethylenediaminetetraacetic acid, 0.1 mmol/L ethylene glycolbis(b-aminoethyl ether)-*N*,*N*-tetraacetic acid, 100 mmol/L dithiothreitol, 0.1% phenylmethylsulfonyl fluoride, 10 mg/mL aprotinin) on ice, incubated for 30 min, and centrifuged at 13000 rpm for 5 min. For each lane, 100 mg of protein was dissolved in 10 mL of 13 sodium dodecyl sulfate loading dye and boiled for 5 min. A biotinylated protein marker (New England Biolabs, Schwalbach, Germany) was added. The samples were separated on a 12% sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore, Bedford, MA) by electroblotting for 2.5 h (Semidry Trans-Blot, BioRad, Hercules, CA). The membrane was blocked in a buffer containing 20 mmol/L Tris-base (pH 7.6), 137 mmol/L NaCl, 3.8 mL 1 mol/L HCl/L, 0.1% Tween (13 TBST), and 5% low-fat dry milk powder for 1 h and incubated with a rabbit polyclonal anti-HO-1 antibody (1:1000 dilution; SPA 895, StressGen, Biotechnologies, Victoria, British Columbia, Canada) in 13 TBST and 5% low-fat dry milk for 2 h at room temperature. After 3 washing steps with 1 X TBST, a secondary anti-rabbit (1:10000 dilution; ECL-detection kit, Amersham Pharmacia, Freiburg, Germany) and horseradish peroxidase- conjugated anti-biotin antibody (1:1000, New England Biolabs) was added and incubated for 1 h in 1 × TBST and 5% low-fat dry milk. Following 2 washing steps with 1 × TBST and 2 washing steps with 1 × TBS, detection was performed by the ECL detection kit (Amersham Pharmacia) according to the manufacturer's instructions. After this the membrane was exposed to a digital camera as part of an electronic imaging system to visualise the proteins bound to the antibody.

Densitometry

This was done by using Adobe Photoshop. The JPEG picture of the western blot was analysed using this software. The maximum density of the film is 255. The density of the background on the film was measured and the density of the HO band obtained for each sample was measured. The density of the background was subtracted from the density of the HO band. The value obtained was subtracted from 255 to obtain the final density of the sample

Elisa for CINC-1

Reagents: One 96 well polystyrene microplate coated with polyclonal antibody specific for rat CINC-1 and 12.5 mL polyclonal antibody against rat CINC-1 conjugated to horseshoeradish peroxidise. Rat CINC-1 Standard**-** 2.5 ng of recombinant rat CINC-1 in a buffered protein base. Rat CINC-1 control-1 vial of recombinant rat CINC-1 in a buffered protein base. Assay diluent RD1W- 12.5 mL of a buffered protein solution with preservatives. Calibrator diluent RD5-4-21 mL of a buffered protein solution with preservatives. Wash buffer concentrate-50 mL of a 25 fold concentrated solution of buffered surfactant with preservatives.

Colour reagent A-12.5 mL of stabilized hydrogen peroxide. Colour reagent B- 12.5 mL of stabilized chromogen (tetramethyl benzidine). Stop solution-23 mL of a diluted HCL. Plate covers- 4 adhesive strips.

Sample preparation: Rat serum samples were diluted 2-fold into calibrator diluent RD5-4 prior to assay.

Assay procedure

50 μL of assay diluent RD1W added to each well. 50 μL of standard, control or sample per well added. The plate was gently tapped for 1 min to mix the contents and covered with adhesive strip. This was followed by incubation for 2 h at room temperature. Each well was aspirated and washed for a total of five times with wash buffer (400 μL). 100 μL of rat CINC-1 conjugate was added to each well. The plate was covered with adhesive strip and incubated for 2 h at room temperature. The wells were aspirated and washed five times. One hundred microliter of substrate solution was added to each well and incubated at room temp for 30 min under protection from light. One hundred microliter of stop solution was added to each well. Plate was gently tapped to ensure mixing. Optical density was measured within 30 min using a microplate reader set to 450 nm. (correction wavelength set at 540 nm or 570 nm).

Statistical analysis

All data are presented as mean plus minus standard error of the mean. Differences were considered

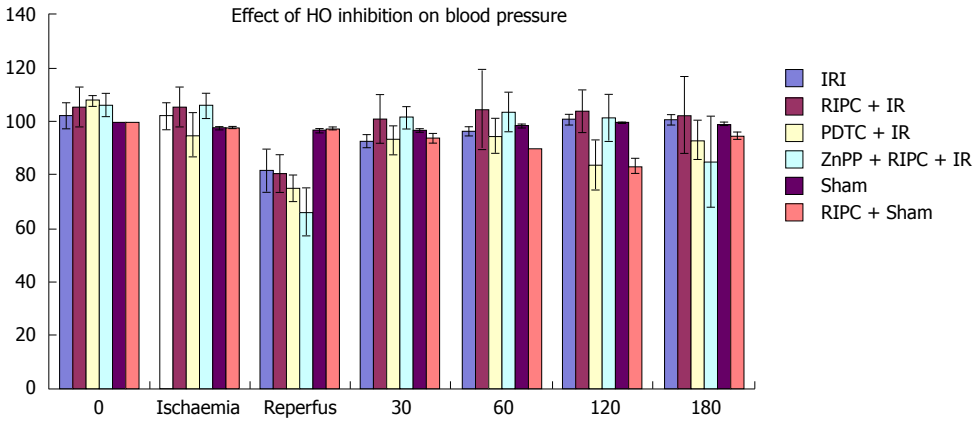

Figure 2 Effect of Haemoxygenase induction and inhibition on blood pressure in hepatic ischemia reperfusion. RIPC prior to IR restores B.P. to baseline rapidly compared to IR only. Inhibition of HO showed a greater fall in B.P but there was no difference in recovery to baseline. Values expressed as mean + SE. RIPC: Remote ischemic preconditioning; HO: Haemoxygenase; IR: Ischemia reperfusion.

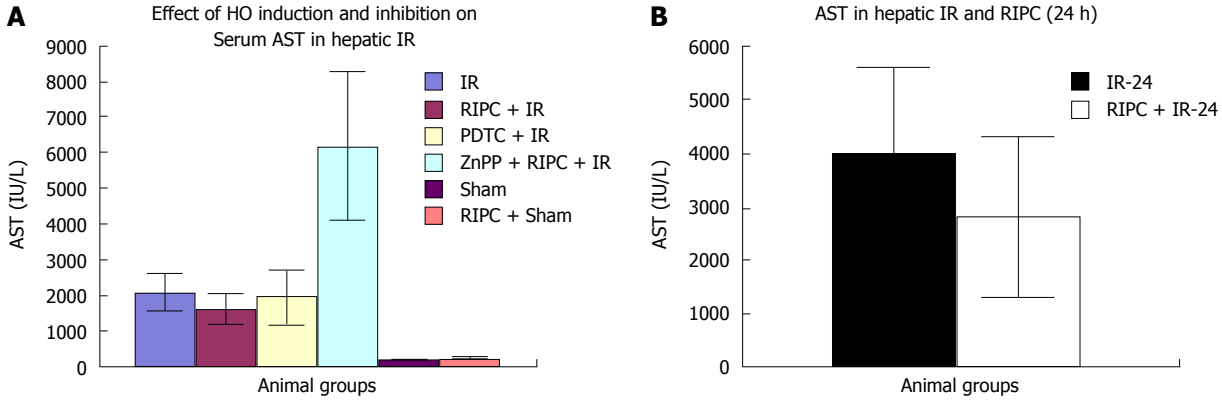

Figure 3 Effect of Haemoxygenase inhibition and induction on hepatic transaminases in the early phase of hepatic ischemia reperfusion. A: Effect of HO inhibition on AST in early phase IR; B: AST in late phase. No significant difference in hepatic transaminase level IR/RIPC + IR. HO inhibition increased transaminase levels RIPC + IR/ZNPP + RIPC + IR. RIPC: Remote ischemic preconditioning; HO: Haemoxygenase; IR: Ischemia reperfusion.

significant for $P < 0.05$.Comparisons between groups were performed by one way analysis (ANOVA). Bonferroni correction was applied for ANOVA.

RESULTS

Haemodynamic measurements (early phase)

RIPC + IR group showed a drop in MAP similar to IR only group however MAP recovered to baseline in RIPC+IR rapidly (30-60 min) compared to IR (60-120 min). HO inhibition caused a further drop in B.P. and delayed recovery of blood pressure (Figure 2). There was no significant difference in pulse rate.

Survival in late phase of IR

Of the six animals in each group, one animal in the IR-24 group died before the end of recovery period due to severe reperfusion injury. This animal was excluded from intravital analysis. All animals in the RIPC + IR-24 group survived after 24 h of reperfusion.

Hepatocellular injury

Effect of IR, RIPC, PDTC and ZnPP on hepatic transaminases (Early phase). Baseline transaminase levels in RIPC+sham were higher than sham. IRI produced a rise in transaminase levels (Figure 3A). Effect of IR and RIPC in the late phase of hepatic IR on hepatic transaminases (Figure 3B).

Microcirculatory changes

The sham group showed constant microcirculatory parameters. RIPC induced a low grade oxidative stress in the liver resulting in lower RBC flow and perfusion as compared to sham. The velocity of flow (160.83 ± 12.24 μm/s), sinusoidal flow (8.42 ± 1.19) and sinusoidal perfusion index (42.12 ± 7.28) in hepatic IR were significantly lower than RIPC + IR.

RIPC significantly increased velocity of flow (328.04 ± 19.13 μm/s), sinusoidal flow (17.75 ± 2.59) and the sinusoidal perfusion index was higher in RIPC group (67.28 ± 1.82). PDTC (HO induction) reproduced the effects of RIPC in hepatic IR. PDTC restored RBC velocity (300.88 ± 22.109 μm/s), sinusoidal flow (17.66 ± 3.71) and sinusoidal perfusion (82.33 ± 3.5) to near sham levels. ZnPP (HO inhibition) significantly reduced velocity of flow of RBC in the RIPC group

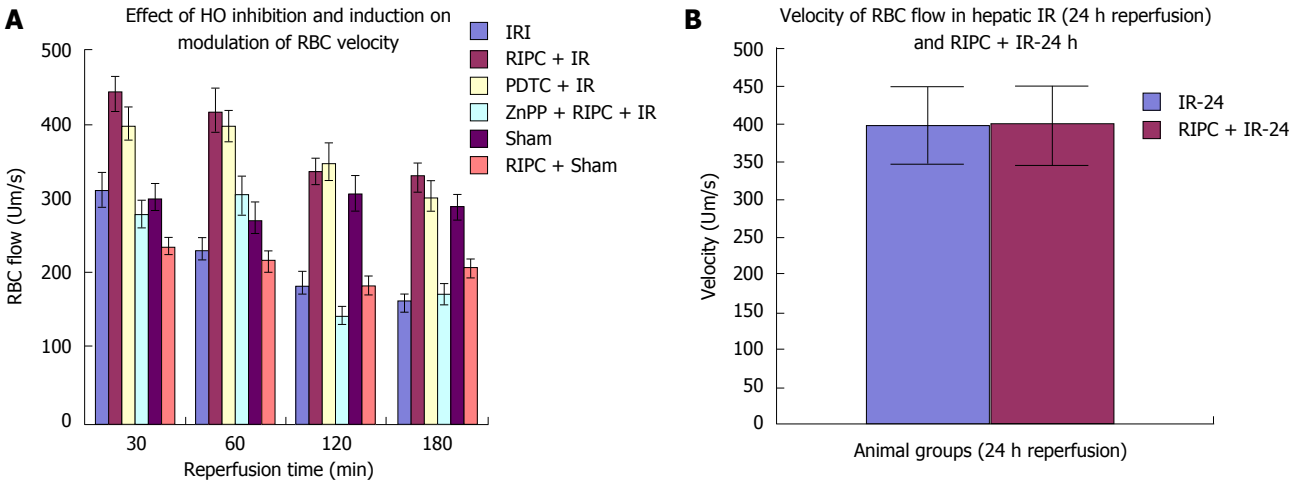

Figure 4 Effect of Haemoxygenase inhibition and induction on velocity of flow in early phase of hepatic ischemia reperfusion (A) and velocity of RBC flow in remote ischemic preconditioning + ischemia reperfusion -24 as compared to ischemia reperfusion -24 (B). Significant increase in velocity in preconditioned animals prior to ischemia reperfusion injury (RIPC + IRI) as compared to IRI at 30, 60 and 120 min of reperfusion. HO inhibition caused a significant fall in velocity of flow in preconditioned animals. Values expressed as mean ± SE. HO: Haemoxygenase; IR: Ischemia reperfusion; RIPC: Remote ischemic preconditioning; IRI: Ischemia reperfusion injury.

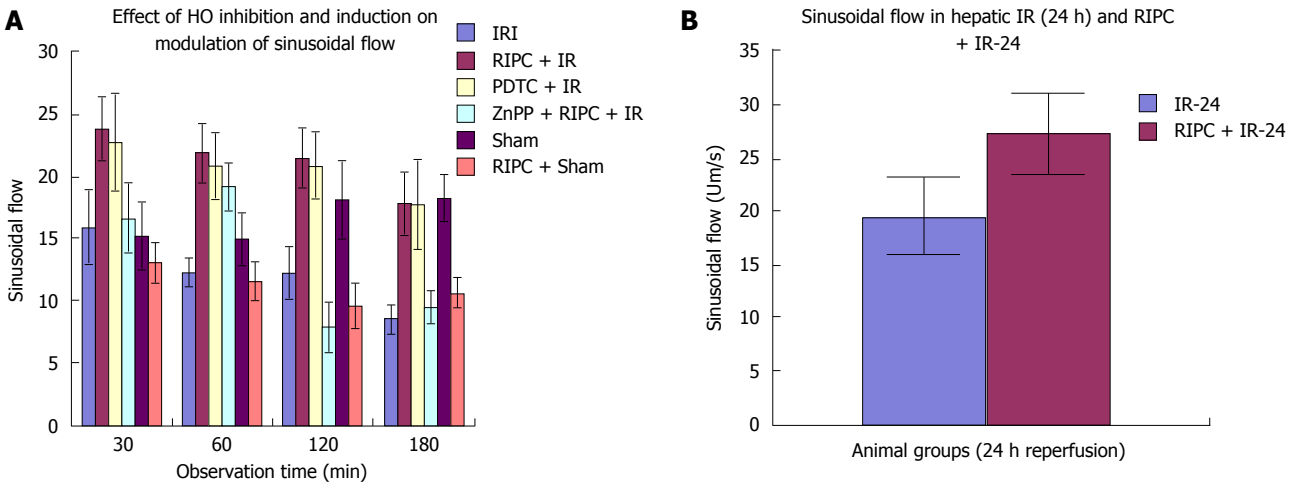

Figure 5 Effect of Haemoxygenase inhibition and induction on sinusoidal flow in early phase of hepatic ischemia reperfusion (A) and sinusoidal flow in late phase of hepatic ischemia reperfusion (B). Sinusoidal flow - V × (D/2) 2 × π = V is velocity of RBC, D is sinusoidal diameter. Significantly better flow in preconditioned animals (RIPC + IRI) as compared to non-preconditioned (IR). HO inhibition significantly inhibits flow in preconditioned animals.Values expressed as mean ± SE. Sinusoidal flow - V × (D/2)2 × π = V is velocity of RBC, D is sinusoidal diameter. Better flow in preconditioned animals (RIPC + IRI-24) as compared to non-preconditioned (IR-24). Values expressed as mean ± SE. HO: Haemoxygenase; IR: Ischemia reperfusion; RIPC: Remote ischemic preconditioning; IRI: Ischemia reperfusion injury.

(170.74 ± 13.43 μm/s and sinusoidal flow in the RIPC group (9.46 ± 1.34) However inhibition of HO by ZnPP in RIPC (60.29 ± 1.82) showed a fall in perfusion only at 180 min of reperfusion (Figure 4A, Figure 5A, Figure 6A and Figure 7A)

Microcirculatory changes in the late phase of hepatic IR

In the late phase of hepatic IR, velocity of flow was similar between preconditioned and IR -24 suggesting recovery of velocity of flow to near normal values after 24 h of reperfusion in the absence of preconditioning. However there was a significant increase in sinusoidal diameter, sinusoidal flow and perfusion in the RIPC + IR-24 group (Figure 4B, Figure 5B, Figure 6B and Figure 7B).

Neutrophil adhesion in venules and sinusoids as seen by IVM

Significantly increased neutrophil adhesion in IR injury is seen in both postsinusoidal venules (769.05 ± 87.48) and sinusoids (97.4 ± 7.49). RIPC significantly reduced neutrophil adhesion in IR injury in both postsinusoidal venules (219.66 ± 93.79) and sinusoids (25.69 ± 9.08).

PDTC significantly reduced neutrophil adhesion in both postsinusoidal venules (89.58 ± 58.32) and sinusoids (17.98 ± 11.01) reproducing the

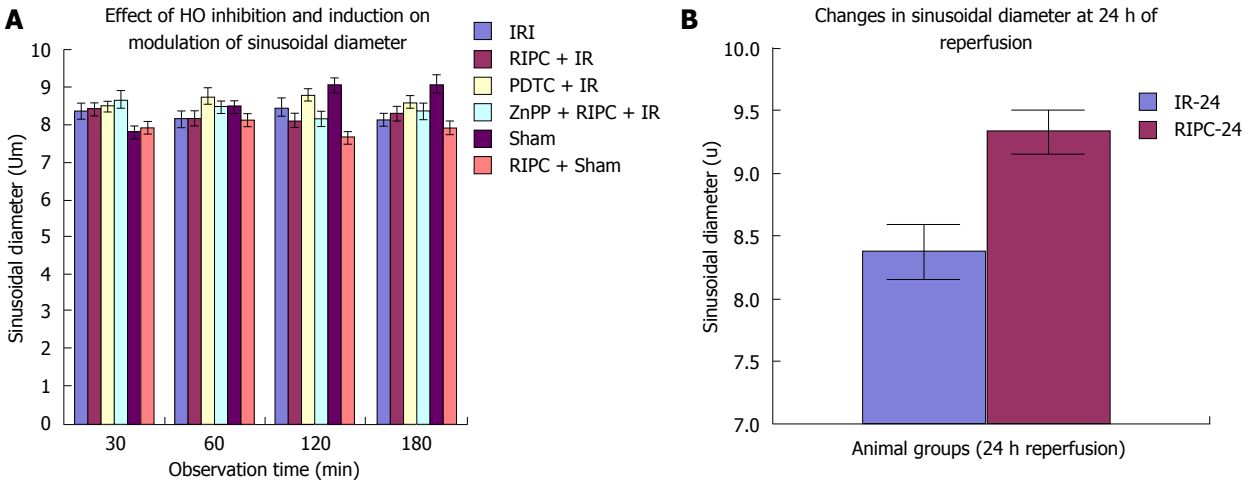

Figure 6 Sinusoidal diameter in late phase of hepatic ischemia reperfusion. A: Sinusoidal diameter early phase; B: Sinusoidal diameter late phase. No significant change ($P > 0.05$) in sinusoidal diameter seen in preconditioned animals and on inhibition of HO. Sinusoidal diameter - Significantly increased diameter in the preconditioned group (RIPC + IR-24). Values expressed as mean ± SE. $P < 0.05$, significantly increased sinusoidal diameter in the preconditioned group. HO: Haemoxygenase; IR: Ischemia reperfusion; RIPC: Remote ischemic preconditioning; IRI: Ischemia reperfusion injury.

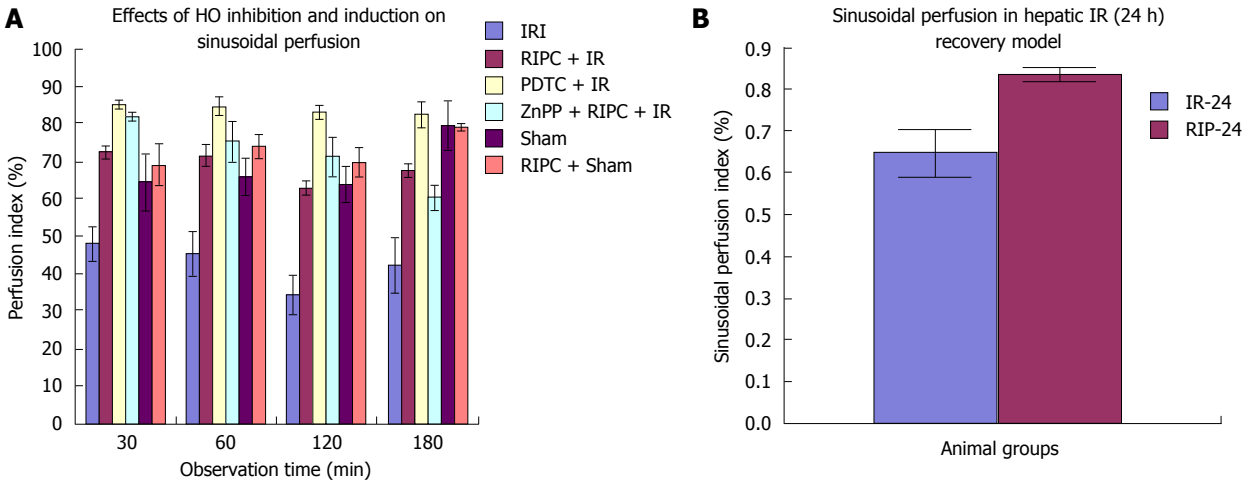

Figure 7 Sinusoidal perfusion in late phase of hepatic ischemia reperfusion. A: Sinusoidal perfusion early phase; B: Sinusoidal perfusion late phase. Perfusion index in remote preconditioned animals (RIPC + IRI) is significantly higher ($P < 0.05$) than non-preconditioned animals (IRI). HO inhibition does not affect sinusoidal perfusion in the early stage of IR however sinusoidal perfusion declines at 180 min of reperfusion. Values expressed as mean ± SE. Sinusoidal perfusion index - The PI in remote preconditioned animals (RIPC + IR-24) is significantly higher than non-preconditioned animals (IR-24). Values expressed as mean ± SE. HO: Haemoxygenase; IR: Ischemia reperfusion; RIPC: Remote ischemic preconditioning; IRI: Ischemia reperfusion injury.

effects of RIPC. HO inhibition with ZnPP significantly increased venular (589.04 ± 144.36) and sinusoidal neutrophil adhesion in preconditioned animals (121.39 ± 30.65). IR after 24 h of reperfusion significantly increased venular and sinusoidal neutrophil adhesion in comparison to the early phase and was significantly reduced by RIPC (Figure 8A and B, Figure 9A and B).

Hepatocellular death

Hepatocellular cell death is significantly less on HO induction (PDTC + IR) in hepatic IR. Preconditioning (RIPC + IRI) reproduces effects of HO induction as Hepatocellular cell death in RIPC+IR is significantly less compared to IRI group. HO inhibition prior to preconditioning (ZnPP + RIPC + IR) significantly enhances hepatocellular death in preconditioned animals.

Histology

IR group showed diffuse and significant periportal congestion. There was severe necrosis in zones 2 and 3 and also sub capsular necrosis. RIPC+IR showed a significantly lower Suzuki score compared to the IR group. On inhibition of HO in RIPC+IR (ZNPP + RIPC + IR group) most of the sections showed diffuse and severe congestion and vacuolation. The necrosis is diffuse and focally associated with acute inflammatory cells. Changes in ZnPp + RIPC + IR were similar to IR group and significantly more compared to RIPC + IR. Only one animal showed extensive congestion and

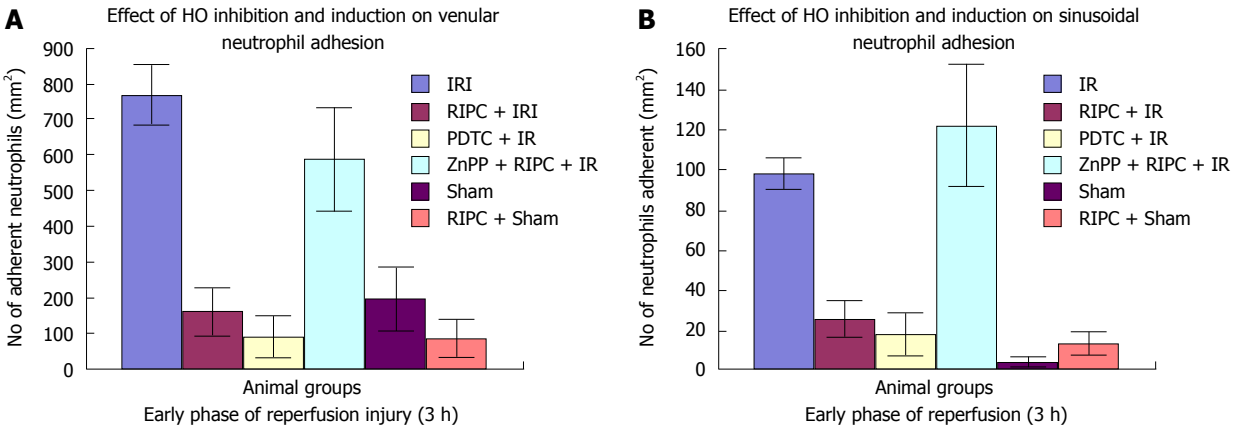

Figure 8 Effect of Haemoxygenase inhibition and induction on Venular neutrophil adhesion in early phase of hepatic ischemia reperfusion. A: Venular neutrophil adhesion early phase; B: Sinusoidal neutrophil adhesion early phase. Significantly reduced venular neutrophil adhesion in preconditioned (RIPC + IRI) group compared to non-preconditioned group (IRI). HO inhibition in RIPC + IR showed significantly increased venular neutrophil adhesion. Values expressed as mean ± SE. Significantly reduced sinusoidal neutrophil adhesion in preconditioned group (RIPC + IRI) compared to non-preconditioned group (IRI). Values expressed as mean ± SE. HO: Haemoxygenase; IR: Ischemia reperfusion; RIPC: Remote ischemic preconditioning; IRI: Ischemia reperfusion injury.

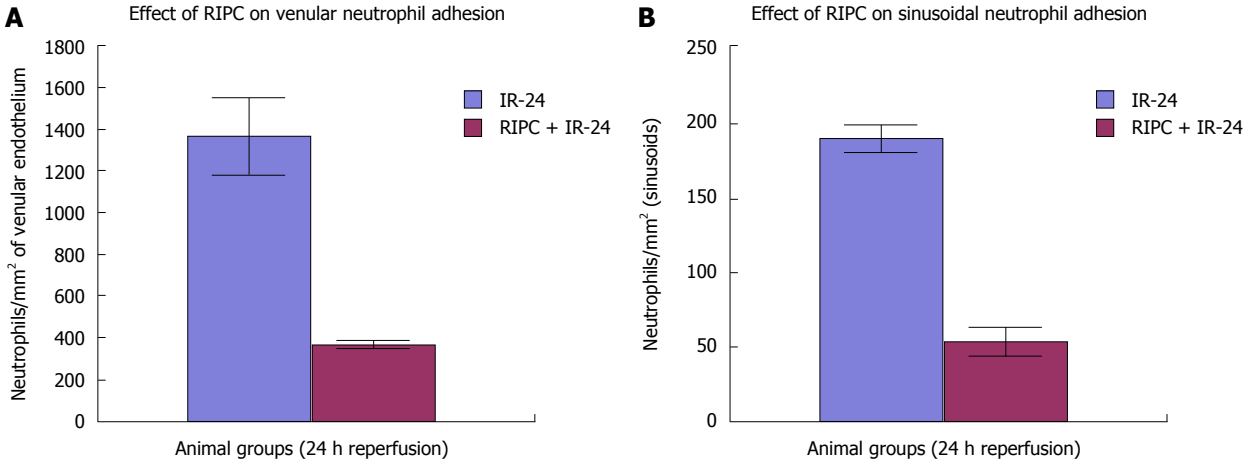

Figure 9 Venular neutrophil adhesion late phase (A) and sinusoidal neutrophil adhesion late phase (B). Significantly reduced venular neutrophil adhesion in preconditioned (RIPC + IR-24) group compared to non-preconditioned group (IR-24). Significantly reduced sinusoidal neutrophil adhesion in preconditioned group (RIPC + IRI) compared to non-preconditioned group (IRI). Values expressed as mean ± SE.

Table 2 Suzuki criteria

Group	IR	RIPC + IR	ZnPP + RIPC + IR	PDTC + IR	Sham	RIPC + Sham
Suzuki score	8.83 ± 0.7	6.2 ± 0.58	8.83 ± 0.6	4.5 ± 0.5	4 ± 0.31	1.5 ± 0.34

RIPC: Remote ischemic preconditioning; IR: Ischemia reperfusion.

vacuolation but minimal necrosis. On HO induction (PTDC + IR) most animals showed diffuse congestion with patchy necrosis except for one which shows mild congestion. Sham animals did not show significant changes apart from congestion in both portal and central vein. Hepatocytes showed vacuolation. RIPC + Sham animals showed moreless similar changes with minimal vacuolation but no necrosis (Figures 10 and 11). Suzuki criteria see Table 2.

Immunohistochemistry for HO

Immunohistochemistry revealed increased HO-1 expression in kupffer cells in RIPC + IR animals in comparison to IR group. Baseline HO expression is seen in kupffer cells in sinusoids of sham group. Haemoxygenase expression in IR (3 h of reperfusion) was more compared to sham. This is due to increased oxidative stress which acts a direct stimulus to HO-1 expression. The increased HO expression was observed in kupffer cells which are the primary source of HO production and degradation of haem to biliverdin and CO. At 24 h of reperfusion more HO was observed in Kupffer cells. PDTC induced HO-1 in macrophages in the early phase of hepatic IR. Inhibition of

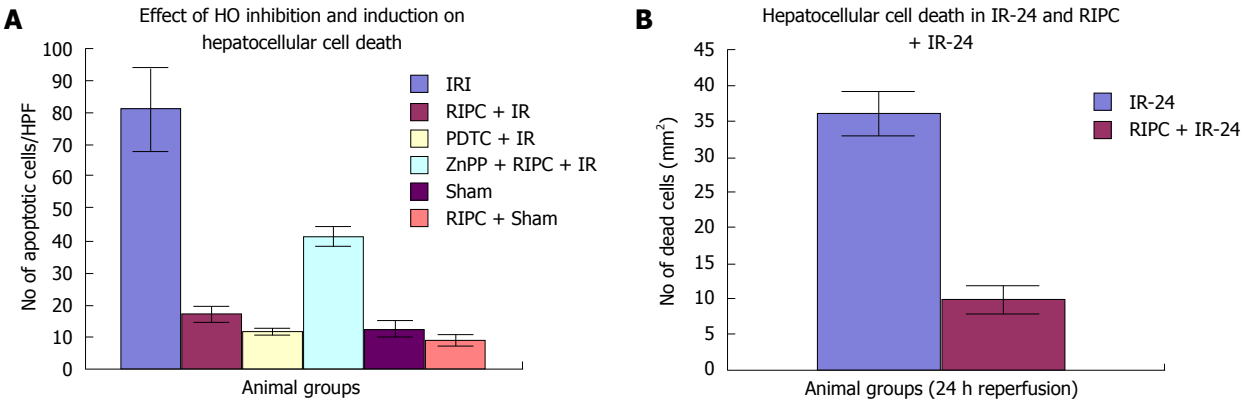

Figure 10 Hepatocellular cell death is significantly less on Haemoxygenase induction (PDTC + ischemia reperfusion) in hepatic ischemia reperfusion. A: Hepatocellular death early phase; B: Hepatocellular death late phase. Preconditioning (RIPC + IRI) reproduces effects of HO induction as Hepatocellular cell death in RIPC + IR is significantly less compared to IRI group. HO inhibition prior to preconditioning (ZnPP + RIPC + IR) significantly enhances hepatocellular death in preconditioned animals. Values expressed as mean ± SE. RIPC: Remote ischemic preconditioning; IRI: Ischemia reperfusion injury.

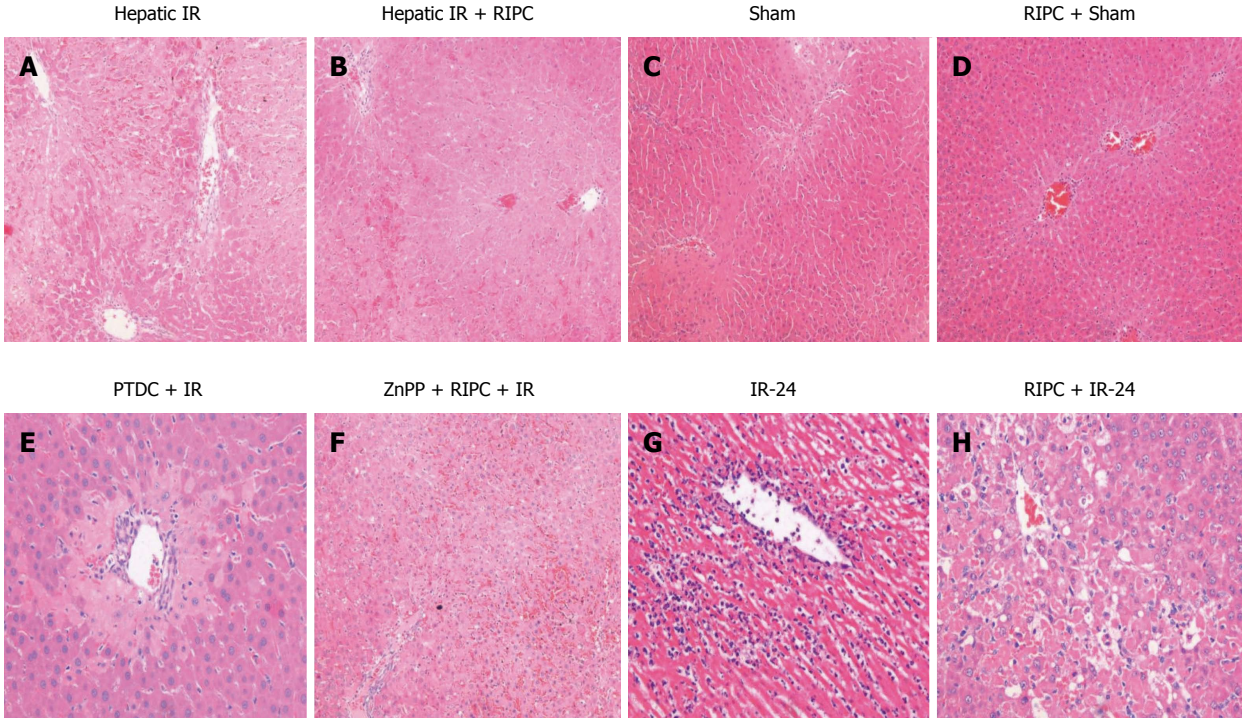

Figure 11 Hepatocytes showed vacuolation. A: IR - the HE section shows large areas of necrosis and sinusoidal congestion, normal residual hepatocytes noted at bottom of the frame; B: RIPC + IR - The HE section shows sinusoidal congestion, some hepatocyte vacuolation but no significant necrosis; C: Sham - The HE section reveals no significant damage; D: RIPC + Sham - The HE section reveals congested central vein but no other significant change; E: PDTC + IR-diffuse congestion and patchy necrosis; F: ZNPP + RIPC + IR - extensive necrosis seen; G: Very severe injury with abundant ballooning degeneration and necrosis is seen in the IR-24 injury group. Very diffuse and significant neutrophil adhesion is seen in the IR group. Apoptosis is evident in the IR group; H: RIPC + IR-24 group shows less injury with some ballooning and degeneration as well as neutrophilic infiltration. RIPC: Remote ischemic preconditioning; IRI: Ischemia reperfusion injury; HO: Haemoxygenase.

haemoxygenase by ZnPP (ZnPP inhibits both HO-1 and HO-2) showed decreased HO expression in macrophages in the early phase of hepatic IR. This explains the intravital findings of increased neutrophil adhesion and hepatocellular death in comparison to preconditioned animals.

In the late phase of hepatic IR (24 h) there was increased HO expression in both kupffer cells and hepatocytes (parenchymal cells) in preconditioned animals in comparison to IR only (Figure 12). Immunohistochemistry score for HO see Table 3.

Western blot analysis

Western blot analysis showed by densitometry significantly more HO expression in RIPC groups as compared to hepatic IR and PDTC reproduced the effects of RIPC. ZnPP inhibited HO expression in the RIPC group. Increased HO expression was seen in

Table 3 Immunohistochemistry score for Haemoxygenase expression

	Sham	RIPC + Sham	IR	RIPC + IR[a]	PDTC + IR[a]	ZnPP + RIPC + IR
Mean	14.0	44.1	28.66	87.5	78.7	25.0
SEM	2.44	7.57	18.77	20.25	8.75	11.83

[a]$P < 0.05$, RIPC + IR and PTDC + IR *vs* IR. RIPC: Remote ischemic preconditioning; HO: Haemoxygenase; IR: Ischemia reperfusion.

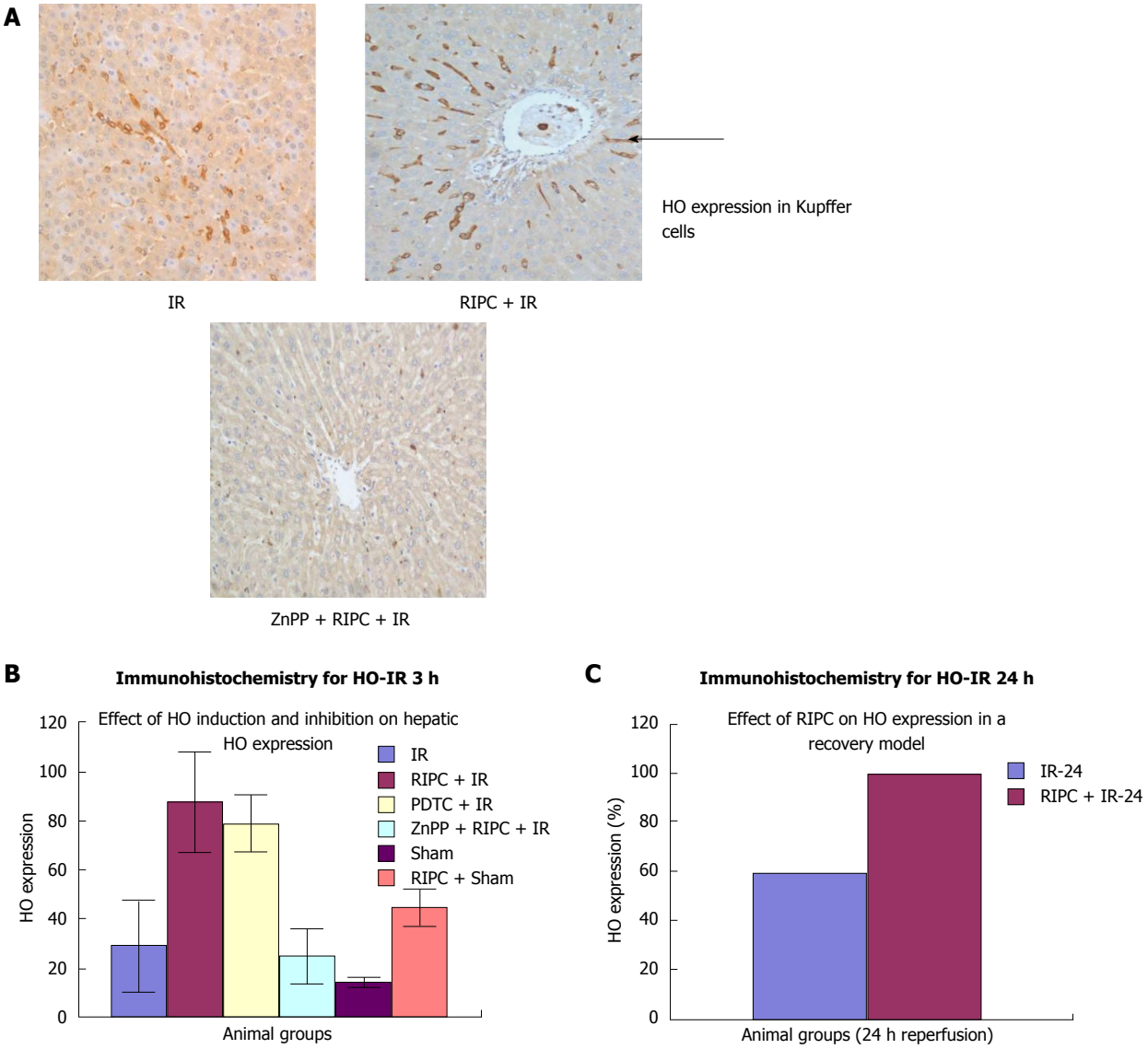

Figure 12 In the late phase of hepatic IR (24 h) there was increased HO expression in both kupffer cells and hepatocytes (parenchymal cells) in preconditioned animals in comparison to IR only. A: Spatiotemporal distribution of HO - There is some HO expression seen in macrophages (kupffer cells) in IR injury in response to oxidative stress. The expression of HO-1 was significantly more in kupffer cells in the RIPC+IR group. ZNPP + RIPC + IR reduced HO-1 expression; B: HO expression in early phase of hepatic IR; C: HO expression in late phase of hepatic IR.

RIPC + IR-24 as compared to IR-24 (Figure 13).

CINC Elisa results

The CINC data shows significant difference between IR injury, RIPC + IR and ZnPP + RIPC + IR in the early phase of hepatic IR. In the late phase of IR there is a significant difference seen between IRI and RIPC+IRI suggesting that preconditioning modulates release of CINC-1 from kupffer cells.

Effect of HO induction and inhibition on CINC

The CINC cytokine levels in sham were low (101.32 ± 6.42). RIPC in sham led to relatively high CINC levels (412.18 ± 65.24) as compared to sham ($P < 0.05$). Hepatic IR injury produced high serum CINC level in comparison to sham animals in the early phase of hepatic IR (644.08 ± 181.24) ($P < 0.05$). RIPC reduced CINC -1 levels in the early phase (401.62 ± 78.56) in comparison to IR only. PDTC (HO inducer)

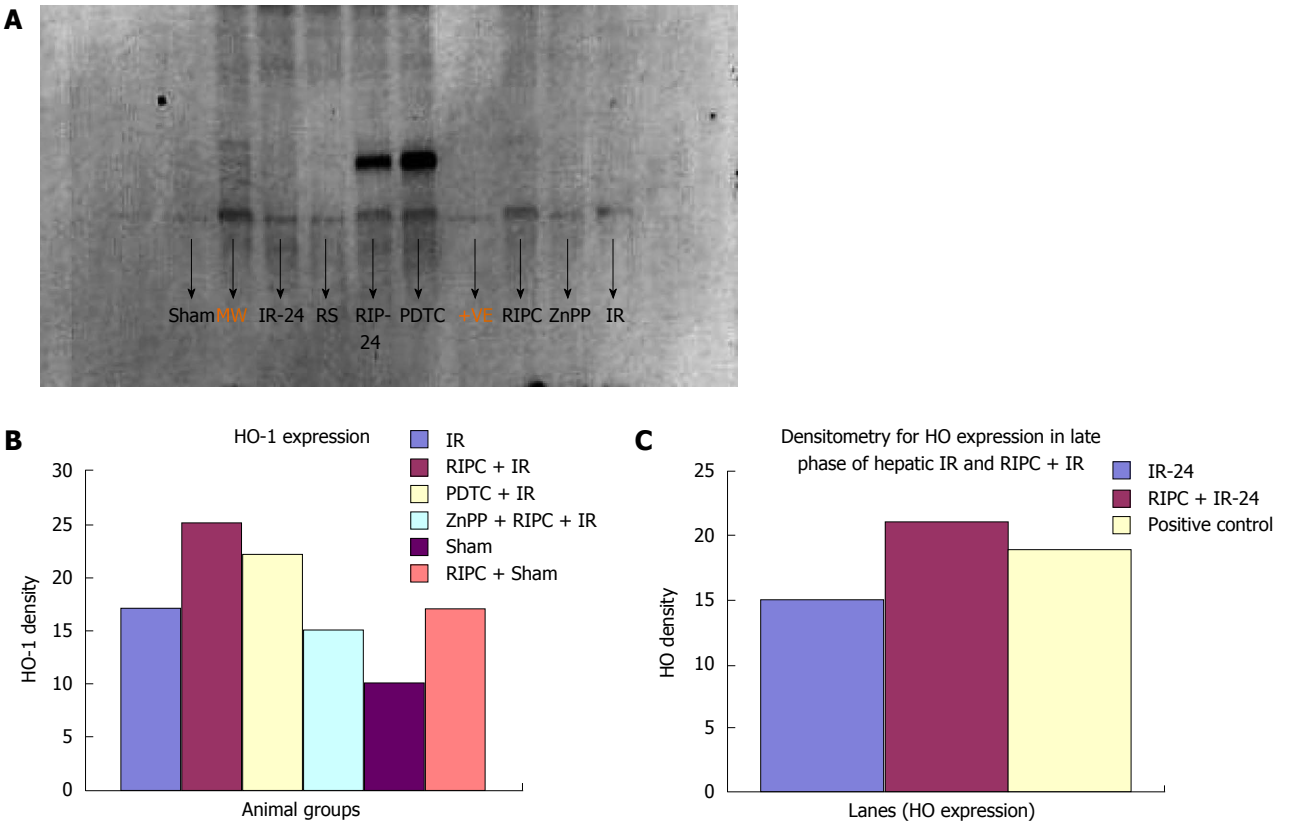

Figure 13 Increased Haemoxygenase expression was seen in all groups + ischemia reperfusion-24 as compared to ischemia reperfusion-24. A: Western blot analysis for HO in early and late phase of hepatic IR. Lane 1: Sham; Lane 2: MW- 32 Kda for HO protein; Lane 3: IR-24 (HO expression in late phase of hepatic IR) Lane 4-RS; Lane 5: RIPC + IR-24 (effect of RIPC on HO expression in late phase of IR); Lane 6: PDTC- HO inducer; Lane 7: Positive control; Lane 8: ZnPP-HO inhibitor; Lane 9: IR (early phase); B: Densitometry HO expression early phase. Early phase of hepatic IR (above). Densitometry for western blots showing increased HO-1 expression in RIPC + IR and PDTC + IR. Prior inhibition of HO (ZnPP + RIPC IR) significantly reduced HO expression in preconditioned animals; C: Densitometry Ho expression late phase. Densitometry for late phase of hepatic IR and RIPC western blot showing increased HO-1 expression in the RIPC group at 24 h. HO: Haemoxygenase; IR: Ischemia reperfusion.

reduced CINC-1 levels in serum in hepatic IR (413.36 ± 63.06). HO inhibition in preconditioned animals with Zinc protoporphyrin increased serum CINC levels (521.81 ± 74.9) ($P < 0.05$) (Figure 14A).

The serum CINC levels were high in the late phase of hepatic IR (15306 ± 1222.04). RIPC reduced CINC levels in the late phase of IR (467.46 ± 26.06, $P < 0.05$) (Figure 14B).

DISCUSSION

New findings

This is the first study to use intravital microscopy and demonstrate the effects of HO inhibition on the protective effect of RIPC in hepatic IR and that RIPC induced HO modulates cytokine release from Kupffer cells and subsequent neutrophil activation.

Haemodynamics

Blood pressure: The model used was haemodynamically stable. However a fall in blood pressure on reperfusion was seen in hepatic IR, RIPC + IR, PDTC + IR and ZnPP group with recovery to baseline in 30-60 min. The blood pressure thereafter was stable throughout the time course of observation. Hence the potential effect of hypotension leading to decreased parenchymal perfusion and confounding results were avoided.

Microcirculatory changes and Role of HO-1

IR, RIPC + IR: RIPC + IR showed an increased velocity of RBC flow, sinusoidal flow and sinusoidal perfusion compared to IR group at all time points of observation and the administration of ZnPP (HO blockade) prior to RIPC abolished all protective effects. The loss of protective effects of RIPC after HO blockade suggests that HO pathways may have a role in modulation of hepatic IR. The lack of any change in sinusoidal diameter on HO inhibition suggests that RIPC modulates IR injury by anti-inflammatory properties of HO rather than sinusoidal dilatation in the early phase of hepatic IR. This observation was made in previous studies on ischaemic preconditioning which have demonstrated no significant variation in sinusoidal diameter between HO induction and controls[26]. Moreover HO produced in the initial phase is in response to stress (inducible HO-1) and is responsible for anti-inflammatory functions[5].

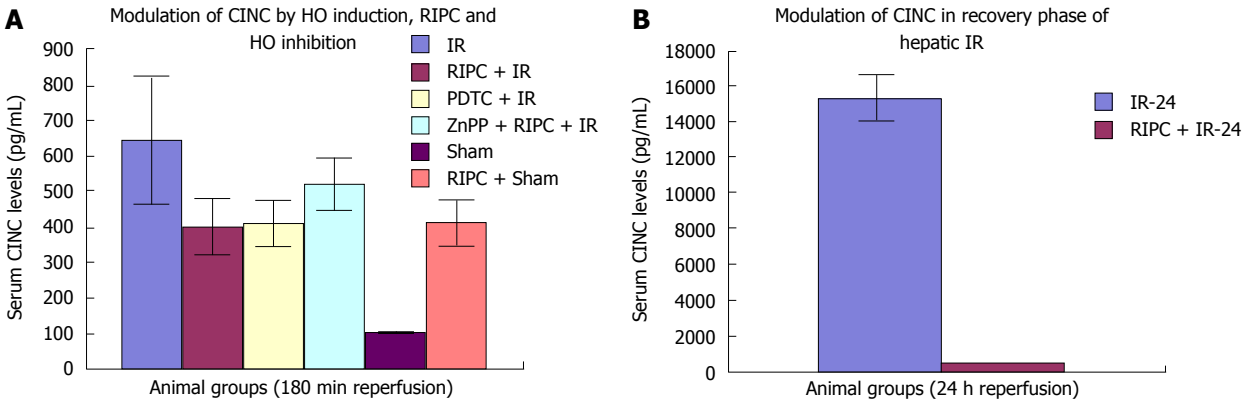

Figure 14 Effect of Haemoxygenase induction and inhibition on CINC. A: The CINC cytokine levels in sham were low (101.32 ± 6.42). RIPC in sham led to relatively high CINC levels (412.18 ± 65.24) as compared to sham ($P < 0.05$). Hepatic. IR injury produced high serum CINC level in comparison to sham animals in the early phase of hepatic IR (644.08 ± 181.24) ($P < 0.05$). RIPC reduced CINC-1 levels in the early phase (401.62 ± 78.56) in comparison to IR only. PDTC (HO inducer) reduced CINC-1 levels in serum in hepatic IR (413.36 ± 63.06). HO inhibition in preconditioned animals with Zinc protoporphyrin increased serum CINC levels (521.81 ± 74.9) ($P < 0.05$); B: The serum CINC levels were high in the late phase of hepatic IR (15306 ± 1222.04). RIPC reduced CINC levels in the late phase of IR (467.46 ± 26.06). HO: Haemoxygenase; IR: Ischemia reperfusion.

Constitutive HO (HO-2) modulates sinusoidal tone and diameter and is produced in parenchmal cells after 6hrs of reperfusion as shown by Goda *et al*[27] and CO derived from an increase in HO-2 (Constitutive HO) in the parenchyma is responsible for modulation of sinusoidal tone and diameter.

HO induction (PDTC + IR) and inhibition (ZnPP + RIPC + IR)

This study shows increased RBC velocity and sinusoidal flow in PDTC+IR group as compared to IR only. The perfusion index in PDTC group is significantly higher than IRI at all time points. Inhibition of HO in preconditioned groups showed a significant fall in RBC velocity and sinusoidal flow in the RIPC + IR + ZnPP group. PDTC is known to induce HO-1, inhibit NF-κB and modulate IRI and increased HO-1 may be responsible for modulation of sinusoidal perfusion. This finding is supported by evidence from studies in animal models which have shown that HO-1 expression is associated with better sinusoidal perfusion[12,28,29]. However there was no significant difference in sinusoidal perfusion between HO induction and HO inhibition (ZnPP group) upto 120 min of reperfusion in our study suggesting that HO pathways maybe responsible for modulation of perfusion after 120 min of reperfusion. Previous studies have shown endogenous haemoxygenase expression to increase sinusoidal perfusion at 120 min of reperfusion[21] in animal models of hepatic IR secondary to prolonged limb ischemia. Hence the initial modulation of sinusoidal perfusion in hepatic IR by PDTC may be due to its anti-inflammatory action and inhibitory effect on NF-κB[29-31].

A previous study in a transplant model by Tsuchihashi demonstrated that PDTC given prior to harvest of liver reduced reperfusion injury after transplantation[30]. ZnPP given just before reperfusion blocked the protective effects of PDTC suggesting the role of PTDC induced HO-1. In our study low doses of PDTC (100 mg/kg) were used and no variation in sinusoidal diameter was observed however, recently Hata *et al*[26] have demonstrated an increase in sinusoidal diameter due to increase in parenchymal HO-1 on administration of higher doses of PDTC (150 mg/kg) intramuscularly in a rat model of hepatic IRI. Hata *et al*[26] has shown that CO derived from PDTC induced increase in parenchymal HO and modulated sinusoidal diameter in the early phase of hepatic IR.

Microcirculatory changes in late hepatic IR and effect of RIPC

RIPC significantly reduced neutrophil adhesion and hepatocellular death and increased sinusoidal perfusion as well as diameter. There was a significant increase in sinusoidal flow due to increase in diameter but not velocity. This increase in sinusoidal diameter has been demonstrated by our group in the late phase of RIPC[32] and other IPC studies.

Haemoxygenase expression

Haemoxygenase expression in the liver was significantly more in the RIPC sham group as compared to shams. Increased HO-1 expression was predominantly observed in Kupffer cells in the early phase with additional parenchymal expression in the late phase of IR. HO-1 pathways are initiated after oxidative stress and previous studies[21] have shown expression of mRNA as early as 2 h after the initial oxidative stress. The increase in velocity and flow seen after 30 min of reperfusion corresponds to 2 h after the first cycle of preconditioning and the time point of earliest HO-mRNA expression suggesting that HO-1 pathways maybe responsible for increased flow seen in this study.

Interestingly the increased HO-1 expression was seen in kupffer cells in RIPC. This observation was also

made by Lai *et al*[15]. Lai *et al*[15] demonstrated lack of increased HO-1 in the peripheral macrophages and hence it is unlikely that the increased haemoxygenase in the liver is from infiltrating macrophages. Hirano *et al*[33] have demonstrated that kupffer cells are the principal source of HO-1 in the liver. Previous studies have demonstrated increased free radicals in the blood following RIPC[34]. Increased ROS may induce a low grade oxidative stress in the liver and since kupffer cells are the key cells in the initial inflammatory response this may explain the increased HO-1 in the kupffer cells.

HO mediates breakdown of haem to biliverdin, iron and CO. Biliverdin and iron have antioxidant functions and CO has both vasodilatory and anti-platelet functions. HO-1 modulates flow, stabilizes membrane potential and most importantly degrades haem. It scavenges free radicals generated in IR injury thus mitigating the effects of IR injury[21]. The protective effects of RIPC, increased HO-1 expression in RIPC group and loss of protective effects of RIPC after inhibition of HO-1 suggest the role of RIPC induced HO-1 pathways in modulation of hepatic microcirculation. In addition the production of CO as a result of haem breakdown maybe responsible for reduced stasis and plugging due to its antiplatelet effects thereby leading to increased RBC flow. CO is known to modulate the function of hepatic sinusoidal pericytes called Ito cells which regulate sinusoidal tone and function. Future studies would need to investigate the role of CO releasing molecules (CORM) in modulation of hepatic IR.

Correlation of histological changes, microcirculatory flow, hepatocellular death, neutrophil adhesion, serum transaminases, HO expression and CINC levels

Hepatic IR/RIPC + IR/PDTC + IR: The modified Suzuki score in IR injury was significantly higher (8.83 ± 0.7) than RIPC+IR group (6.2 ± 0.58) or PDTC+IR group (4.5 ± 0.5). This correlated with decreased flow and sinusoidal perfusion, increased hepatocellular death observed by propidium iodide staining under IVM in hepatic IR, increased sinusoidal and venular neutrophil adhesion, increased CINC levels and increased serum transminase levels. Increased HO expression was seen in kupffer cells in hepatic IR as compared to sham group. This is due to oxidative stress of IR and has been observed in hepatic IR in previous experiments by Katori *et al*[4] and Hata *et al*[26]. Both preconditioned animals (RIPC + IR) and PTDC+IR group showed improved blood flow, sinusoidal perfusion, decreased hepatocellular death , sinusoidal and venular neutrophil adhesion, decreased CINC levels and serum transaminase levels. Lai *et al*[15] showed that preconditioned animals (RIPC+IR) showed decreased histological evidence of injury and serum transminases supporting our findings in this study although they did not investigate microcirculatory flow. Increased HO expression was seen in RIPC as compared to IR only. Previous studies have shown by intravital microscopy improved flow and sinusoidal perfusion and decreased hepatocellular death following PDTC induced HO expression in the liver[12,20].

ZnPP + RIPC + IR: Inhibition of HO by ZnPP in the preconditioned group showed a significantly high Suzuki score (8.83 ± 0.6) suggestive of increased cell necrosis and congestion which correlated with decreased sinusoidal flow and perfusion, increased hepatocellular death on IVM, increased neutrophil adhesion, CINC levels and serum transaminase levels suggestive of significantly increased hepatocellular injury. Decreased HO expression was seen on immunohistochemistry and Western blot analysis in comparison to RIPC group. Previous studies by Katori *et al*[35] have shown that HO inhibition of the donor with chromium mesoporphyrin prior to harvest of the liver was associated with decreased recipient animal survival following subsequent implantation in recipient liver transplant models. Lai *et al*[15] showed that inhibition of HO by ZnPP in RIPC was associated with significantly increased histological evidence of cell necrosis and congestion and increased serum transaminases.

IR-24 and RIPC + IR-24: Increased HO protein correlated with decreased sinusoidal and venular neutrophil adhesion in RIPC in the late phase, decreased histological necrosis and congestion and hepatocellular death as observed by propidium iodide staining in the late phase of RIPC + IR. This suggests that HO protein is responsible for protection of hepatic parenchyma and hepatic microcirculation due to reduced injury in delayed phase of hepatic IR. Sinusoidal diameter increased in the late phase of hepatic IR suggesting that HO may be responsible for modulation of sinusoidal dilatation. Interestingly the difference in flow between IR and RIPC groups was due to modulation of flow due to changes in sinusoidal diameter and not RBC velocity.

Drug dose selection and toxicity

ZnPP and its hepatotoxicity: Previous experiments in sham animals have shown that ZnPP inhibits haemoxygenase if used in the darkness. If ZnPP is exposed to light its effect of HO inhibition is lost however, ZnPP inhibits hepatic artery dilatation when exposed to light and this potentially leads to hepatotoxicity[36,37]. Hepatoxicity can confound the experimental data and hence in order to avoid this confounding effect ZnPP was prepared in the dark and administered through a syringe covered with silver foil with minimal laboratory light. The dose used was 1.5 mg/kg (2.5 μmol/kg) and this is much lower than the hepatotoxic toxic dose of ZnPP (5-10 μmol/kg) as shown by Greenbaum *et al*[36]. Amersi *et al*[3] (1999) showed that ZnPP treatment in shams led to

undetectable baseline HO and reduced portal flow and bile flow.

PDTC and toxicity

Tsuchihashi *et al*[30] (2003) demonstrated in an animal model that doses in excess of 600 mg/kg killed animals but animals did not die by injection of any dose upto 600 mg/kg. Doses more than 200 mg/kg offset the beneficial effects of PDTC as demonstrated by Liu *et al*[29] (1999). Hence we chose a dose of 100 mg/kg as used in experiments by Tsuchihashi *et al*[30]. PDTC shows a dose dependent relationship with regard to its beneficial effects. At doses of 100 mg/kg PDTC induces HO in kupffer cells but does not influence sinusoidal dilatation. Hata *et al*[26] showed that at doses of 150 mg/kg PDTC induced sinusoidal dilatation in sham animals, increased HO-1 with peak mRNA levels detectable at 3 h after PDTC injection and then declined. HO Protein expression peaked at 24-48 h after injection and was dose dependent. Immunohistochemistry revealed that PDTC at higher doses induced HO-1 in both periportal kupffer cells and hepatocytes in pericentral areas.

In a recent animal rat model of orthotopic liver transplantation RIPC applied to the recipient in the anhepatic recipient phase significantly improved liver graft function[38].

This study has demonstrated the hepatic micro-circulatory changes which are associated with the early and late phases of liver warm IR injury. RIPC induced increased sinusoidal perfusion and sinusoidal flow in both phases of IR but increased velocity of RBCs in the early phase in contrast to increased sinusoidal diameter in the late phase. The effect of RIPC was reproduced by HO-1 induction prior to the IR injury and inhibited by a HO-1 blocker in the early phase of hepatic IR. Increased HO production was observed in the late phase of hepatic IR both in kupffer cells and parenchymal cells.

These data would suggest that HO-1 is a key pathway responsible for modulation of microcirculatory changes and protection from the deleterious effects of IR. In the early period inducible HO-1 rather than constitutive HO-1 through its antinflammatory actions is likely to be the key candidate molecule responsible for protection of microcirculation. In the late phase it is likely that parenchymal HO is responsible for sinusoidal dilatation and protection of hepatic microcirculation. This study could not distinguish between the subtypes of HO.

Another Key finding of the study was demonstration of CINC modulation and neutrophil activation by HO.

Further investigation in HO knockout mice to clarify the mechanism of HO and the role of different subtypes is needed. The role of HO inducers in the clinical setting of hepatic IR and use of CORM (CO releasing molecules), curcumin and simvastatin (HO inducer) needs to be further investigated. Translation to clinical application would be the next step.

COMMENTS

Background

This original research article has focussed on investigating the role of haemoxygenase pathways in remote ischemic preconditioning (RIPC) induced modulation of hepatic ischemia reperfusion injury. Intravital microscopy in this study has demonstrated significantly improved hepatic microcirculation following haemoxygenase (HO) induction and loss of protective effects of RIPC following HO inhibition.

Research frontiers

This study is Novel in demonstrating the protective effect of RIPC on hepatic IR through modulation of haemoxygenase pathways and Serum CINC (Cytokine induced neutrophil chemoattractant) levels. Significantly increased flow, perfusion and decreased neutrophil adhesion as well as hepatocellular death co-related with decreased CINC levels and increased HO production in RIPC.

Innovations and breakthroughs

This study highlights the modulation of CINC by RIPC induced HO. CINC is responsible for neutrophil activation and modulation of neutrophil activation is a key mechanism in protection of hepatic microcirculation.

Applications

RIPC can be translated to donor preconditioning and preconditioning prior to liver resection to minimise ischemia reperfusion injury and manage small for size syndrome.

Peer-review

This article is to study the hepatic microcirculatory changes due to HO induced by RIPC, effect of HO inhibition and modulation of CINC. The study finding is new and interesting.

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ORIGINAL ARTICLE

Basic Study

Impact of *Helicobacter pylori* on the healing process of the gastric barrier

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Author contributions: Mnich E, Chmiela M substantially contributed to the conception and design of the research, performed analysis and data interpretation; Mnich E participated in all experiments; Kowalewicz-Kulbat M and Sicińska P performed the cell cycle and apoptosis assay; Gajewski A participated in DAPI staining; Hinc K, Obuchowski M and Moran AP provided essential reagents: subunit A of urease, *H. pylori* LPS; Mnich E and Kowalewicz-Kulbat M performed statistical analysis; all authors drafted the article and made critical revisions related to the intellectual content of the manuscript, and approved the final version of the article to be published.

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Abstract

AIM

To determine the impact of selected well defined *Helicobacter pylori* (*H. pylori*) antigens on gastric barrier cell turnover.

METHODS

In this study, using two cellular models of gastric epithelial cells and fibroblasts, we have focused on exploring the effects of well defined *H. pylori* soluble components such as glycine acid extract antigenic complex (GE), subunit A of urease (UreA), cytotoxin associated gene A protein (CagA) and lipopolysaccharide (LPS) on cell turnover by comparing the wound healing capacity of the cells in terms of their

proliferative and metabolic activity as well as cell cycle distribution. Toxic effects of *H. pylori* components have been assessed in an association with damage to cell nuclei and inhibition of signal transducer and activator of transcription 3 (STAT3) phosphorylation.

RESULTS

We showed that *H. pylori* GE, CagA and UreA promoted regeneration of epithelial cells and fibroblasts, which is necessary for effective tissue healing. However, *in vivo* increased proliferative activity of these cells may constitute an increased risk of gastric neoplasia. In contrast, *H. pylori* LPS showed a dose-dependent influence on the process of wound healing. At a low concentration (1 ng/mL) *H. pylori* LPS accelerated of healing epithelial cells, which was linked to significantly enhanced cell proliferation and MTT reduction as well as lack of alterations in cell cycle and downregulation of epidermal growth factor (EGF) production as well as cell nuclei destruction. By comparison, *H. pylori* LPS at a high concentration (25 ng/mL) inhibited the process of wound repair, which was related to diminished proliferative activity of the cells, cell cycle arrest, destruction of cell nuclei and downregulation of the EGF/STAT3 signalling pathway.

CONCLUSION

In vivo H. pylori LPS driven effects might lead to the maintenance of chronic inflammatory response and pathological disorders on the level of the gastric mucosal barrier.

Key words: *Helicobacter pylori*; Wound healing; Gastric barrier dysfunction

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Core tip: This manuscript focused on the impact of *Helicobacter pylori* (*H. pylori*) antigens to the gastric mucosal barrier. We evaluated the effects of *H. pylori* antigens using *in vitro* two cellular models of gastric epithelial cells and fibroblasts, which had been independently exposed to *H. pylori* components. In this study, we showed different effects of subunit A of urease, cytotoxin associated gene A protein, lipopolysaccharide (LPS) as well as compounds included in a glycine acid extract on the regenerative activity of gastric epithelial cells and fibroblasts. Our results indicate deleterious, dose dependent influence of *H. pylori* LPS on this process.

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INTRODUCTION

The gastric mucosal barrier (GMB) is composed of a pre-epithelial layer (mucus and bicarbonate), a tight epithelial component, the post-epithelial layer (fibroblasts and immune cells), microcirculation (blood flow) and nerves[1]. Epithelial cells are responsible for gastric barrier integrity and function[2]. Any disruption of GMB, due to infectious agents or inflammation, leads to a variety of disorders, including gastritis or even gastric cancer. In order to establish and develop a disease, infectious agents must overcome GMB[3]. Among bacterial pathogens, a Gram-negative, spiral-shaped bacterium *Helicobacter pylori* (*H. pylori*) has been shown to play a crucial role in the development of gastritis and gastric as well as duodenal ulcers[4] due to various mechanisms to evade host's responses[5]. In 10%-15% individuals this infection can lead to severe inflammation, peptic ulcer disease (10%), mucosa-associated lymphoid tissue (MALT) lymphoma (0.1%), or gastric adenocarcinoma (1%-3%)[6-9]. *H. pylori* induces histological gastritis associated with an infiltration of gastric mucosa with immune cells[10]. However, other microorganisms or even non-infectious agents such as corticosteroids, nonsteroidal anti-inflammatory drugs, aspirin and excessive alcohol consumption can play a role in the development of gastritis[11-13]. *H. pylori* antigens, which are translocated through the gastrointestinal tract in the Payer's patches, induce specific immune response[14]. Small molecular weight antigens including LPS enter the lamina propria *via* goblet cells. Moreover, the epithelial cells villi can also internalize particles of antigens such as bacterial cell debris, which can be found co-localized with $CD11^+$ dendritic cells in the lamina propria[15].

The infection begins by mucus colonization, which is followed by the attachment of bacteria to the underlying epithelial cells and extracellular matrix proteins[16-18]. The bacteria also interact with infiltrating immune cells *via* Pathogen Recognition Receptors (PRR) stimulating them to cytokine secretion or can even enter the bloodstream[19,20]. *H. pylori* urease protects the pathogens from gastric acid and degrades of intracellular tight junctions[21-23]. Adhesins representing outer membrane proteins such as Hop proteins and blood antigen binding adhesins mediate *H. pylori* binding to GMB[16,18]. Other factors, such as cytotoxin-associated gene A (CagA) protein and vacuolating toxin A (VacA) are able to trigger inflammatory responses in host gastric tissues and predispose to gastric ulcer and cancer[6,24]. The CagA is delivered into the host cells by the type IV secretion system (T4SS)[25-27] where it interferes with host signalling pathways and cellular functions[28,29]. However, CagA may also interact with the host cells in a soluble form[30,31] or as phospholipid vesicles[32,33], which have been indentified to attach to and to be taken up by

human epithelial cells[34-36]. Furthermore, it has been found that gastric epithelial cells inducibly expressing CagA secrete exosomes containing CagA, which can be distributed by circulation[37]. By using the *H. pylori* G27 strain (*cagA*+/*vacA*+) and two isogenic mutants defective in *cagA* (G27 *cagA*-/*vacA*+) or *vacA* (G27 *cagA*+/*vacA*-)[38], we showed that CagA present in the cytoplasmic fraction of bacterial cells was responsible for the inhibition of proliferation of T lymphocytes[28].

Among *H. pylori* virulence factors, LPS has a unique status since modifications of lipid A lead to reduction of endotoxic properties, whereas O-specific chains structurally similar to human Lewis (Le) blood-group antigens are responsible for molecular mimicry[39,40], which allow *H. pylori* to persist[41-43]. This is by reducing the host immune response mechanisms including phagocytosis[44], Natural Killer cells activity[45] and proliferation of T lymphocytes[46-48]. LPS through binding with dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) may interfere with the development of specific immune response[49,50]. The biological actions of LPS are mediated by CD14 and Toll-like receptors (TLR) 4 and TLR2, scavenger receptors, β2 integrins and LPS-binding protein (LBP)[51,52].

The long-term inflammation can increase the gastric barrier permeability as well as further damage to lamina propria[5,50] and might promote different extragastric disorders[53-55]. Although several *H. pylori* factors engaged in gastric lining disruption have been identified, the mechanisms of tissue damage are still not well known. We hypothesised that gastric epithelial barrier disruption could result in either epithelial cell loss due to ulceration or excessive epithelial cell growth predisposing to gastric neoplasia. The aim of this study was to explore the ability of gastric epithelial cells and fibroblasts to heal wound after the challenge with selected *H. pylori* antigens: glycine acid extract antigenic complex (GE), subunit A of urease (UreA), CagA and LPS. We used *in vitro* cellular models to assess the effectiveness of the cells in the wound healing by monitoring the cell migration in association with cell metabolic activity, proliferation, cell cycle distribution, as well as damage to cell nuclei.

MATERIALS AND METHODS

Cell culture

The human AGS (CRL-1739) gastric adenocarcinoma epithelial cell line[56] and guinea pig fibroblasts (CRL-1405)[57] were obtained from the American Type Culture Collection (ATCC, Rockville, Md.). The cells were routinely grown as a monolayer in complete RPMI-1640 medium (cRPMI; Sigma St. Louis, MI, United States), containing 10% heat inactivated Fetal Bovine Serum (FBS; CytoGen, Łódź, Poland), 1% penicillin/streptomycin (Gibco, Zug, Switzerland), at 37 ℃ in a humidified atmosphere containing 5% CO_2. The cells were passaged every seven days with 0.25% trypsin/0.02% EDTA (HyClone, Thermo Fisher Scientific, Waltham, MA, United States) and the medium was changed every 3-4 d.

Stimuli

GE from the reference *H. pylori* strain CCUG 17874 (Culture Collection University of Gothenburg, Sweden), at 10 μg/mL was used in the experiments. Surface *H. pylori* antigens were extracted using 0.2 mol/L glycine buffer, pH 2.2, as previously described[58,59] with the evaluation of protein composition by SDS-PAGE electrophoresis and Western blot - Immuno blot (Milenia® Blot *H. pylori*, DPC Biermann, GmbH, Bad Nauheim, Germany). Serological detection of antigens was performed with reference serum samples from patients infected with *H. pylori*[60]. Major proteins in GE recognized by sera from *H. pylori* infected patients were: 120 kDa (CagA), 87 kDa (VacA), 66kDa (UreB), 60 kDa (Hsp), 29 kDa (UreA), between 66-22 kDa. The protein concentration in GE was 600 μg/mL (NanoDrop 2000c Spectrophotometer, ThermoScientific, Wlatman, WY, United States). GE contained < 0.001 EU/mL of LPS, as shown by the chromogenic *Limulus amebocyte* lysate test (Lonza, Braine-Alleud, Belgium).

Recombinant CagA protein - rCagA (a kind gift from Antonello Covacci, IRIS, Siena, Italy) was used at the concentration of 1 μg/mL. A recombinant fragment of the CagA antigen of *H. pylori*: nt 2777 to nt 3465 of *cagA* gene was used. It was expressed (QIAexpress System, Qiagen, Hilden, Germany) in *E. coli* as a fusion protein (about 26 kDa size) with a 6 His-tail in front of a 230 aa polypeptide of CagA. The protein was purified by Ni^{2+}-NTA agarose column[61] and checked for serological activity in the enzyme immunoassay[62].

Based on the common substrate activity and high homology of urease produced by the species of the genus *Helicobacter* in this study the UreA subunit from *H. acinonychis* isolated from the acidic environment of cheetah stomach was used as a homologue of *H. pylori* UreA protein (97% homology). The urease gene was amplified by a polymerase chain reaction (PCR), as previously described[63] using chromosomal DNA as a template and oligonucleotides hisureA-up and hisureA-dn as primers. DNA encoding six histidines (His6-tag) was carried by oligonucleotide hisure-A-dn. The obtained PCR product of 737 bp was digested with enzymes *KpnI* and *NheI* and cloned into the commercial vector pBAD (Stratagene, California, United States). The resulting plasmid, pMD1, was verified by restriction analysis and nucleotide sequencing. pMD1 was used to transform the *E. coli* strain DH5α and the recombinant strain was used to overproduce UreA by the addition of arabinose 0.05%. A 27 kDa protein was visualized on a coomassie blue stained gel and purified on Ni-NTA superflow agarose (Qiagen) followed by gel filtration on Superose 6 resin. UreA was used at 5 μg/mL.

LPS from the reference strain of *H. pylori* CCUG

17874 was prepared by hot phenol-water extraction as previously described[64]. Whole cell lysates were pretreated with proteinase K (Sigma, St Louis, MI, United States)[65]. Crude extraction of LPS from bacteria was performed with 45% aqueous phenol at 68 ℃ for 30 min[66]. The LPS preparations were purified by treatment with RNase A, DNase II and proteinase K (Sigma) and by ultracentrifugation at 100000 × *g* at 4 ℃ for 18 h[67]. *H. pylori* LPS was used at two concentrations: 1 ng/mL and 25 ng/mL. Standard *E. coli* LPS was used at 1 ng/mL or 25 ng/mL, as a control of *H. pylori* LPS (serotype O55: B5; Sigma).

In order to study the synergistic or antagonistic effects, the following combinations of antigens were used: (1) *H. pylori* LPS (25 ng/mL) + CagA (1 μg/mL); and (2) *H. pylori* LPS (25 ng/mL) + CagA (1 μg/mL) + UreA (5 μg/mL) + GE (10 μg/mL). The antigen concentrations were adjusted experimentally or adopted from previously performed experiments[44,47,48,68].

Scratch wound assay

Cell migration was evaluated based on the ability of the cells to migrate into an empty space created by an *in vitro* scratch wound as previously described[69]. AGS cells or guinea pig fibroblasts were seeded in six-well plates at the density of 1 × 10^6 cells/well in 1 mL/well RPMI-1640 medium supplemented with 2% FBS/1% standard antibiotics and cultured until reaching 100% confluence. A lower percentage of FBS was used to minimize cell proliferation, and sufficient to prevent apoptosis and/or cell detachment. The cell monolayer in each well was physically disrupted with a sterile 200 μL pipette tip, and designated as time 0 h of wound repair. The stimuli described above were added to the cells. The control for a migratory assay consisted of untreated cells alone, which exhibited the normal capacity to migtate. Twenty-four hours after the challenge, antigens were removed from the cell cultures and the cells were washed twice with culture medium/2%FBS. Wound images were taken at 0, 24, 48 and 72 h by a digital camera (Nikon P20, Tokyo, Japan) at the same positions. Areas were measured using the software ImageJ version 1.48v (National Institute of Health, United States). Each wound was measured four times and the average value was assessed. The wound healing in the milieu of antigens was expressed as a percentage of cells migrating to the wound zone in comparison to untreated cells.

Cell viability assay

Cytotoxic effects of stimulators used in this study were evaluated using a tetrazolium yellow dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], which is reduced by living cells to yield soluble purple formazan crystals that can be detected colorimetrically. AGS cells and guinea pig fibroblasts (1 × 10^6 cells/well) were placed in 96-well plates in a volume of 100 μL and left to adhere overnight. Subsequently, all cells were washed with cRPMI and incubated for further 24 h with the described stimuli. Fresh MTT solution (5 mg/mL in sterile PBS; Sigma St. Louis, MI, United States) was added to each well and the plates were incubated for 2 h at 37 ℃, 5% CO_2. Formazan crystals were dissolved with acidic isopropanol (0.1 mol/L HCl in absolute isopropanol). Absorbance at 570 nm was estimated with a plate reader Victor2 (Wallac, Oy, Turku, Finland). All results were presented as the percentage means ± SD (standard deviation) relative to untreated cells of at least four independent experiments performed in triplicates. The effectiveness of MTT reduction was calculated based on the following formula: MTT reduction relative to untreated cells (%) = (absorbance of treated cells/absorbance of untreated cells × 100%) - 100%.

Cell proliferation study

A radioactive proliferation assay based on the measurement of the tritiated thymidine ([^{3}H]TdR) incorporation during DNA synthesis was used for the quantification of AGS cells and guinea pig fibroblasts proliferation. These cells were seeded at a density of 1 × 10^6 cells/well into 96-well microplates in 100 μL/well of cRPMI medium and preincubated overnight in order to obtain a monolayer of adherent cells. Further, the cells were stimulated for 24 h in the presence of bacterial antigens or in culture medium alone (as a control of spontaneous proliferation). After stimulation, the antigens were removed by washing the cells with cRPMI. In all experiments, wells containing the cells alone (without any antigens) were included as a control. At 18 h before the end of cultivation, 1 μCi of [^{3}H]TdR (Lacomed, Prague, Czech Republic) was added to each well to estimate cell proliferation. The incorporation of thymidine was measured using a MicroBeta 2 scintillation counter (Wallac Oy, Turku, Finland) after harvesting the cells on fibre filters. All cultures were settled in six repeats. The results were expressed as mean counts per minute (cpm)/culture ± SD of six independent experiments, performed in triplicates. The stimulation index (SI), expressing the relative cpm ratio, was calculated by dividing the counts/min for the cell cultures with a stimulator by the cpm counts/min for the cell cultures without a stimulator. SI values higher than or equal to 1.0 (*cut-off*) were considered as a positive result in the proliferation assay.

Cell cycle analysis

The cell cycle was assessed as previously described[70,71]. Briefly, AGS cells or guinea pig fibroblasts (1 × 10^6 cells/mL) were seeded in 6-well plates (NUNC, Denmark) in 1 mL/well of cRPMI. After overnight preincubation, the cells were cultured for 24 h in the presence of bacterial stimuli or in culture medium alone (as a control). After stimulation, the cells were harvested by trypsinization

and fixed in 70% ice-cold ethanol. The cells were stained for the total DNA content with a solution containing 75 μmol/dm^3 propidium iodide (PI) and 50 IU Kunitz/mL of DNase-free RNase (Sigma St. Louis, MI, United States) in PBS for 30 min, at 37 ℃. The cell cycle distribution was then analyzed in an LSR II Flow Cytometer (Becton Dickinson, Mountain View, CA, United States). The percentage of cells in G_1, S and G_2/M phases of the cell cycle, and the percentage of cells undergoing apoptosis were determined with the FlowJo analytical software.

DAPI staining of cell nuclei

Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, MI, United States), a fluorescent dye which has a strong affinity to the AT base pair in DNA. The cells, after a 24 h stimulation with bacterial antigens, were fixed with 4% formaldehyde, and stained with DAPI solution (2.5 μg/mL) for 15 min at room temperature. Preparations were viewed under a fluorescent microscope (Zeiss, Axio Scope, A1) at a wavelength of 358 nm (excitation) and 461 nm (emission). We evaluated the percentage of the cells with damaged nuclei.

Comet assay

The comet assay was used to detect DNA damage. It was performed under alkaline conditions (pH > 13) as previously described[72]. Briefly, AGS and fibroblast cell suspensions were separately mixed with low-melting point agarose at 1 × 10^4 cells/mL, at 37 ℃ and evenly pipetted onto the microscope slides pre-coated with 250 μL of 0.5% normal melting point agarose. The slides were maintained on ice for 10 min to solidify. All the steps were conducted in the dark or under reduced light to prevent additional DNA damage. The remaining cells were exposed to bacterial stimuli for 24 h. After incubation, the treated cells were washed with ice-cold PBS and spread on the slides as described above. The slides were then immersed in a chilled lysis solution (2.5 mol/L NaCl, 100 mmol/L EDTA, 10 mmol/L Tris-HCl, 1% Triton X-100 and 1% N-lauroylsarcosine sodium, pH 10.0) for 1 h at 4 ℃ in the dark. Thereafter, the slides were rinsed in freshly prepared and chilled electrophoresis buffer (1 mmol/L EDTA, 300 mmol/L NaOH and pH > 13) at 4 ℃ for 40 min to allow DNA unwinding. Electrophoresis was then performed at 25 V, 300 mA (0.86 V/cm) for 23 min at 4 ℃. The slides were washed with a neutralizing buffer (0.4 mol/L Tris-HCl and pH 7.5) and then DNA was stained with DAPI (2 μg/mL). Images of the comets were captured under a fluorescence microscope (Zeiss, Axio Scope. A1) at × 400 magnification. For each sample, a minimum of 100 comets were randomly selected and the percentage of DNA in the comet tail (% tail DNA) was analyzed using the Comet Assay Software Project (CASP) as recommended by Końca *et al*[73].

Apoptosis detection assay

The binding of annexin V-fluorescein isothiocyanate (Ann-V) to externalized phosphatidylserine was used as a marker of apoptotic AGS cells and fibroblasts detected by flow cytometry as previously described[70,71]. Cells were seeded at a density of 1 × 10^6 cells/well into 6-well microplates (NUNC, Denmark) in 1 mL/well of cRPMI. After overnight preincubation, the cells were cultured for a further 24 h in the presence of bacterial stimuli or in culture medium alone (as a control). The fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit (Becton Dickinson, San Jose, CA, United States) was used for the differentiation of apoptotic and necrotic cells. Briefly, after stimulation, the cells were harvested by gentle trypsynization and washed with cold PBS. The cells were resuspended in 1 mL of 1 × binding buffer. Next, 100 μL was transferred to a 5 mL flow cytometry tube, and incubated with 5 μL of Annexin V and 5 μL of PI for 15 min, at room temperature in the dark. Next, 400 μL of 1 × binding buffer was added to each tube. Flow cytometric analysis was performed immediately after staining.

Annexin V/PI fluorescence was analysed for each sample; 10000 events were collected and fluorescence was detected using FlowJo software. The results are presented as percentages of cells that were viable (Ann-V$^-$/PI$^-$), early apoptotic cells (Ann-V$^+$/PI$^-$), the cells in the late stages of apoptosis (Ann-V$^+$/PI$^+$) or necrotic cells (Ann-V$^-$/PI$^+$).

ELISA assays

The epidermal growth factor (EGF) concentration was evaluated in supernatants from AGS cell cultures untreated or stimulated for 24 h with bacterial antigens, according to the manufacturer's protocol (Human EGF ELISA Kit, Elabscience Biotechnology Co., Ltd, China). Since there is a positive correlation between the concentration of EGF and the signal transducer and activator of transcription 3 (STAT3) signalling pathway, we also assessed the percentage of phospho-STAT3. The procedure detecting phospho-STAT3 in AGS cells was used according to the manufacturer's instructions (Human/Mouse phospho-STAT3 (Y705) Cell-Based ELISA, R&D Systems, Minneapolis, United States). Briefly, 100 μL of 2 × 10^4 AGS cells was seeded into each well of a black 96-well microplate with a clear bottom, and incubated overnight at 37 ℃. The cells were then treated with bacterial antigens for 24 h as previously described. AGS cells stimulated with EGF (0.125 ng/mL) were used as a positive control. Following the treatments, the cells were tested with the cell-based ELISA kit.

Statistical analysis

All values were expressed as the mean ± SD. The differences between antigen activities were tested using the non-parametric Mann-Whitney *U* test. For

statistical analysis the Statistica 12 PL software was used. Results were considered statistically significant when $P < 0.05$.

RESULTS

Kinetics of wound healing in response to H. pylori antigens

The percentages of gastric epithelial cells and fibroblasts migrating to the wound zone are presented on Figure 1A(i) and B(i), respectively, and visualised on images showing the influence of selected antigens which interfered with cell migration and wound healing process [Figure 1A(ii) and B(ii)].

The motility of untreated AGS cells increased with time and the percentages of cells migrating to the "wounded zone" were: 62.3%, 80.8% and 100% after 24, 48 and 72 h, respectively. The rate of wound healing accelerated after 24-h of the cell cultures treatment with GE (10 μg/mL), UreA (5 μg/mL), CagA (1 μg/mL), and *H. pylori* LPS as well as *E. coli* LPS at 1 ng/mL ($P = 0.03$) (Figure 1A). On the other hand, *E. coli* LPS at 25 ng/mL affected cell migration up to 45.4% confluence and 46.1% in 24 and 48-h cell cultures, respectively. By comparison, *H. pylori* LPS at the same concentration (25 ng/mL) inhibited completely the wound healing in 24-, 48- and 72-h cell cultures, which was correlated with a decrease in the cell adhesion (100% lack of confluence). This effect was abolished in the cell cultures exposed for 24 h, but not in those exposed for 48 and 72 h, to *H. pylori* LPS (25 ng/mL) in combination with CagA. Prolonged cell exposure to *H. pylori* LPS (25 ng/mL) and CagA resulted in a complete loss of cell adherence. Interestingly, the inhibitory effect of *H. pylori* LPS on cell migration was abolished in the cell cultures exposed for 24, 48 and 72 h to *H. pylori* LPS (25 ng/mL) in the presence of CagA, UreA and GE.

The results indicate that *H. pylori* compounds differ in terms of their impact on cell migration. *H. pylori* LPS at a high concentration inhibited wound healing, while GE, UreA, CagA as well as *H. pylori* LPS at a low dose accelerated cell motility.

Similarly to AGS cells, the wound healing rate of untreated fibroblasts increased with the time by 10.8%, 42.8% and 68.7% in 24-, 48- and 72-h cell cultures, respectively (Figure 1B). Cell migration increased in cell cultures exposed to UreA ($P = 0.03$) for 24 and 48 h as well as to CagA ($P = 0.03$) or GE ($P = 0.03$) for 24, 48 and 72 h. In contrast, 24, 48 and 72-h incubation of cell cultures with *H. pylori* LPS at 1 ng/mL resulted in a significant decrease in the extent of wound recovery. By comparison, *E. coli* LPS at 1 ng/mL did not affect cell migration 24 and 48 h after the challenge. The percentage of wound confluence was even higher in 72-h cell cultures exposed to the same concentration of *E. coli* LPS as compared to untreated cells. The percentage of wound confluence in 24-h cell cultures, but not in 48- and 72-h cell cultures exposed to *H. pylori* LPS at 25 ng/mL slightly increased when *H. pylori* LPS (25 ng/mL) was combined with CagA. Also, a mixture of *H. pylori* LPS (25 ng/mL) with the following antigens: CagA, UreA and GE minimized the loss of cell confluence. However, these *H. pylori* antigens were not able to completely neutralize the effect of *H. pylori* LPS used at high concentration (25 ng/mL).

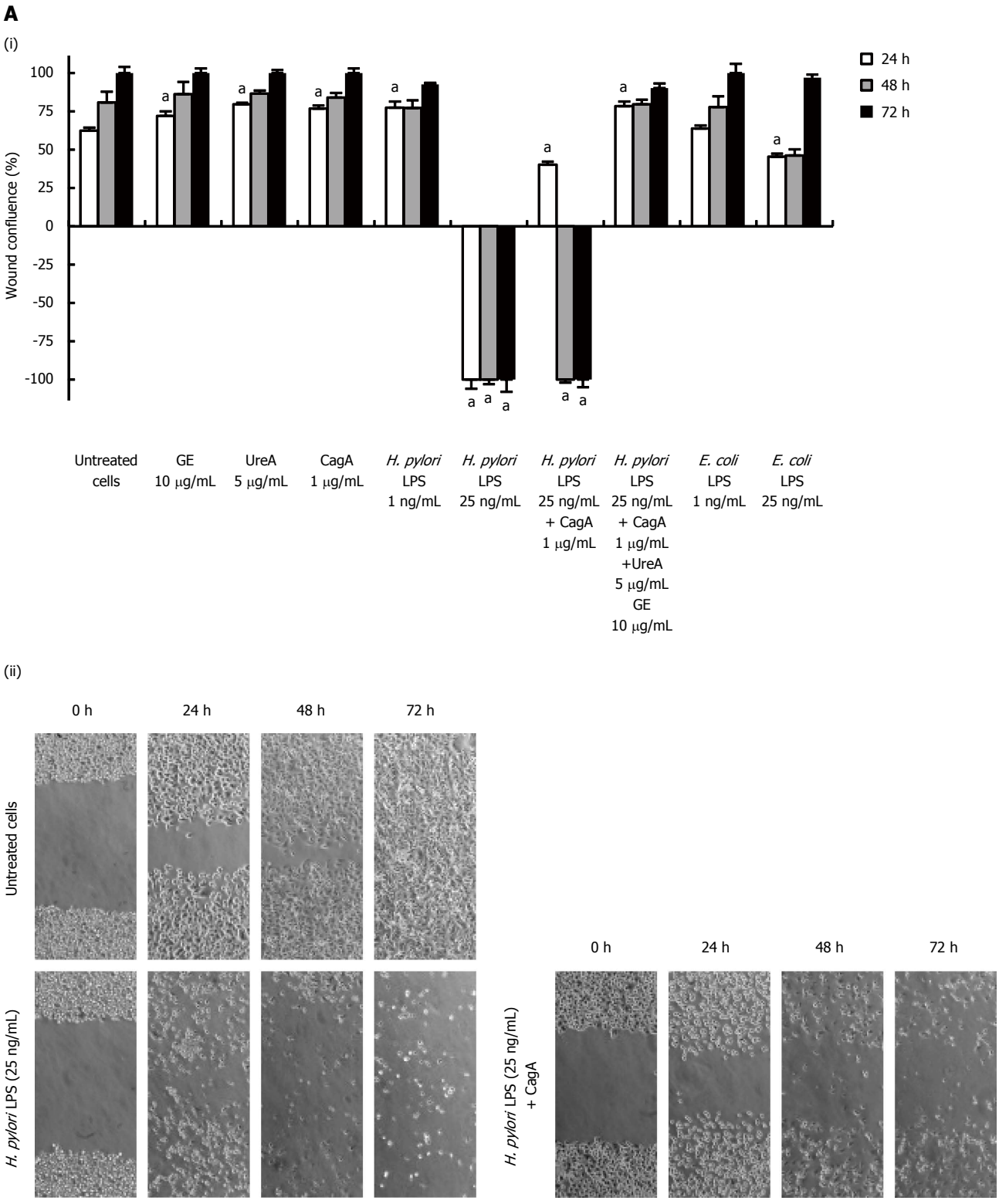
These results show inhibitory effect of *H. pylori* LPS used both at high and low concentrations on the wound healing process in fibroblasts and up-regulation of cell migration after the challenge with other *H. pylori* compounds used separately in the study.

Cell migration vs cell proliferation, cell cycle and metabolic activity in response to H. pylori antigens 24 h after the challenge

In order to specify the mechanism of wound healing in response to activity of *H. pylori* antigens, we compared the impact of *H. pylori* compounds on proliferation, cell viability and cell cycle of AGS cells and fibroblasts 24 h after the challenge. The increased wound closure observed in AGS cell cultures exposed to GE, UreA, CagA or *H. pylori* LPS (1 ng/mL) (Figure 1A) was related to the increased proliferation ($P = 0.03$; Figure 2A) and enhanced ability of the cells to reduce MTT ($P = 0.02$; Figure 2B). The *E. coli* LPS at 1 ng/mL did not influence the cell movement (Figure 1A), proliferation (Figure 2A) or viability (Figure 2B). Despite the reduced wound confluence in response to *E. coli* LPS at 25 ng/mL (Figure 1A), the proliferative activity and cell viability were not affected (Figure 2A and B). By comparison, *H. pylori* LPS at 25 ng/mL downregulated cell migration (100% lack of cell confluence, $P = 0.03$; Figure 1A) and proliferation (SI = 0.3, $P = 0.03$; Figure 2A) as well as cell viability ($P = 0.02$; Figure 2B). A similar effect was observed when *H. pylori* LPS (25 ng/mL) was used in combination with CagA (SI = 0.5, $P = 0.03$). In these conditions also the cell ability to reduce MTT was reduced ($P = 0.02$; Figure 2B). However, when *H. pylori* LPS (25 ng/mL) was used together with CagA, UreA and GE, it lost its inhibitory potential in terms of cell migration as well as proliferative and metabolic activity ($P < 0.05$; Figure 1A, Figure 2A and B).

The obtained results indicate that the ability of *H. pylori* LPS at a high concentration to reduce cell migration is associated with the inhibition of cell spreading and metabolic activity. By comparison, epithelial cells recovered their activity after the exposure to *H. pylori* GE, UreA, CagA or *H. pylori* LPS used at a low dose.

To determine whether the impaired ability of AGS cells to proliferate was related to the cell cycle arrest, we analyzed the cell cycle phase distribution in 24-h cell cultures untreated or pulsed with *H. pylori* antigens. Cells were stained with PI and subjected to


A
(i)
Wound confluence (%)
100
75
50
25
0
-25
-50
-75
-100
24 h
48 h
72 h
a
Untreated cells
GE 10 μg/mL
UreA 5 μg/mL
CagA 1 μg/mL
H. pylori LPS 1 ng/mL
H. pylori LPS 25 ng/mL
H. pylori LPS 25 ng/mL + CagA 1 μg/mL
H. pylori LPS 25 ng/mL + CagA 1 μg/mL +UreA 5 μg/mL GE 10 μg/mL
E. coli LPS 1 ng/mL
E. coli LPS 25 ng/mL
(ii)
0 h
24 h
48 h
72 h
Untreated cells
H. pylori LPS (25 ng/mL)
H. pylori LPS (25 ng/mL) + CagA

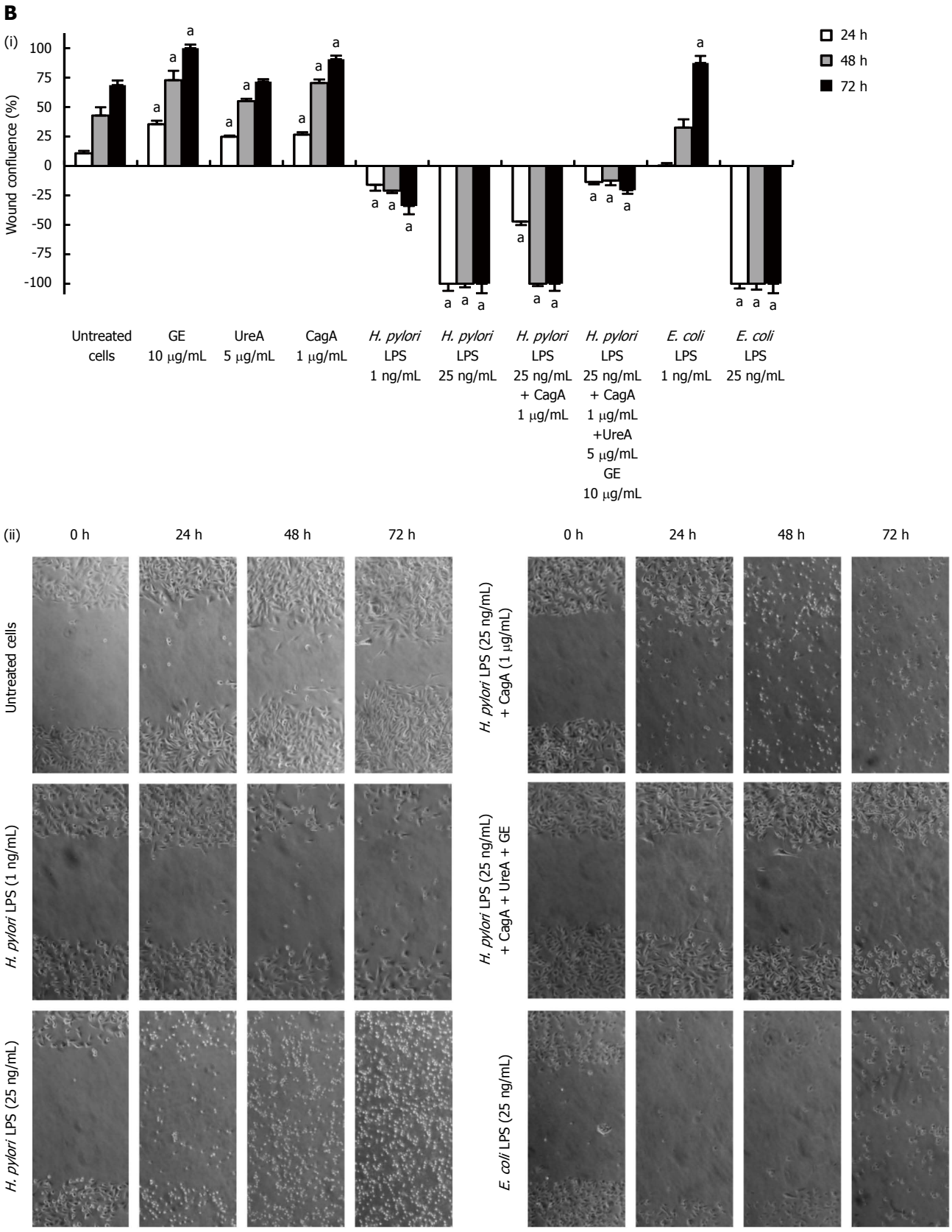


Figure 1 Migration effectiveness of human epithelial AGS cells and guinea pig fibroblasts assessed in a scratch assay. (i) AGS cells (A) and fibroblasts (B) were grown to confluence and incubated overnight in RPMI-1640 medium/2% FBS/1% standard antibiotics. A wound was then made in a cell monolayer and culture medium alone or solutions of bacterial antigens were added. Wound areas were measured at 0, 24, 48 and 72 h after the challenge. Graphs of the average wound size against time, in which the results are shown for cells incubated alone (culture medium) or treated with GE (10 μg/mL), UreA (5 μg/mL), CagA (1 μg/mL) and *Helicobacter pylori* (*H. pylori*) LPS as well as *Escherichia coli* (*E. coli*) LPS (1 ng/mL or 25 ng/mL) or with a combination of *H. pylori* compounds: *H. pylori* LPS (25 ng/mL) and CagA (1 μg/mL) or *H. pylori* LPS (25 ng/mL), CagA (1 μg/mL), UreA (5 μg/mL) and GE (10 μg/mL). $P = 0.03$ *vs* untreated cells; (ii) Phase-contrast microscopy images were taken at the indicated time points and the extent of wound closure for each treatment variant was calculated as a percentage of migrating cells. Representative photos of each time point are shown (magnification × 200). [a]$P = 0.03$ *vs* untreated cells (according to the time of stimulation).

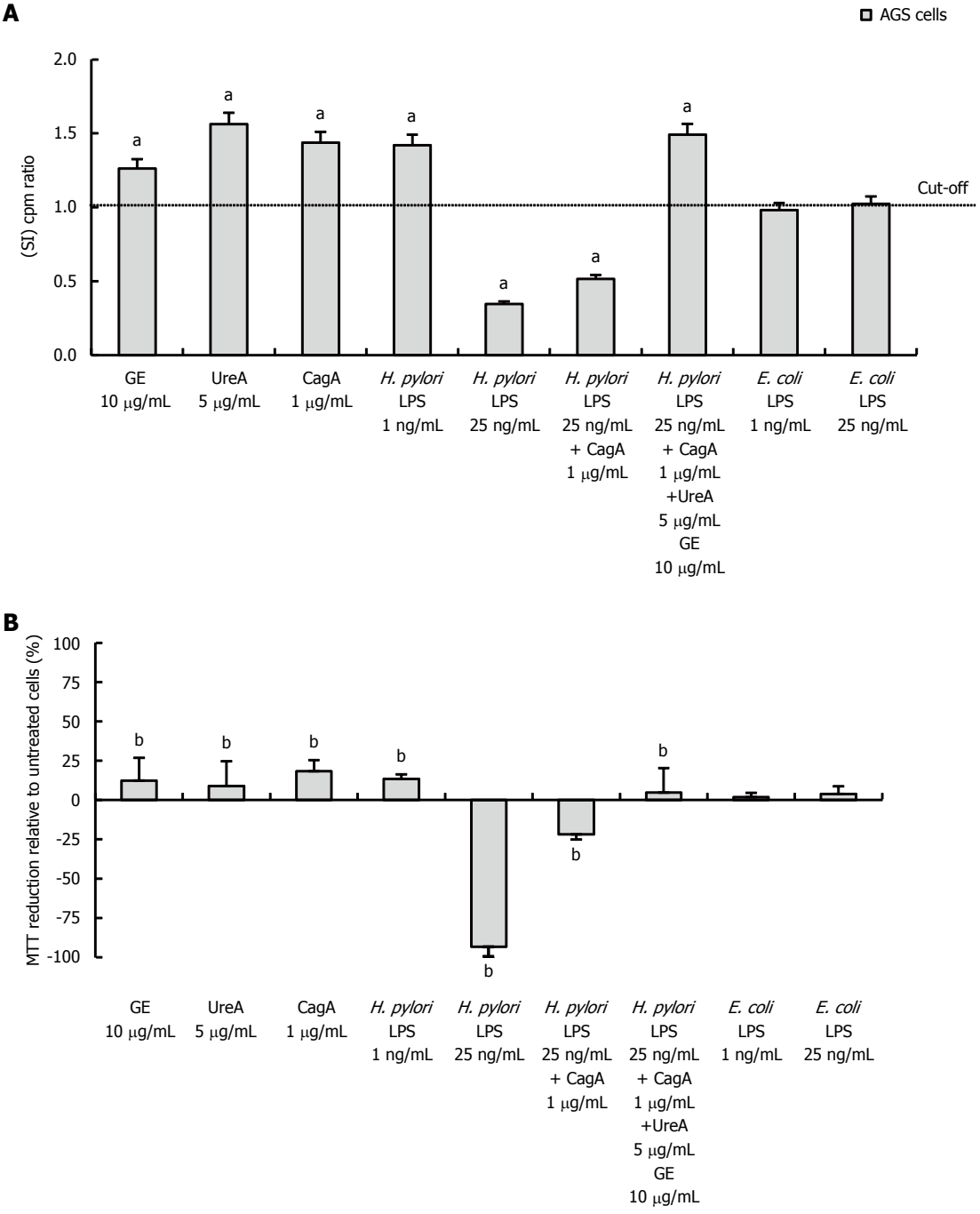


Figure 2 Influence of bacterial antigens on AGS cell proliferation and ability to reduce MTT. A: The proliferative activity of AGS cells was estimated in cell cultures non-stimulated or stimulated for 24 h with bacterial antigens. After incubation, [^{3}H]-thymidine incorporation into cellular DNA was analyzed. The graph shows the stimulation index (SI), which was calculated by dividing the radioactivity counts (cpm/min) for the cell cultures in the presence of a stimulus by the counts for control cell cultures in RPMI-1640 medium alone. The results are shown as SI ± SD of six independent experiments, performed in triplicates. [a]*P* = 0.03 *vs* untreated cells; B: AGS were treated for 24 h with bacterial antigens. After incubation, the ability of cells to reduce MTT was estimated. The graph shows the percentage of MTT reduction ± SD relative to untreated cells. The data represent the average values of four independent experiments performed in triplicates. The values have been normalized to those of the untreated cells. [b]*P* = 0.02 *vs* untreated cells.

flow cytometric analysis. As shown in Figure 3, 63%, 21% and 13% of untreated AGS cells were in the G_1, S and G_2 phase, respectively. It was shown that *H. pylori* LPS at 25 ng/mL, 24 h after the challenge, prevented AGS cells from entering the G_2 phase, resulting in the accumulation of AGS cells in the S phase (37%, *P* = 0.01). Similarly, *H. pylori* LPS (25 ng/mL) in the presence of CagA blocked the cell cycle and caused an increase in the cell number in the S phase (34%), (*P* = 0.03). A mixture of *H. pylori* LPS (25 ng/mL), CagA, UreA and GE did not induce any block in the cell turnover. By comparison, *H. pylori* LPS at 1 ng/mL and *E. coli* LPS at both low and high concentrations as well as other *H. pylori* antigens used separately did not affect the cell cycle.

These results reveal that the disturbance in epi-

	Cell cycle of AGS cells			
	G_1	S	G_2	Apoptosis
Untreated cells	63%	21%	13%	3%
GE (10 μg/mL)	64%	20%	14%	2%
UreA (5 μg/mL)	62%	22%	15%	1%
CagA (1 μg/mL)	61%	20%	16%	3%
H. pylori LPS (1 ng/mL)	62%	22%	15%	1%
H. pylori LPS (25 ng/mL)	49%	**37**% P = 0.01	9%	5%
H. pylori LPS (25 ng/mL) + CagA (1 μg/mL)	50%	**34**% P = 0.03	7%	9%
H. pylori LPS (25 ng/mL) + CagA (1 μg/mL) + UreA (5 μg/mL) + GE (10 μg/mL)	67%	14%	12%	7%
E. coli LPS (1 ng/mL)	53%	22%	12%	13%
E. coli LPS (25 ng/mL)	63%	20%	13%	2%

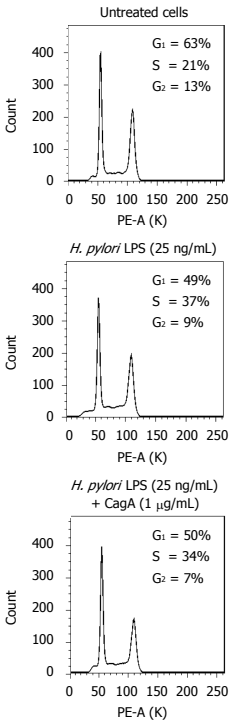


Figure 3 Effect of *Helicobacter pylori* antigens on the epithelial cell cycle profile. AGS cells were incubated for 24 h in RPMI-1640 medium alone or in the presence of bacterial antigens. The cell cycle profile was determined by propidium iodide (PI) staining and the analysis was performed by flow cytometry. The data represent the percentage of cells in each cycle phase of six experiments. Statistically significant differences are indicated as $P < 0.05$ *vs* untreated cells and included in DNA histograms.

thelial cell migration induced by high dose of *H. pylori* LPS is a result of the cell cycle arrest.

The similar comparisons were made for the wound healing capacity of fibroblasts (Figure 1B), their proliferative activity (Figure 4A), the ability to reduce MTT (Figure 4B) and cell cycle distribution (Figure 5), in response to *H. pylori* antigens 24 h after the challenge. As shown in Figure 1B, migration of fibroblasts was modulated positively or negatively depending on the *H. pylori* antigen. The percentage of wound closure increased in response to single *H. pylori* antigens: GE, UreA or CagA, 24 h after the challenge (Figure 1B). In the case of GE, this effect was related to proliferation enhancement (P = 0.01) and MTT reduction (P = 0.02), whereas in response to UreA both activities were on the level of untreated cells (Figure 4A and B). In the cell cultures exposed to CagA the enhanced MTT reduction was shown (P = 0.01, Figure 4B). Reduced cell migration (-16%), which was observed in response to *H. pylori* LPS at 1 ng/mL, was not related to a decrease in MTT reduction and cell proliferation (Figure 1B, Figure 4A and B). However, even at the low concentration, *H. pylori* LPS caused an accumulation of cells in the S phase (25%), (P = 0.05; Figure 5). The strongest effect on fibroblasts was induced by *H. pylori* LPS at 25 ng/mL. At this concentration, *H. pylori* LPS completely abrogated the process of wound healing (Figure 1B), significantly diminished the proliferative activity of the cells, (P = 0.02; Figure 4A), and MTT reduction (P = 0.001; Figure 4B). This phenomenon was related to the increased number (16%) of cells in the G_2 phase of the cell cycle (P = 0.02; Figure 5). It was also shown that *H. pylori* LPS at 25 ng/mL even in combination with CagA downregulated cell migration (P = 0.03; Figure 1B), which was followed by the inhibition of proliferative response (P = 0.02; Figure 4A), MTT reduction, (P = 0.001; Figure 4B) and the cell cycle arrest in the S phase (28%), (P = 0.03; Figure 5). Stimulation of fibroblasts with the combination of *H. pylori* compounds (CagA, UreA, GE) in the presence of *H. pylori* LPS at 25 ng/mL resulted in the inhibition of the cell functions: migration (P = 0.03; Figure 1B), proliferation (P = 0.02; Figure 4A), MTT reduction (P = 0.001; Figure 4B) and the cell cycle arrest in the S phase (21%, P = 0.05; Figure 5). However, none of these antigens used alone (without *H. pylori* LPS at 25 ng/mL) affected any of these functions. By comparison, standard *E. coli* LPS at 1 ng/mL did not affect cell migration, proliferation or metabolic activity, whereas at 25 ng/mL it downregulated the cell movement (P = 0.03; Figure 1B), proliferation (P = 0.02; Figure 4A) and MTT reduction (P = 0.00002; Figure 4B). These effects were associated with the cell

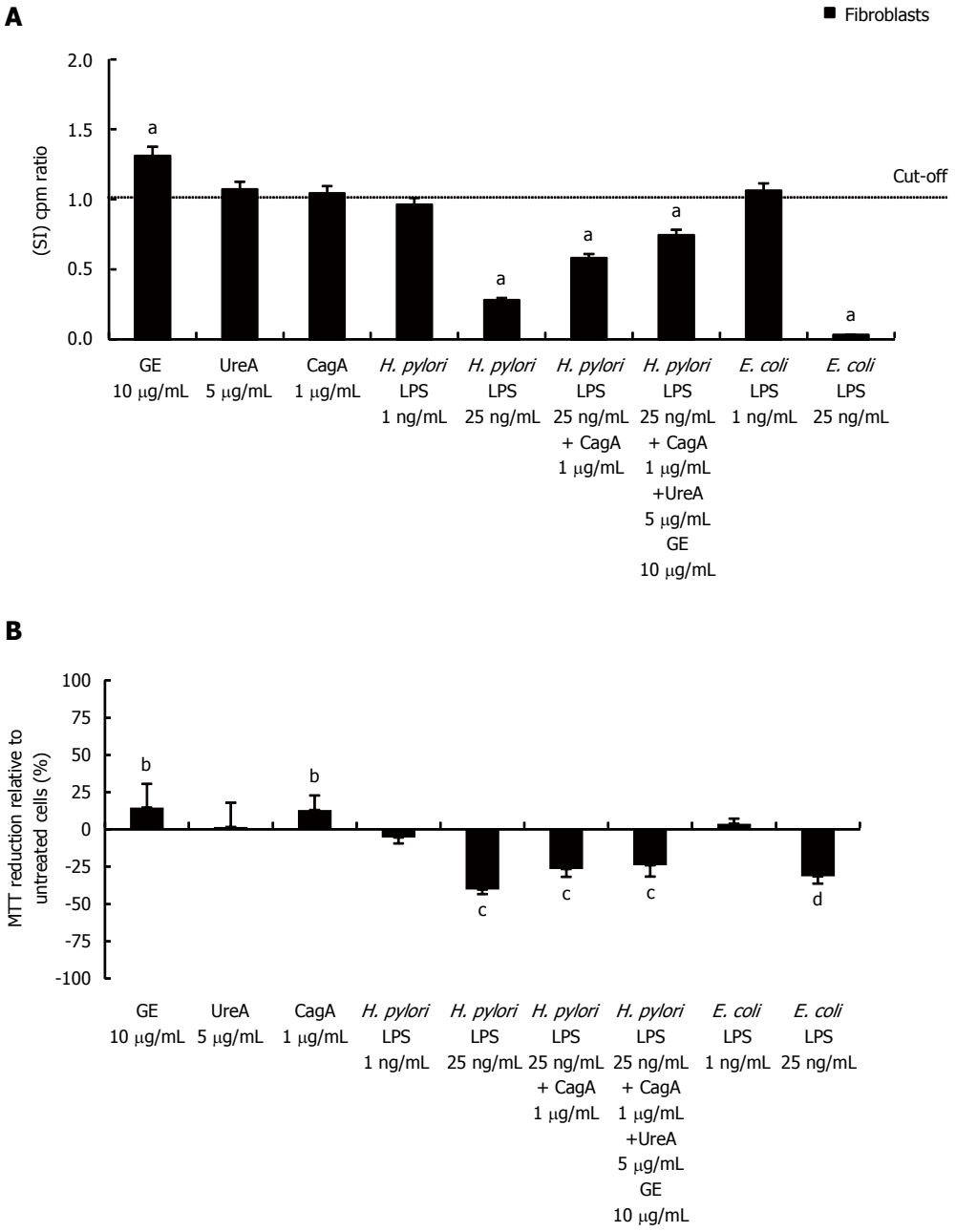


Figure 4 Influence of bacterial antigens on the ability of fibroblasts to proliferate and reduce MTT. A: The proliferative activity of fibroblasts was estimated in cell cultures non-stimulated or stimulated for 24 h with bacterial antigens. After incubation, [^{3}H]-thymidine incorporation into cellular DNA was analyzed. The graph shows the stimulation index (SI), which was calculated by dividing the radioactivity counts (cpm/min) for the cell cultures in the presence of a stimulus by the counts for control cell cultures in RPMI-1640 medium alone. The results are shown as SI ± SD of six independent experiments, performed in triplicates. $^aP = 0.02$ *vs* untreated cells; B: Fibroblasts were treated for 24 h with bacterial antigens. After incubation, the ability of cells to reduce MTT was estimated. The graph shows the percentage of MTT reduction ± SD relative to untreated cells. The data represent the average values of four independent experiments performed in triplicates. The values have been normalized to those of the untreated cells. $^bP = 0.01$; $^cP = 0.001$; $^dP = 0.00002$ *vs* untreated cells.

cycle arrest (17%) in the G_2 phase, $P = 0.01$ (Figure 5).

These studies show that fibroblasts similarly to the epithelial cells are sensitive to high concentration of *H. pylori* LPS, but in contrast to epithelial cells fibroblasts are also affected by low concentration of *H. pylori* LPS as well as high dose of *E. coli* LPS.

H. pylori LPS-induced cell dysfunction vs DNA disintegration, cell apoptosis or necrosis

The results presented above prompted us to search for a deeper explanation of the nature of *H. pylori* LPS-dependent negative modulation of the cell functions, on the level of DNA integrity and signs of cell death. To examine whether *H. pylori* LPS at 25 ng/mL may induce DNA damage in AGS cells and fibroblasts, we performed DAPI staining and a comet assay. The results of these assays are shown in Figure 6A and B for AGS cells and fibroblasts, respectively. *H. pylori* LPS at 25 ng/mL and in combination with CagA (but not CagA alone) induced significant DNA condensation

	Cell cycle of fibroblasts			
	G1	S	G2	Apoptosis
Untreated cells	72%	16%	11%	1%
GE (10 μg/mL)	71%	15%	13%	1%
UreA (5 μg/mL)	69%	18%	11%	2%
CagA (1 μg/mL)	71%	12%	15%	2%
H. pylori LPS (1 ng/mL)	63%	25% *P* = 0.05	12%	0%
H. pylori LPS (25 ng/mL)	68%	12%	16% *P* = 0.02	4%
H. pylori LPS (25 ng/mL) + CagA (1 μg/mL)	58%	28% *P* = 0.03	11%	3%
H. pylori LPS (25 ng/mL) + CagA (1 μg/mL) + UreA (5 μg/mL) + GE (10 μg/mL)	67%	21% *P* = 0.05	11%	1%
E. coli LPS (1 ng/mL)	65%	20%	11%	4%
E. coli LPS (25 ng/mL)	69%	13%	17% *P* = 0.01	1%

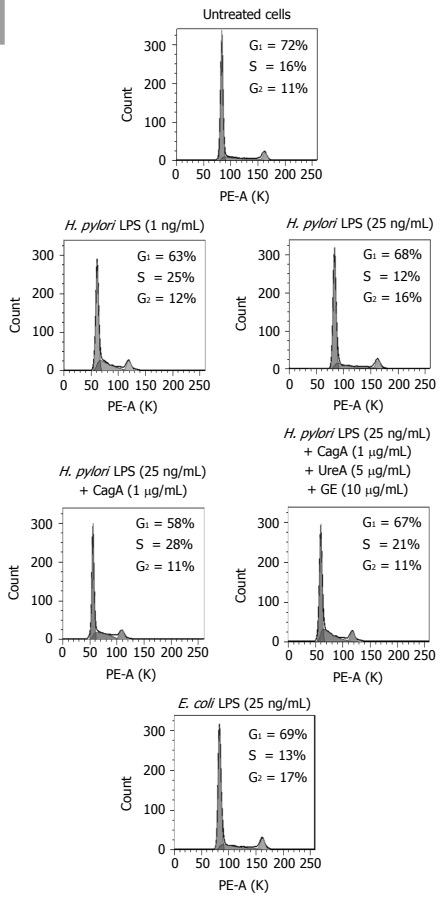
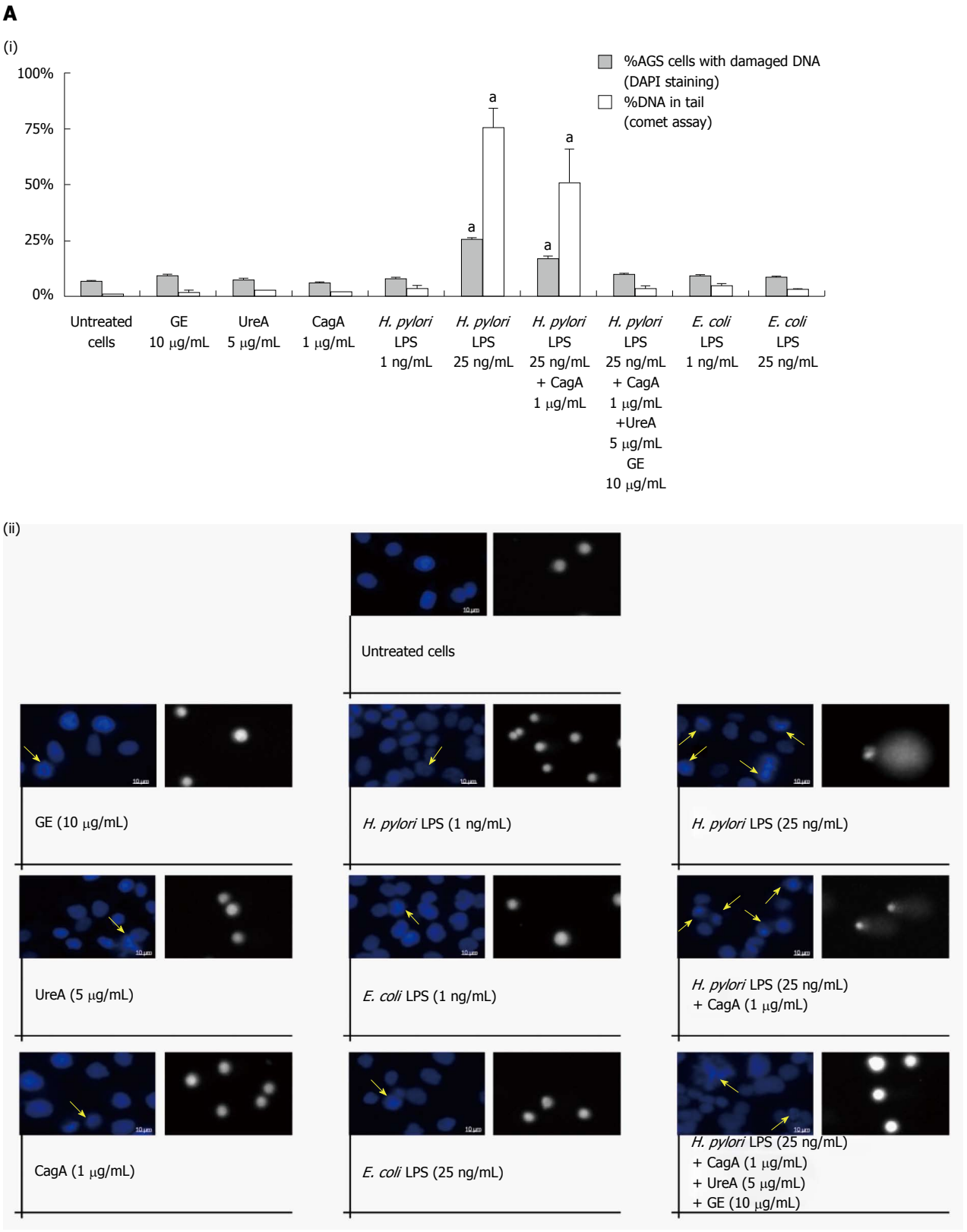


Figure 5 Effect of *Helicobacter pylori* antigens on the fibroblast cell cycle profile. Fibroblasts were incubated for 24 h in RPMI-1640 medium alone or in the presence of bacterial antigens. The cell cycle profile was determined by propidium iodide (PI) staining and the analysis was performed by flow cytometry. The data represent the percentage of cells in each cycle phase of six experiments. Statistically significant differences are indicated as $P < 0.05$ *vs* untreated cells and included in DNA histograms.

in 24.7% and 16.3% of AGS cells, respectively, which was shown by the percentage of cells with damaged DNA and visualized by an increased fluorescence intensity (Figure 6A). Furthermore, *H. pylori* treatment led to longer DNA smears: 75% and 49.7% DNA in tail, respectively ($P < 0.0001$; Figure 6A).

In a cell culture of fibroblasts, *H. pylori* LPS induced dose dependent DNA damage as assessed by DAPI staining and a comet assay (Figure 6B). The percentages of fibroblasts treated with *H. pylori* LPS at 25 ng/mL or 1 ng/mL with the signs of DNA damage detected by DAPI staining were 45.7% and 12.7%, respectively ($P = 0.03$). A higher concentration of *H. pylori* LPS (25 ng/mL) caused longer DNA smears, whereas a lower concentration (1 ng/mL) resulted in the formation of shorter comet tails, 78.7% ($P = 0.000001$) and 6.4% ($P = 0.0001$), respectively (Figure 6B). DNA damage was also detected in cell cultures treated with *H. pylori* LPS (25 ng/mL) and CagA (23.7%, $P = 0.03$) or *H. pylori* LPS (25 ng/mL) and other *H. pylori* compounds: CagA, UreA and GE (14%, $P = 0.03$). However, cellular DNA damage induced by a combination of *H. pylori* antigens was lower than in the cells treated with *H. pylori* LPS (25 ng/mL) alone (45.7%, $P = 0.03$). All these results are in accordance with the results obtained in a comet assay (Figure 6B). By comparison, in cell cultures treated with standard *E. coli* LPS at 25 ng/mL DNA damage was detected in 24% fibroblasts ($P = 0.03$) and the percentage of DNA in the tail was 72.4%, $P < 0.05$ (Figure 6B). *E. coli* LPS at 1 ng/mL did not induce any significant DNA damage.

In order to clarify whether the reduction of cells viability, and the inhibition of cell migration are related to apoptosis or necrosis, FACS analysis was performed. The results are presented in Figure 7A and B for AGS cells and fibroblasts, respectively. AGS cells were cultured with or without *H. pylori* LPS (25 ng/mL) or *H. pylori* LPS in combination with CagA for 24 h and subjected to flow cytometry analysis after staining with Ann-V-FITC and PI. As shown in Figure 7A, 91% of uninfected AGS cells were viable (Ann-V^- PI^-), 3% were early apoptotic, 4% were late apoptotic (Ann-V^+ PI^-) and 2% were necrotic (Ann-V^- PI^+). In cell cultures of AGS treated with *H. pylori* LPS alone at 25 ng/mL an increase in the percentage of cells undergoing


A
(i)
100%
75%
50%
25%
0%
%AGS cells with damaged DNA (DAPI staining)
%DNA in tail (comet assay)
a
a
a
a
Untreated cells
GE 10 μg/mL
UreA 5 μg/mL
CagA 1 μg/mL
H. pylori LPS 1 ng/mL
H. pylori LPS 25 ng/mL
H. pylori LPS 25 ng/mL + CagA 1 μg/mL
H. pylori LPS 25 ng/mL + CagA 1 μg/mL +UreA 5 μg/mL GE 10 μg/mL
E. coli LPS 1 ng/mL
E. coli LPS 25 ng/mL
(ii)
Untreated cells
GE (10 μg/mL)
H. pylori LPS (1 ng/mL)
H. pylori LPS (25 ng/mL)
UreA (5 μg/mL)
E. coli LPS (1 ng/mL)
H. pylori LPS (25 ng/mL) + CagA (1 μg/mL)
CagA (1 μg/mL)
E. coli LPS (25 ng/mL)
H. pylori LPS (25 ng/mL) + CagA (1 μg/mL) + UreA (5 μg/mL) + GE (10 μg/mL)

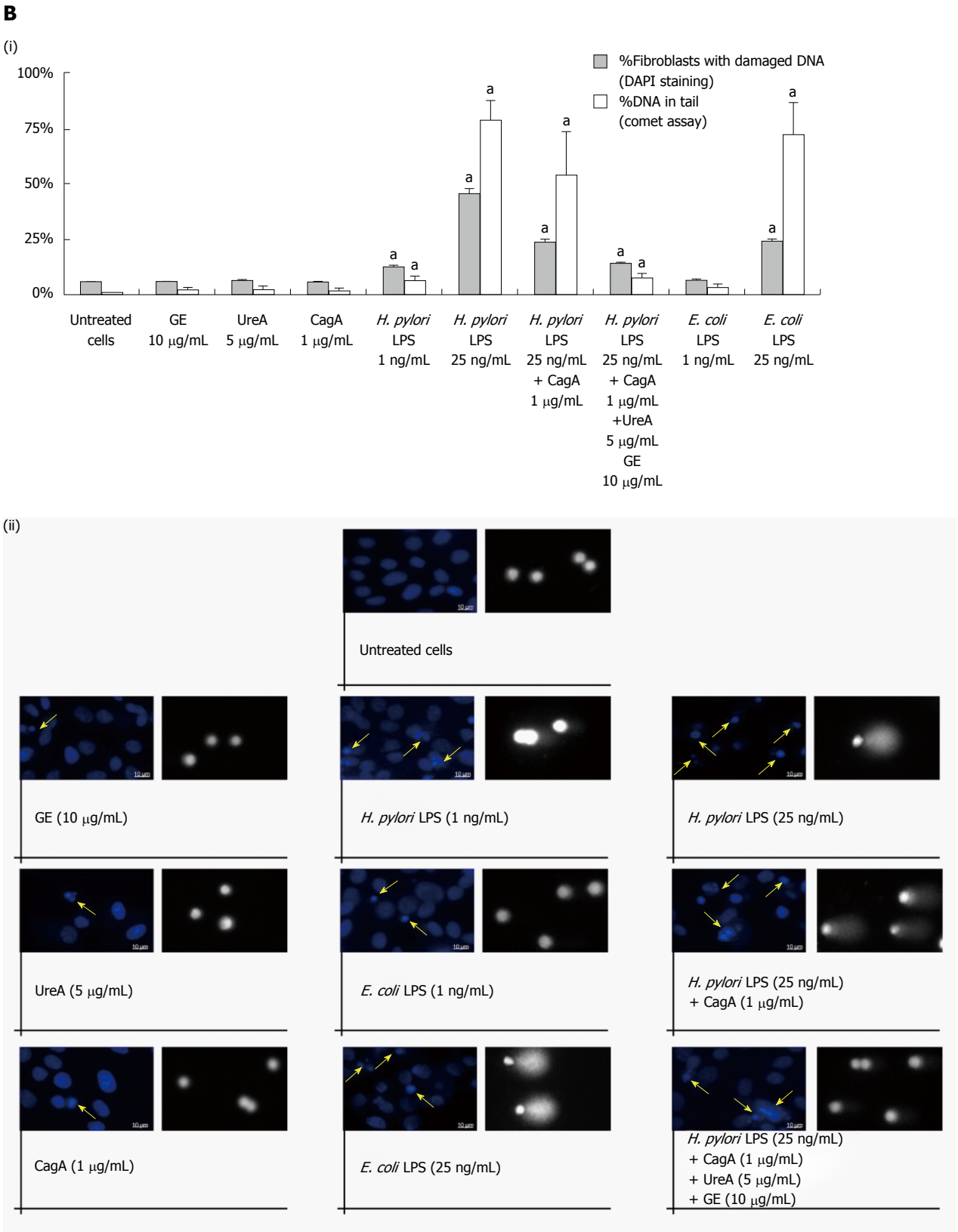


Figure 6 Genotoxic properties of *Helicobacter pylori* antigens assessed by 4',6-diamidino-2-phenylindole staining and a comet assay. The influence of *Helicobacter pylori* or *E. coli* antigens on DNA stability was estimated in AGS cells (A) and fibroblasts (B) 24 h after the challenge. A 4',6-diamidino-2-phenylindole (DAPI) staining assay was used to visualize DNA changes in cell nuclei and a comet assay was applied to confirm DNA damage by the measurement of the percentage of DNA in the comet tail. Mean values were replicated of 50 comets each. The values are the means ± SD. $^{a}P < 0.05$ *vs* untreated cells. (i) the graphs indicate the percentage of cells with DAPI stained nuclei (blue bars) and the percentage of nuclei with DNA in the comet tail (grey bars); and (ii) visualization of morphological changes in the cell nuclei after the treatment with bacterial antigens followed by DAPI staining and a comet assay. Imaging was performed using a fluorescent microscope (Axio Scope A1, Zeiss, Germany). The arrows indicate the damaged cell nuclei (magnification, × 1000). DNA tails were measured using the CASP software (latest beta version 1.2.3.beta2). Representative results of the comet assay were selected (magnification, × 400).

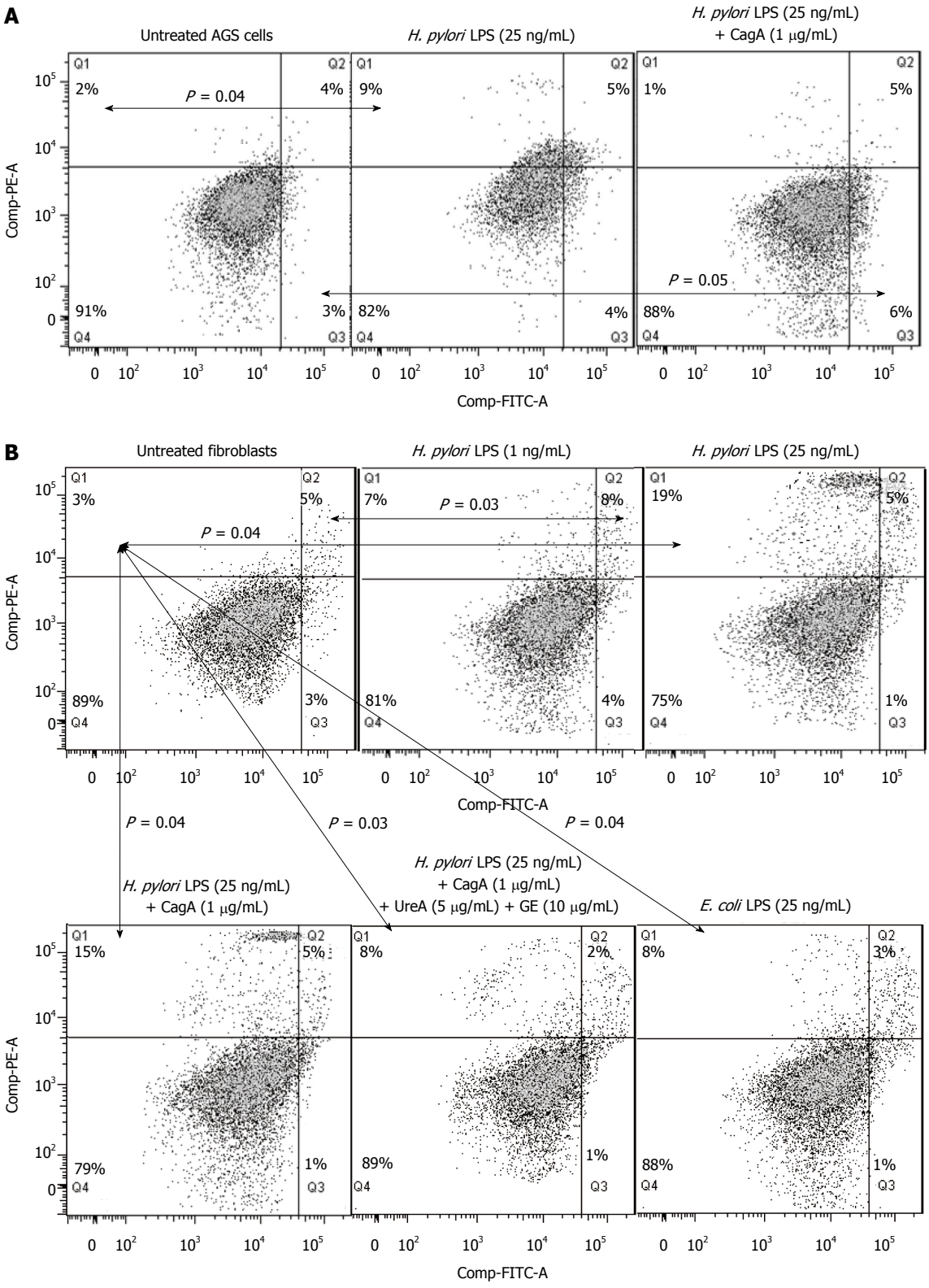


Figure 7 Type of cell death in response to *Helicobacter pylori* antigens. Effects of bacterial antigens on AGS cells (A) and fibroblasts (B) concerning cell death were measured 24 h after the challenge by double staining of the cells with isothiocyanate fluorescein (FITC)-conjugated annexin V and propidium iodide (PI) using flow cytometry. Quadrants were designed as follows, Q4: Ann-V$^-$/PI$^-$ - viable cells; Q3: Ann-V$^+$/PI$^-$ - cells with the signs of early apoptosis; Q2: Ann-V$^+$/PI$^+$ - cells with the signs of late apoptosis; Q1: Ann-V$^-$/PI$^+$ - necrotic cells. All dot plots are a representation of equal cell populations (the fluorescence of 10000 cells was gated and counted using the FlowJo software). The data represent the average values of six independent experiments. Statistically significant differences are indicated as $P < 0.05$ *vs* untreated cells.

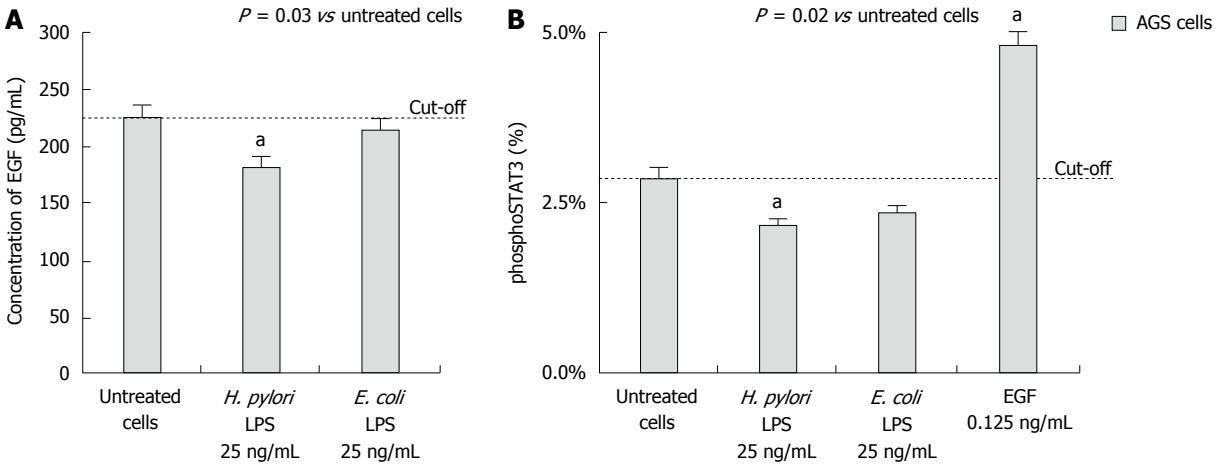


Figure 8 ***Helicobacter pylori*** **lipopolysaccharide-driven inhibition of signal transducer and activator of transcription 3 phosphorylation and epidermal growth factor production.** A: The impact of *Helicobacter pylori* or *E. coli* lipopolysaccharide (LPS) on the secretion of epidermal growth factor (EGF) by AGS cells; B: the percentage of phospho-signal transducer and activator of transcription 3 (phosphoSTAT3) in AGS cells measured by cell-based ELISA. Cells treated with EGF (0.125 ng/mL) were used as a positive control. The *cut-off* value was related to the response of untreated cells. The data represent the average values of four independent experiments. Statistically significant differences are indicated as $^{a}P < 0.05$ *vs* untreated cells.

necrosis was observed for 9% of all cells as compared to untreated cells, $P = 0.04$. However, there were no significant differences between the percentages of early and late apoptotic cells. When the cells were treated with the combination of *H. pylori* LPS (25 ng/mL) and CagA, an increased number of early apoptotic cells was detected (6%). In comparison to untreated cells with signs of early apoptosis (3%) this difference was of low significance $P = 0.05$.

As shown in Figure 7B, 89% untreated fibroblasts were viable (Ann-V$^-$ PI$^-$), 5% were late apoptotic and 3% were necrotic. Nevertheless, 8% fibroblasts showed the signs of late apoptosis ($P = 0.03$) after a treatment with *H. pylori* LPS at 1 ng/mL. In the cell cultures incubated in the milieu of *H. pylori* LPS at 25 ng/mL, only 75% fibroblasts were viable, while 19% underwent necrosis ($P = 0.04$). In the presence of standard *E. coli* LPS at 25 ng/mL, 8% of fibroblasts underwent necrosis ($P = 0.04$). Challenging the cells with *H. pylori* LPS (25 ng/mL) in combination with CagA or CagA, UreA and GE resulted in a decreased percentage of necrotic fibroblasts (15%, $P = 0.04$ and 8%, $P = 0.03$, respectively).

H. pylori LPS induces a decrease in EGF secretion and phosphorylated Y(705)STAT3 concentration in gastric epithelial cells

The Janus kinase (JAK)/STAT3 pathway is one of the major signal transduction pathways. STAT3 is activated through phosphorylation in response to various cytokine and growth factors including EGF. STAT3 mediates the expression of a variety of genes in response to cell stimuli, and thus plays a key role in many cellular processes such as cell growth and apoptosis. In order to specify the mechanism of *H. pylori* LPS induced inhibition of wound healing, we examined the ability of the cells to secrete EGF in relation to the concentration of phosphorylated STAT3. For this purpose we used supernatants obtained from AGS cell cultures untreated or stimulated with bacterial antigens. Only, in response to *H. pylori* LPS at 25 ng/mL, the concentration of EGF was decreased as compared to untreated cells ($P = 0.03$; Figure 8A). The decrease in EGF secretion was correlated with a reduced amount of phospho-STAT3 ($P = 0.02$; Figure 8B). Other bacterial antigens did not cause a decrease in the concentration of EGF and phospho-STAT3 (data not shown).

DISCUSSION

H. pylori colonization of the gastric tissue promotes excessive inflammation, which exerts harmful effects on the gastric mucosa. However, precise mechanisms of tissue destruction are not well understood. *H. pylori* produce many virulence factors responsible for cell damage, which facilitate the survival of these pathogens on the surface of gastric mucosa and evasion of the immune response of the host[74-77]. In this study, we used human gastric epithelial AGS cells and guinea pig fibroblasts as an *in vitro* model for the assessment of the effects of interactions between *H. pylori* and the gastric epithelial barrier, which might also take place *in vivo*. Gastric epithelial cells constitute the first protective barrier responsible for the maintenance of local homeostasis. Fibroblasts were chosen for several reasons: *in vivo* they are present in the sub-epithelial mucosa and can be targeted by *H. pylori* compounds leaking through epithelial lesions or transferred there through the epithelium[78]. Fibroblasts are involved in the wound healing and participate in many immunological processes including direct response to pro-inflammatory cytokines[79,80]. In order to restore gastric epithelial homeostasis, gastric tissue ulceration initiated

via H. pylori - host cell interaction should be followed by a healing process. In this study, we monitored the influence of *H. pylori* antigens on the healing process by the assessment of the wound repair using a scratch assay mimicking an ulcer lesion. The ability of the cells to heal an injury was monitored in relation to cell viability, proliferating activity and the cell cycle as well as genotoxicity of bacterial compounds used for the cell challenge: *H. pylori* GE, which is a complex of surface antigens, UreA urease subunit, CagA protein and LPS. All of these components are present in the environment inhabited by *H. pylori*. In this study, we have shown that *H. pylori* antigens may differ in their effects towards epithelial cells as well as fibroblasts and that both cell types differ in their susceptibility to various *H. pylori* antigens. Epithelial AGS cells after the challenge with GE, UreA and CagA were able to repair the wound, which was associated with an increased number of viable cells and intensified cell proliferation while in fibroblast cultures these parameters were elevated after the challenge with GE and CagA but not with UreA. Increased cell migration and proliferation are necessary to heal tissue damage. It has been suggested that a possible molecular mechanism of wound healing during *H. pylori* infection could be related to *H. pylori*-derived RpL1 aa 2-20 peptide (Hp 2-20), which by interacting with formyl peptide receptors induces cell migration and proliferation, as well as the expression of the vascular endothelial growth factor, thereby promoting gastric mucosal healing[81]. However, Pousa *et al*[82] have suggested that early angiogenesis required for the repair process during *H. pylori* infections is inhibited probably due to the anti-proliferating properties of the bacteria.

While increased proliferation is essential for gastric tissue healing, an uncontrolled proliferative activity can promote the accumulation of harmful mutations and cancerogenesis[83]. The mechanism preventing these processes is apoptosis. In our study, increased proliferation of AGS cells in response to CagA, UreA and GE as well as enhanced proliferation of fibroblasts after the challenge with GE were not accompanied by a parallel increase in cell apoptosis. Several studies have shown that *cagA*$^+$ strains have a greater carcinogenic potential than *cagA*$^-$ strains[30]. Increased cellular proliferation in response to *H. pylori* infection, especially with *cagA*+ strains, has been confirmed *in vivo* both in humans and laboratory animals[84,85]. It has been shown that the functional *cag* secretion system is required for the induction of phosphatidylinositol 3-kinase, an integral component of a signal transduction pathway, which leads to an increase in the cell proliferation and inhibition of apoptosis[86,87]. However, in this study we observed increased epithelial cell proliferation also in response to soluble CagA, which *in vivo* might be present in the inflammatory milieu and translocated to cytosol by phagocytosis or endocytosis. Also, interaction *via* surface receptors cannot be excluded. It is worth mentioning that, while CagA enhances proliferation of epithelial cells, it inhibits the division of peripheral blood *H. pylori*-reactive T lymphocytes possibly allowing these bacteria to survive in the host[31]. *In vivo*, enhanced gastric mucosal proliferation and the low apoptosis rate were positively correlated with the severity of acute gastritis, which means that hyperproliferation not balanced by cell death might contribute to neoplasia[30]. The relation between increased expression of the cellular proliferation marker Ki-67 (also known as MKI67) in gastric tissue sections and severity of inflammatory response has been shown in *H. pylori*-infected guinea pigs[85].

Recently, it has been indicated that not only CagA but also other *H. pylori* compounds such as adhesin BabA and JHP0290 protein, may contribute to *H. pylori*-associated diseases by promoting gastric epithelial cell proliferation and increased resistance to apoptosis[88,89]. In our study elevated gastric epithelial cell expansion was observed after exposure to *H. pylori* GE, UreA and a low dose of *H. pylori* LPS. Moreover, *H. pylori* GE increased also proliferation of fibroblasts. It may lead to either tissue regeneration or neoplasia. In our study, *H. pylori* LPS used at a low concentration (1 ng/mL) accelerated wound repair, which was associated with significantly enhanced cell proliferation and MTT reduction, whereas *H. pylori* LPS at a high concentration (25 ng/mL), inhibited the wound repair process. Also another study revealed that *H. pylori* LPS (1 μg/mL) accelerated the proliferation rate of gastric epithelial cells *via* TLR2, and a MEK-1/2-ERK-1/2 (mitogen-activated protein (MAP) kinase activating extracellular-signal-regulated kinases) MAP kinase cascade[90]. Similar dose-dependent effects were found for *Pseudomonas aeruginosa* LPS in a model of pulmonary epithelial cell damage[91]. AGS cell response to the low concentration of *H. pylori* LPS observed in our study suggests that gastric epithelium is able to react to deleterious signals by the activation of host defence mechanisms. However, this barrier was destabilised after exposure to a high dose of *H. pylori* LPS. In this case, during natural infection, the *in vivo* effects of *H. pylori* on the gastric barrier may depend on the type and local concentration of bacterial compounds. It is worth mentioning that the colonization of *H. pylori* is not homogenous, but is stratified by a gastric site and acuteness of inflammation. This allows increasing the concentrations of the individual on-site components. Interestingly, *E. coli* LPS used at a concentration of 25 ng/mL did not affect the process of wound repair in the AGS cell monolayer. This could be due to the lack of MD2 protein in these cells, which is involved in the recognition of *E. coli* LPS[92]. Therefore, the migration of AGS cells may not be inhibited in response to this type of LPS. Different effect of *H. pylori* LPS could be due to its engagement in the signaling pathway mainly with the participation of TLR2 but not TLR4

host cell receptors[90]. Various activities of *H. pylori* LPS and *E. coli* LPS may be the result of their different chemical structures. *H. pylori* LPS contains Le^x and Le^y determinants, which make the bacteria less visible to the immune cells and therefore it might exert different effects on the level of gastric epithelial cells[43,50,93].

In the case of fibroblasts, which in the natural environment constitute the deeper layers of the gastric mucosa, *H. pylori* LPS impaired wound healing regardless of its concentration. Even an addition to the cell culture of other *H. pylori* antigens (UreA, GE, CagA), which alone intensified the migration of fibroblasts and wound healing, did not result in neutralizing the inhibitory effect of *H. pylori* LPS at a high concentration. The motility and viability of fibroblasts were also inhibited after the challenge of the cells with *E. coli* LPS used at the concentration of 25 ng/mL, indicating that generally fibroblasts are more susceptible to various bacterial LPS than AGS epithelial cells and that similar cell signaling pathways can be involved in the response to different types of LPS. *In vivo*, such deep disruption of the epithelial barrier by *H. pylori* components and inflammatory mediators may facilitate damage to lamina propria, and promote the development of both local and systemic inflammatory response. *H. pylori* antigens penetrating across the gastric epithelium, can be processed in lamina propria by macrophages *via* PRR and presented to T lymphocytes[8,19,20]. However, it has been shown that *H. pylori* LPS is able to downregulate the lymphocyte blastogenic response, probably due to the interference with the process of macrophage maturation[46-48,94]. It also downregulates the phagocytic potential of macrophages[44] and decreases the cytotoxic activity of NK cells[45,68]. In the gastric epithelium, colonized by pathogenic microorganisms, including *H. pylori*, probably IL-33 acting as an alarming molecule can induce the signalling beneficial for tissue recovery due to a short-term increase in endothelial permeability. However, under some circumstances it may cause the aggravation of inflammation and tissue dysfunction by attracting Th2 lymphocytes, promotion of cell apoptosis and maintenance of tissue dysfunction[95]. *In vivo*, during *H. pylori* infection different environmental conditions (*e.g.,* stress, nutrients, pH), time of the cell exposure to bacterial antigens and their concentration may also determine the fate of different cell types. Other inflammatory mediators such as matrix metalloproteinases by suppression of apoptosis and preservation of the cells with damaged DNA may influence the rate of epithelial cell growth or cell loss[96-98].

We have shown that CagA, UreA, GE and *H. pylori* LPS at low concentration increased the proliferative activity of AGS cells, which was not accompanied by a parallel increase in cell apoptosis. Cell viability was not altered by these *H. pylori* antigens. *In vivo* loss of the cell integrity could be attributed to other mechanisms such as a breakdown of tight junctions in *H. pylori* infection[3,99,100]. Furthermore, high-temperature requirement A (HtrA) protease secreted by *H. pylori* as well as inflammatory factors induced by *H. pylori* such as interferon gamma and tumour necrosis factor alpha (TNF-α) might favour bacterial attachment to epithelial cells[101-104]. *In vivo*, recruitment of mesenchymal stem cells (MSC) in response to TNF-α secreted by *H. pylori* colonized epithelial cells is necessary to balance *H. pylori*-related apoptosis. However, MSC fusion with epithelial cells may render them more susceptible to neoplastic transformation[105].

We have demonstrated that *H. pylori* LPS at high concentration reduced viability of both AGS cells and fibroblasts, which was associated with the accumulation of AGS cells in the S and fibroblasts in the G_2 cell cycle phase, and with an increased frequency of cell death. These data provide strong evidence for the cell cycle arrest in response to *H. pylori* LPS and, for the inhibition of the cell growth. However, the suppressed cell proliferation could also be attributed to the cell death apart from to the cell cycle arrest. These effects were not observed when AGS cells were treated with *E. coli* LPS at a high dose, which is consistent with the observations made by Peek *et al*[106]. In our study, *H. pylori* LPS at a high dose inhibited the AGS cell cycle in the S phase even in a combination with CagA, which promoted cell proliferation. In the case of fibroblasts, *H. pylori* LPS or *E. coli* LPS at the high concentration arrested the cell cycle in the G_2 phase, whereas a low dose of *H. pylori* LPS inhibited the cell cycle progression in the S phase. In other studies, the *H. pylori*-induced cell cycle arrest of gastric epithelial cells as well as fibroblasts in the G_1 phase was reported[107-111]. It has been shown that also *H. pylori* L-asparaginase inhibited the cell cycle of gastric epithelial cells and fibroblasts and recurrent infections might influence the cell cycle[109]. It means that cellular effects are antigen-, dose- and cell type-dependent.

In general, cells are arrested in the S phase due to the depletion of the substrates required for DNA synthesis, whereas the entry to mitosis is blocked by the G_2 checkpoint mechanism when DNA is damaged[112]. In our study, we used AGS cells and fibroblasts that were not serum starved (unsynchronized cells) in order to mimic events that occur in the naive gastric mucosa. However, treatment of these cells with *H. pylori* LPS at a high concentration resulted in nuclear morphology changes in both cell types, which were visualised by DAPI staining and a comet assay. It confirmed the genotoxic properties of *H. pylori* LPS used at a higher concentration.

The results obtained in this study and data of other authors indicate that *H. pylori* may initiate damage to gastric epithelium directly through its components such as urease, VacA and LPS[100,113-115]. Several mechanisms can drive epithelial cell damage after the challenge with *H. pylori* LPS. It can be due to direct cytotoxic effect

associated with lipid A binding *via* TLR4/TLR2. Handa *et al*[116] have shown that *H. pylori* LPS increased NADPH oxidase (NOX; nicotinamide adenine dinucleotide phosphate oxidase) and TLR4 expression on gastric epithelial cells leading to elevation of deleterious oxidase stress. In this study we used an Le^{xy} positive *H. pylori* strain. Despite TLR4/TLR2 surface receptor binding, such variants can interact with surface lectins *via* Le carbohydrate moieties[52]. It has been shown that Le positive *H. pylori* variants can bind DC-SIGN C-type lectin, which *in vivo* is present on gastric dendritic cells[41,50].

H. pylori may affect gastric homeostasis also indirectly by the interaction with the angiogenesis process and over-expression of inflammatory response. It has been shown that early angiogenesis, which is necessary for epithelial reconstruction[117], is inhibited during *H. pylori* infection probably due to anti-proliferative and pro-apoptotic activity of the bacteria[82,100,113]. *H. pylori* LPS, when transferred into the sub-epithelial space, can hinder polymorphonuclear leukocyte (PMNL) apoptosis[31,118,119]. In order to prevent PMNL apoptosis and increase the cell survival, epithelial cells secrete pro-inflammatory cytokines such as IL-8 and the granulocyte macrophage colony stimulating factor. However, it is followed by an enhanced epithelial injury due to the excess of proteinases and oxidative stress compounds, which are tolerated by *H. pylori* equipped with neutralizing enzymes[77].

An important aspect of wound repair is the ability of cells to respond to EGF which promotes cell migration and wound healing[120]. It has been shown that treatment of AGS cells with non-phosphorylated CagA protein leads to the activation of the JAK/STAT3 signalling pathway. By comparison, phosphorylated CagA has been observed to alter cell morphology, polarity, growth and activation of β-catenin, which is implicated in cancerogenesis[86,121]. In order to determine whether *H. pylori* LPS-driven inhibition of AGS cell proliferation observed in our study was associated with alternation in EGF concentration we evaluated quantitatively both EGF and phospho-STAT3. A high dose of *H. pylori* LPS has been shown to decrease the amount of EGF and phospho-STAT3 in the epithelial cell cultures. This observation might explain the mechanism of *H. pylori* LPS-mediated disturbance in wound healing process.

CONCLUSION

This study shows that *H. pylori* soluble components may affect the balance between proliferation of surface epithelial and lamina propria cells and cell death. This balance is crucial for the renovation of the epithelium, wound healing and protection against neo-plastic transformation. Our results allow suggesting that *in vivo*, during acute or chronic *H. pylori* infection, various cellular effects might depend on target cells, the bacterial antigen and its concentration. Domination of antigens capable of stimulating cell proliferation such as UreA, CagA or surface antigens (GE) can lead to the epithelial renewal, although their excessive activity may pose an increased risk of developing cancer. In contrast, domination of antigens such as LPS with cytotoxic and anti-proliferative activity towards mucosal cells may promote chronic inflammation, and, in the case of immune cells, inhibition of antibacterial response, resulting in the maintenance of *H. pylori* infection.

COMMENTS

Background

The human gastric mucosal barrier is permanently exposed to various infectious agents and their soluble components. Disruption of this barrier homeostasis results in the inflammatory response and may lead to a variety of pathological effects. Among bacterial pathogens, Gram-negative *Helicobacter pylori* (*H. pylori*) rods play a crucial role in the development of gastritis, gastric and duodenal ulcers and even gastric cancers.

Research frontiers

H. pylori demonstrates affinity to gastric epithelium, resulting with an excessive inflammation, peptic ulcers and cancers. Strong inflammation and metaplasia suggest that *H. pylori* interfere with cell growth and initiate different disorders. The research hotspot is to further clarify the mechanisms used by *H. pylori* to maintenance chronic infection.

Innovations and breakthroughs

The authors found that *H. pylori* soluble antigens such as subunit A of urease (UreA), cytotoxin associated gene A protein (CagA) and glycine acid extract antigenic complex (GE) are capable to stimulate cell proliferation leading to the epithelial renewal, although their excessive activity *in vivo* may increase the risk of cancer development. In contrast, domination of antigens such as *H. pylori* lipopolysaccharide (LPS) with cytotoxic and anti-proliferative activity towards mucosal cells may promote chronic inflammation and the maintenance of *H. pylori* infection.

Applications

The results of this study improve the knowledge about the mechanisms used by *H. pylori* to maintenance chronic infection and disrupt barrier function of gastric mucosa.

Terminology

Two independent cell lines: gastric epithelial AGS cells and fibroblasts (challenged with *H. pylori* soluble antigens), were used by the authors in terms of mimicking the interaction of *H. pylori* compounds with gastric mucosal barrier.

Peer-review

This *in vitro* cellular study provides new data about the impact of *H. pylori* soluble antigens such as CagA, UreA, GE and LPS to the gastric mucosal barrier.

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ORIGINAL ARTICLE

Basic Study

Melatonin modulates adiponectin expression on murine colitis with sleep deprivation

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Abstract

AIM

To determine adiponectin expression in colonic tissue of murine colitis and systemic cytokine expression after melatonin treatments and sleep deprivation.

METHODS

The following five groups of C57BL/6 mice were used in this study: (1) group Ⅰ, control; (2) group Ⅱ, 2% DSS induced colitis for 7 d; (3) group Ⅲ, 2% DSS induced colitis and melatonin treatment; (4) group Ⅳ, 2% DSS induced colitis with sleep deprivation (SD) using specially designed and modified multiple platform water baths; and (5) group V, 2% DSS induced colitis with SD and melatonin treatment. Melatonin (10 mg/kg) or saline was intraperitoneally injected daily to mice for 4 d. The body weight was monitored daily. The degree of colitis was evaluated histologically after sacrificing the mice. Immunohistochemical staining and Western blot analysis was performed using anti-adiponectin antibody. After sampling by intracardiac punctures, levels of serum cytokines were measured by ELISA.

RESULTS

Sleep deprivation in water bath exacerbated DSS induced colitis and worsened weight loss. Melatonin injection not only alleviated the severity of mucosal injury, but also helped survival during stressful condition. The expression level of adiponectin in mucosa was decreased in colitis, with the lowest level observed in colitis combined with sleep deprivation. Melatonin injection significantly ($P < 0.05$) recovered the expression of adiponectin. The expression levels of IL-6 and IL-17 were increased in the serum of mice with DSS colitis but decreased after melatonin injection.

CONCLUSION

This study suggested that melatonin modulated adiponectin expression in colonic tissue and melatonin and adiponectin synergistically potentiated anti-inflammatory effects on colitis with sleep deprivation.

Key words: Colitis; Melatonin; Adiponectin; Sleep deprivation

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Core tip: We report this first study that melatonin and sleep deprivation are related to adiponectin expression in the colonic mucosa of murine colitis. C57BL/6 mice were feeding with 2% DSS for inducing colitis and using specially designed multiple platform water baths for sleep deprivation. Immuno-histochemical staining and Western blot analysis was performed using anti-adiponectin antibody. The expression level of adiponectin in mucosa was decreased in colitis, with the lowest level observed in colitis combined with sleep deprivation. Melatonin injection significantly ($P < 0.05$) recovered the expression of adiponectin. This study suggests that melatonin and adiponectin synergistically potentiate the anti-inflammatory effects in murine colitis.

Kim TK, Park YS, Baik HW, Jun JH, Kim EK, Sull JW, Sung HJ, Choi JW, Chung SH, Gye MC, Lim JY, Kim JB, Kim SH. Melatonin modulates adiponectin expression on murine colitis with sleep deprivation. *World J Gastroenterol* 2016; 22(33): 7559-7568 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i33/7559.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i33.7559

INTRODUCTION

Inflammatory bowel disease (IBD) is caused by multiple genetic, environmental, and host factors[1]. Environmental factors such as stress and sleep disturbance can affect the progression and relapse of patients with IBD[2,3]. Stress frequently influences sleep quality. IBD patients have significant sleep disturbance even during inactive state. Sleep disturbance might affect the quality of life and gastrointestinal symptoms. In addition, it can increase the risk of flare-up of IBD[4]. Inflammatory cytokines such as tumor necrosis factor, interleukin-1 (IL-1), and IL-6 are known as significant contributors to sleep disturbances. On the other hand, sleep disturbances can upregulate these inflammatory cytokines[5].

Melatonin is secreted by pineal gland. It functions as a regulator of circadian rhythms and an antioxidant[6]. Melatonin levels in the gut are independent of pineal production. Pinealectomy has no influence on gut melatonin concentrations in rats[7]. At any time of the day or night, the gut contains at least 400 times more melatonin than that of the pineal gland, emphasizing the functional importance of melatonin in the gut[8]. The melatonin in GI tract has anti-inflammatory effect in experimental models of colitis in many previous reports[9-13]. Our previous study also shows that mRNA level of adiponectin is down regulated by sleep deprivation but up-regulated by melatonin based on microarrays and real-time PCR analysis of mice colon tissues[14]. Currently, the relationship between melatonin and adiponectin on colitis with sleep deprivation remains unknown. It is meaningful to evaluate the level of adiponectin expressed in colon tissue of mice by immunohistochemical staining and western blotting to understand the pathogenesis of inflammatory bowel disease. Therefore, the objective of this study was to investigate tissue expression of adiponectin in colitis with sleep deprivation and melatonin injection. The expression levels of cytokines in mouse during sleep deprivation with melatonin injection were also analyzed in this study.

MATERIALS AND METHODS

Animals

A total of 30 eight-week-old C57BL male mice with body weight of 20-25 g were purchased from Samtako Inc. (Gyunggido, Korea). They were randomly assigned

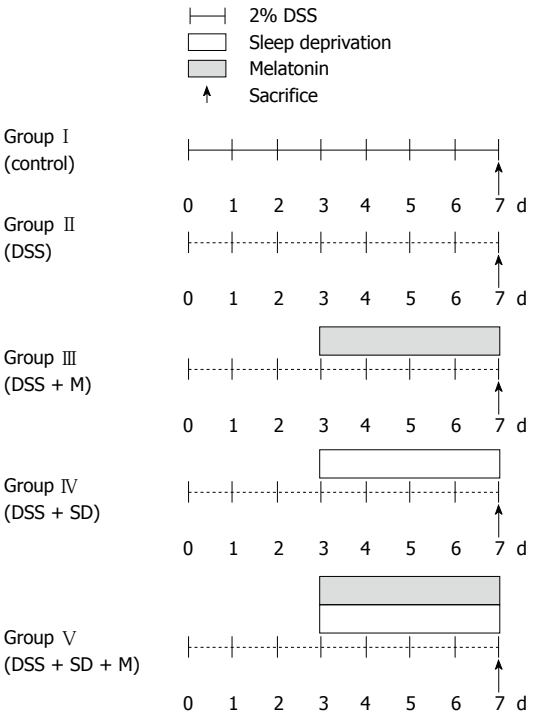

Figure 1 Experimental procedure. Experimental colitis of mice was induced by feeding mice with 2% DSS in group Ⅱ. Melatonin was administered to mice for 4 d with 2% DSS in group Ⅲ. Sleep deprivation was induced partially with 2% DSS in group Ⅳ. 2% DSS with partial sleep deprivation and melatonin was administered to mice in group Ⅴ. M: Melatonin; SD: Sleep deprivation.

to the following five groups (6 mice per group): (1) Group Ⅰ, control; (2) Group Ⅱ, 2% Dextrose Sodium Sulfate(DSS) induced colitis for 7 d; (3) Group Ⅲ, 2% DSS induced colitis and melatonin treatment; (4) Group Ⅳ, 2% DSS induced colitis with sleep deprivation (SD) (20 h wakening/d) using specially designed and modified multiple platform water baths; and (5) Group V, 2% DSS induced colitis with SD and melatonin (Figure 1). Temperature of 22-24 ℃ and humidity of 55%-60% were maintained with a 12-h light/dark cycle (lights on at 08:00). During the experiment period, mice had free access to food and water. Their body weights were measured daily. All procedures in experiments were conducted according to the Animal Care Guidelines of the National Institutes of Health and Korean Academy of Medical Sciences. The Institutional Review Board of Eulji Hospital at Eulji University College of Medicine approved the study protocol (Approval number: EUIACUC12-11).

Experimental procedures

2% DSS induced colitis model: For 7 d, mice were fed with 2% DSS (Sigma-Aldrich, Inc., United States) solution to induce colitis.

Partial sleep deprivation: On the 3rd day, all mice were moved to water bath. Water bath had either wide platforms for normal sleep or narrow platforms for sleep deprivation. Partial sleep deprivation of mice was achieved by using a modified multiple platform water bath specifically designed for sleep deprivation. In a water tank, 4 platforms and 2 mice were placed. Every mouse in the water bath could move from one platform to another by jumping. The water bath was filled with water for 4 cm from the base (enough to drown). When mice started to sleep, muscle atonia led mice to fall down into the water, waking up the mice. They would try to climb up the platform to avoid being drowned. Through all experiments, water was changed every day to clean water. Partial sleep deprivation of mice began from 2 PM to 10 AM (20 h) for 4 d. Mice had 4 h of sleep daily from 10 AM to 2 PM during the 4 d. After finishing the 4 d of partial sleep deprivation, mice were sacrificed for analysis.

Administration of melatonin: After induction of colitis with 2% DSS, melatonin was administered to mice intraperitoneally at a dose of 10 mg/kg for 4 d. Normal saline was administered to mice intraperitoneally as control.

Assessment of the severity of colitis: The severity of colitis was assessed by measuring weight loss and histological analysis. Colon tissues were fixed with 10% formaldehyde solution. Fixed tissues were dehydrated with alcohol and embedded with paraffin. Tissues were sliced into 4-μm thick sections. These sections were stained with hematoxylin and eosin to evaluate inflammatory change. The severity of inflammation in colon was determined, including loss of mucosal structure (score 0-3), crypt abscess (score 0-1), thickened muscle (score 0-3), cellular infiltration (score 0-3), and depletion of goblet cell (score 0-1). The final score for the severity of inflammation ranged from 0 to 11[15].

Immunohistochemical staining of adiponectin: Immunohistochemistry procedures were carried out using DAKO Autostainer plus (DAKO Cytomation, Carpinteria, CA, United States). Briefly, 4-μm sections of formalin-fixed and paraffin-embedded tissues were positioned onto poly-L-lysine slides. After deparaffinization and rehydration, antigen retrieval was performed using citrate buffer solution (pH 6.0) at 121 ℃ for 10 min. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 5 min. The sections were then incubated with specific antibodies against Adiponectin (Abcam, Cambridge, United Kingdom, 1:500). Slides were then stained with 3,3′-diaminobenzidine and counterstained with hematoxylin. Immunoreactivity was determined based on the percentage of stained cells: 1 + for less than 10%, 2 + for 10%-50%, and 3 + for over 50%.

Quantification of adiponectin by western blot analysis: Tissues were homogenized in 10 volumes of extraction buffer (20 mmol/L Tris-HCl, pH 7.5, 1% Triton X-100) containing protease inhibitor cocktail (Complete™, Roche, Germany) on ice.

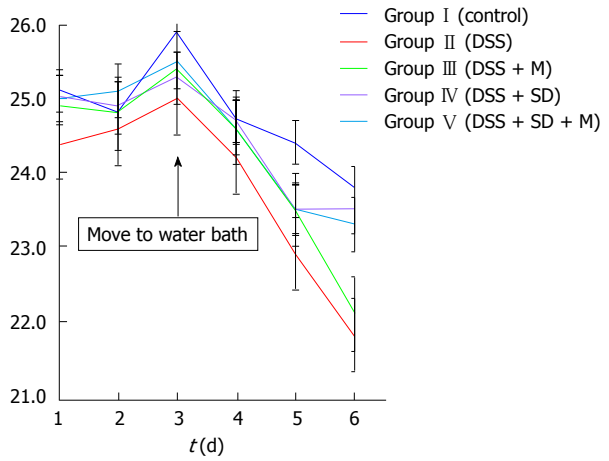

Figure 2 Changes in body weight of each group. Group Ⅰ: control, Group Ⅱ: 2% DSS induced colitis, Group Ⅲ: 2% DSS induced colitis with melatonin treatment, Group Ⅳ: 2% DSS induced colitis with sleep deprivation, Group Ⅴ: 2% DSS induced colitis with sleep deprivation and melatonin treatment). Data were presented as mean ± SD. M: Melatonin; SD: Sleep deprivation.

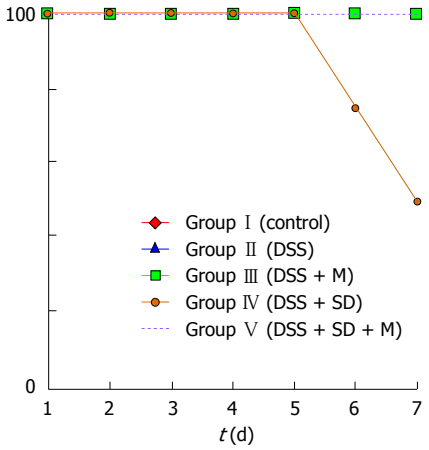

Figure 3 Survival rate of each group. Group Ⅰ: control, Group Ⅱ: 2% DSS induced colitis, Group Ⅲ: 2 % DSS induced colitis with melatonin treatment, Group Ⅳ: 2 % DSS induced colitis with sleep deprivation, Group Ⅴ: 2% DSS induced colitis with sleep deprivation and melatonin treatment). M: Melatonin; SD: Sleep deprivation.

Homogenates were centrifuged at 12000g for 1 h at 4 ℃. The supernatant was collected and its protein concentration was determined using a commercial protein assay kit (Bio-Rad, CA). Protein samples were mixed with 2X sample buffer (Laemmli, 1970), boiled for 5 min, and cooled at room temperature. After briefly spinning, clear supernatants were resolved on 8% SDS-polyacrylamide gels. Proteins were electrophoretically transferred to nitrocellulose membranes (GE Healthcare, United Kingdom) and blocked with Tris-buffered saline (TBS) containing 1% bovine serum albumin overnight at 4 ℃. After rinsing three times with TBS/0.1% Tween 20 (TBST) for 10 min each, protein blots were incubated with rabbit anti-adiponectin polyclonal antibody (ab62551, abcam, United Kingdom) diluted 1:1000 in TBS for 2 h at room temperature. After rinsing with TBST three times for 10 min each, the blots were incubated with peroxidase-labeled goat anti-rabbit IgG (Invitrogen, OR, United States) diluted 1:1000 in TBST for 1 h. The blots were washed with TBST for 10 min followed by washing with TBS for 10 min. Signals of protein bands were detected using an ECL kit (GE Healthcare).

Measurement of serum cytokines: Blood were collected by intracardiac sampling from the experimental mice just before sacrificing. Serum was prepared to measure cytokine levels. Levels of IL-17 (Ray Bio ELISA Kit Mouse IL-17; RayBiotech, Norcross, GA, United States), IL-6 (Ray Bio ELISA Kit Mouse IL-6; RayBiotech), and TNF-α (Ray Bio ELISA KitMouse TNF-alpha; RayBiotech) were evaluated using commercially available kits following the manufacturer's instructions.

Statistical analysis

All data were represented as mean ± SD. Statistical Package for Social Sciences software (SPSS; Korean version 18.0) was used for all statistical analyses. One way analysis of variance (ANOVA) was used to calculate the statistical significance. Significance was considered when *P* value was less than 0.05.

RESULTS

Effect of sleep deprivation and melatonin treatment on body weight and survival

On the 3rd d, mice were moved to water bath. From that time, the body weight of all mice started to decrease due to stressful water bath condition. The body weight of 2% DSS induced colitis group was significantly ($P = 0.005$) lower than that of the control group (Figure 2). Sleep deprivation induced severe weight loss. Half of mice were dead during sleep deprivation (Figure 3). Melatonin failed to significantly recover the weight loss, but it could help all mice survive during sleep deprivation.

Effect of sleep deprivation with melatonin treatment on colonic inflammation

In histological analysis, the colon of 2% DSS induced colitis mice showed edema and infiltration of inflammatory cells into the mucosa compared to that of the control group. Melatonin treatment reduced inflammation in the colon of 2% DSS induced colitis in group Ⅲ. The number of infiltrating cells and mucosal injury such as ulcer or necrosis were increased by sleep deprivation in the colon of 2% DSS induced colitis mice. Melatonin treatment also diminished the inflammation and erosion in the colon of 2% DSS induced colitis mice with sleep deprivation. The microscopic inflammatory score of the colitis group (Group Ⅱ) was 6.5 ± 0.6. It was significantly ($P = 0.003$) decreased to 4.4 ± 0.8 by melatonin treatment (Group Ⅲ). However, it was significantly ($P = 0.018$) increased to 8.5 ± 0.5 by sleep deprivation (Group Ⅳ). The histologic score of 8.5 ± 0.5 of 2%DSS induced colitis mice with sleep

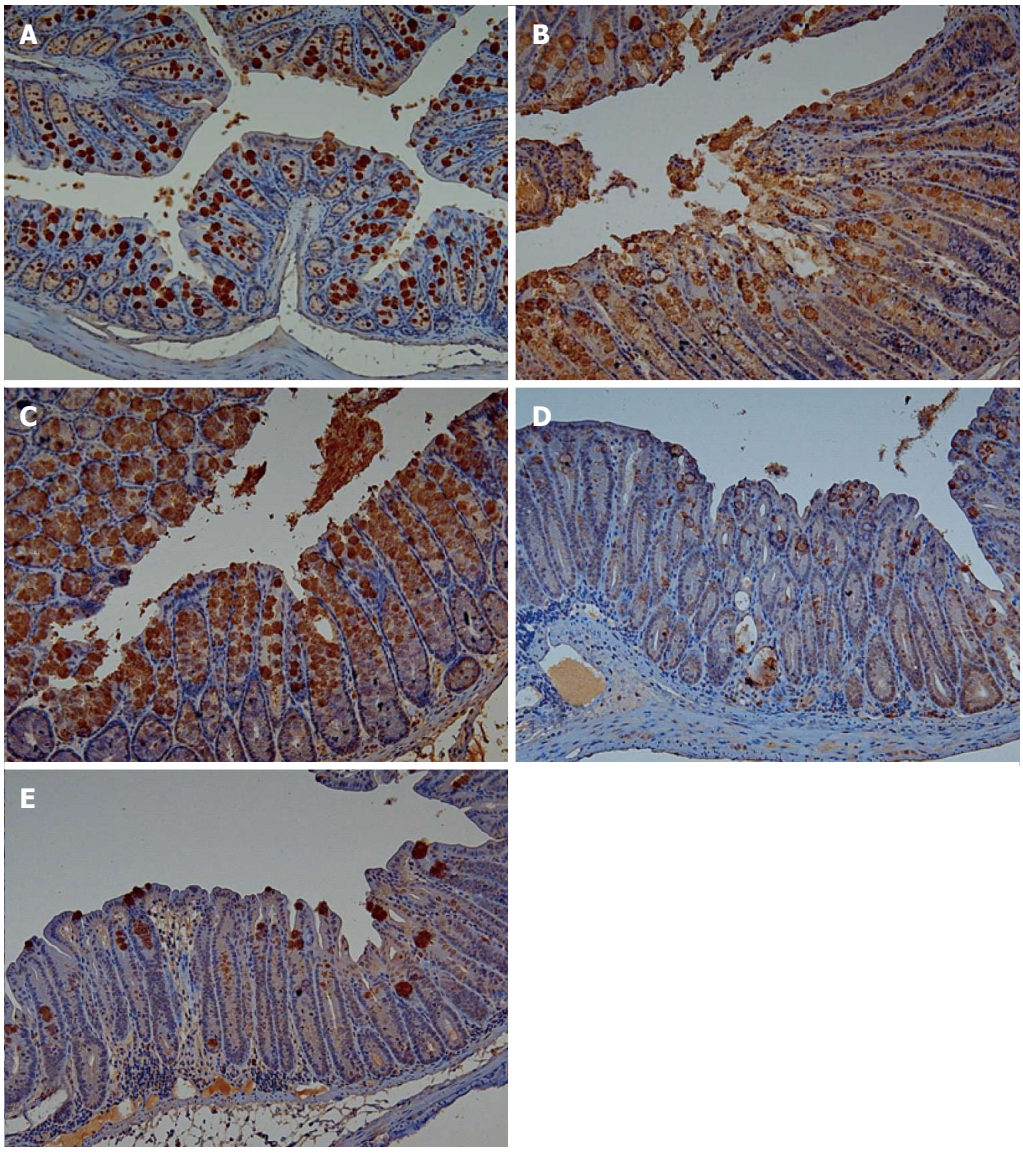

Figure 4 Immunohistochemical staining of adiponectin (A: × 100, B-E: × 200). A: control; B: DSS: 2% DSS induced colitis; C: DSS + M: 2% DSS induced colitis with melatonin treatment; D: DSS + SD: 2% DSS induced colitis with sleep deprivation; E: DSS + SD + M: 2% DSS induced colitis with sleep deprivation and melatonin treatment.

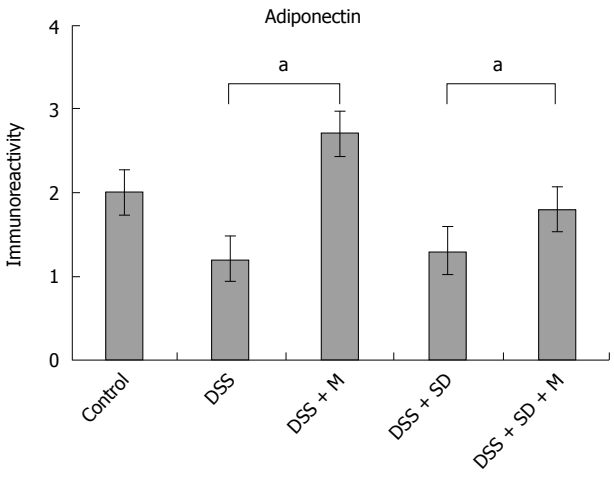

Figure 5 Adiponectin immunoreactivity based on immunohistochemical staining. Melatonin injection significantly recovered adiponectin expression, $^{a}P < 0.05$ *vs* control. M: Melatonin; SD: Sleep deprivation.

deprivation (group Ⅳ) was decreased ($P = 0.067$) by melatonin treatment (group Ⅴ, score of 7.0 ± 1.0). Changes of inflammatory score in all groups were statistically significant ($P < 0.01$).

Immunohistochemical staining of adiponectin expression by melatonin treatment

Adiponectin expression in colonic mucosa was determined by immunohistochemical staining (Figure 4). Colitis mice and colitis mice with sleep deprivation showed decreased adiponectin immunoreactivity compared to mice of the control group. In melatonin injection group, mice showed significant recovery in adiponectin immunoreactivity (Figure 5).

Western blotting of adiponectin expression after melatonin treatment

Based on western blot analysis, the expression levels

Table 1 Effect of melatonin and sleep deprivation on inflammatory change of 2% DSS induced colitis mice based on histology

	Control	DSS	DSS + M	DSS + SD	DSS + SD + M	*P* value
Microscopic scores	0 ± 0	6.5 ± 0.6	4.4 ± 0.8	8.5 ± 0.5	7.2 ± 1.0	< 0.01

M: Melatonin; SD: Sleep deprivation.

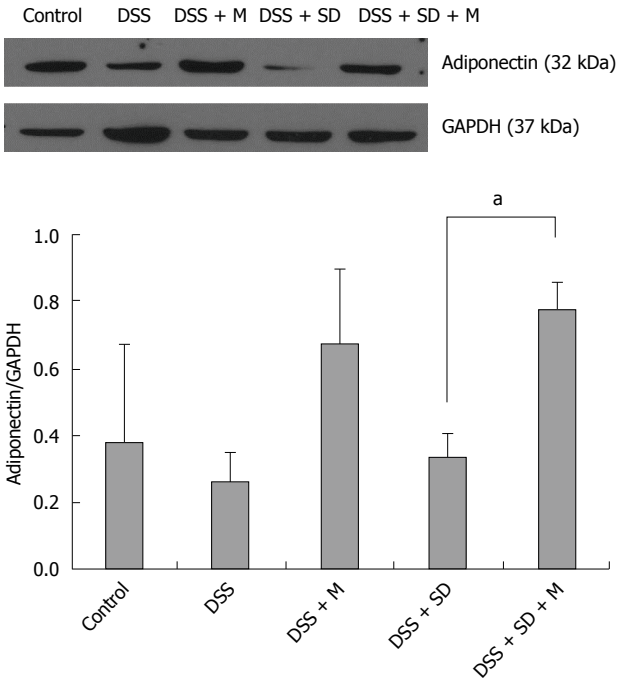

Figure 6 Western blot analysis of adiponectin. The expression of adiponectin was reduced in DSS colitis and DSS colitis with sleep deprivation. Melatonin injection significantly recovered adiponectin expression, $^{a}P < 0.05$ *vs* control. M: Melatonin; SD: Sleep deprivation.

of adiponectin were decreased in the colitis group and the colitis with sleep deprivation group compared to that in the control group (Figure 6). In the group of colitis with sleep deprivation, adiponectin expression was significantly ($P < 0.05$) recovered by melatonin injection (Figure 6). These results are consistent with results from immunohistochemical staining.

Estimation of inflammatory cytokines in the serum of mice

The levels of pro-inflammatory cytokines such as IL-6, IL-17, and TNF-α in the serum of mice were increased in DSS colitis group and colitis with sleep deprivation group compared to those of the control group. Their levels were decreased after melatonin injection. Even in the control group, the levels of these pro-inflammatory cytokines were relatively higher level than that in the melatonin treatment group. This could be due to the fact that all mice were moved to water bath at the 3rd experimental day when control mice got stressed out due to their fear of water. The expression levels of IL-6, IL-17, and TNF-α were significantly ($P < 0.05$) decreased after melatonin treatment (Figure 7). The level of anti-inflammatory cytokine IL-10 was lower in colitis. However, it had a tendency of increase after melatonin injection, indicating that melatonin treatment might have systemic anti-inflammatory properties.

DISCUSSION

Melatonin plays various important roles in the GI tract. As a physiological antagonist to serotonin, it decreases peristalsis and stimulates the secretion of mucosal bicarbonate mediated by MT2 receptor. An important receptor independent action of melatonin in the GI tract is that it is a free radical scavenger. The preventive role of melatonin against ulcer formation for healing has been well established[16]. Previous studies have suggested that melatonin administration is beneficial for IBD *via* its antioxidant, anti-apoptotic, and anti-inflammatory properties.

In this study, pathologic examination showed that melatonin could attenuate inflammation and decrease the severity of inflammation under sleep deprivation (Table 1). During our experiment, moving to water bath was stressful to mouse. Control mice also lost weight in the water bath. The sleep deprivation group showed aggravated weight loss and half of this group could not survive until the end of experiments. However, melatonin injection could help the survival of mice under sleep deprivation.

In our previous study, we found the genetic expression of adiponectin after melatonin injection with sleep deprivation using microarray and PCR[14]. Here, we tried to confirm the tissue expression of adiponectin after melatonin injection through immunohistochemical staining (Figure 4) and Western blotting (Figure 6). Our results showed that adiponectin expression was decreased in both colitis and colitis with sleep deprivation condition. However, melatonin injection significantly recovered the adiponectin expression in colon tissues, in consistent with genetic change that we found earlier.

Adiponectin is mainly expressed by mature adipocytes. It circulates at high levels in the blood stream[17]. There are some reports about the anti-inflammatory effect of adiponectin. Adiponectin could inhibit the expression of NF-κB, TNF-α, IL-6, and IL-1b but induce the expression of anti-inflammatory cytokines such as IL-10 and IL-1 receptor antagonists[18]. Adiponectin can also induce the production of IL-10 and IL-1Ra in human PBMC, macrophages, and DC while impairing the production of IFNγ in macrophages[19-21]. Overexpressed adiponectin in mice can decrease

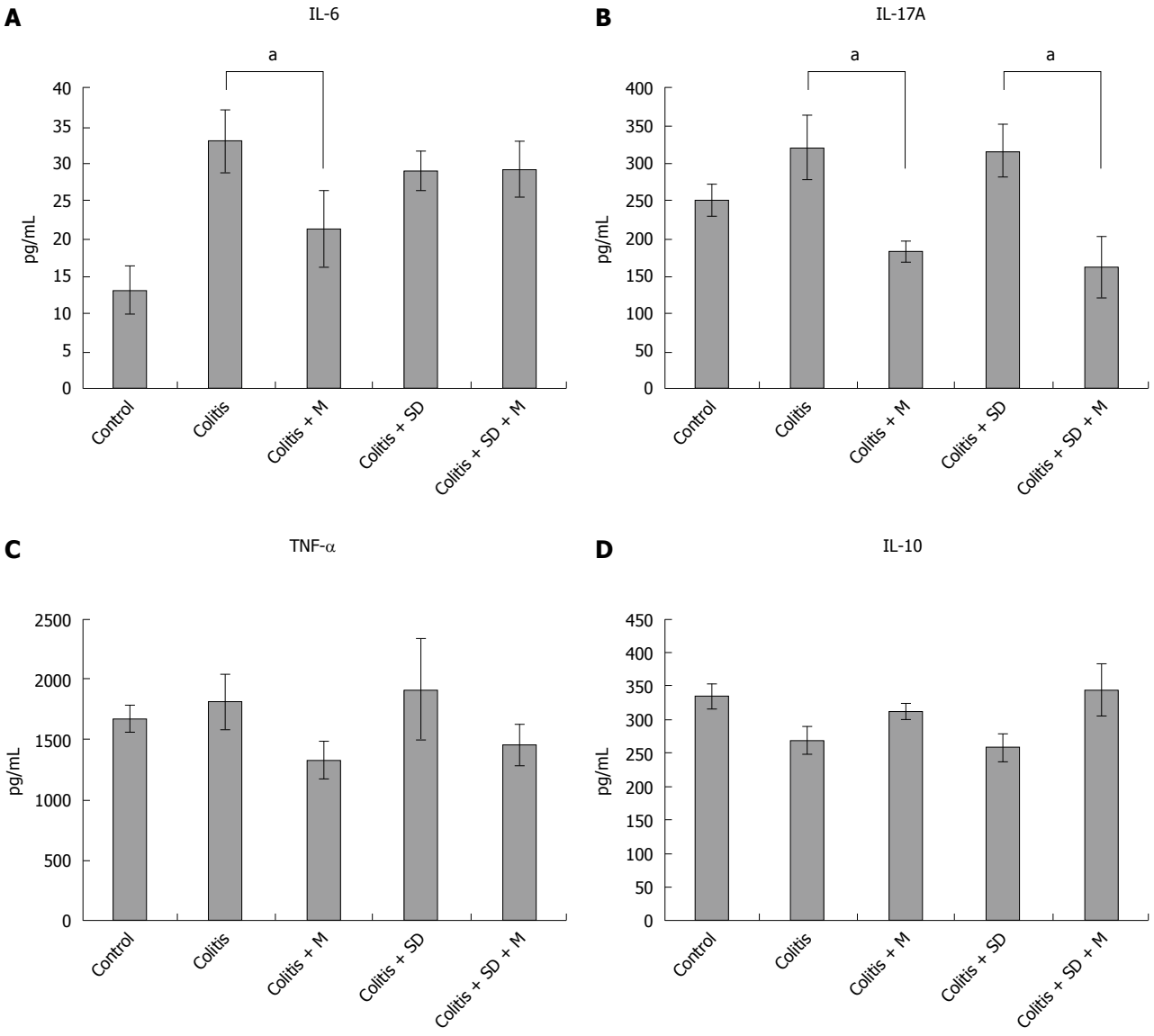

Figure 7 Cytokine levels in serum of mice. Pro-inflammatory cytokines such as IL-6 (A), IL-17 (B), and TNFα (C) were reduced by melatonin injection; IL-10 (D) expression was not significantly changed. [a]*P* < 0.05 *vs* control. M: Melatonin; SD: Sleep deprivation.

pro-inflammatory cytokines including cellular stress markers, TNF, IL-6, and IL-1b in mice[22,23]. Our study showed that mucosal expression of adiponectin was significantly decreased in DSS-induced colitis compared to that in the control. This result was consistent with other reports. Adiponectin mRNA level is reported to be decreased in inflamed colonic mucosa of DSS induced murine colitis[24]. Adiponectin can inhibit chemokine production in intestinal epithelial cells. It has been reported that adiponectin KO mice has much more severe colitis compared to wild type mice[25]. Recently, adiponectin expression in human tissue has been identified. In patients with ulcerative colitis, the mRNA expression of adiponectin in colonic mucosa is decreased[24]. Adiponectin concentration in mesenteric adipose tissue of CD patients has been found to be significantly lower in patients with internal fistula compared to that in patients without fistula[26].

Interaction of melatonin with adiponectin was first reported by Ríos-Lugo *et al*[18]. They showed that melatonin attenuated body weight increase and hyperglycemia, but increased mean plasma adiponectin in high fat-fed rats. The high-fat diet disrupted the normal 24-h pattern of adiponectin, which was counteracted by melatonin[18]. Direct effect of melatonin on adiponectin has not been clarified yet. An *in vitro* study using pre-adipocytes has shown an inhibitory effect of melatonin on adiponectin synthesis[18,27]. Another study has demonstrated that chronic melatonin administration to rats mainly affects the 24-h rhythm of adiponectin secretion[28]. A recent study has shown a significant increase in median plasma adiponectin levels and insulin sensitivity as early as 4 weeks after melatonin administration in cohort of patients with non-alcoholic steatohepatitis[29].

In this study, the expression level of adiponectin was lower in DSS-induced colitis than that in the control, with the lowest level in the sleep deprivation group. After melatonin injection, the expression level of adiponectin was significantly increased, even higher than that in the control. Based on these results, adiponectin expression in colonic tissue might be modulated by melatonin injection. These results are comparable to genetic expression of adiponectin after melatonin treatment in our previous study[14].

To understand the mechanism of inflammation, the levels of serum cytokines of mice were determined. Pro-inflammatory cytokines such as IL-6, IL-17A, and TNF-α were increased in colitis. As one of mechanisms to control inflammation, melatonin can modulate a variety of molecular targets, including NF-κB, cyclooxygenase-2, interleukin 17, matrix metalloproteinase-9, and connective tissue growth factor[30]. We also found that the levels of pro-inflammatory cytokines such as IL-6, IL-17A, and TNF-α were decreased while the level of IL-10 was increased after melatonin treatment (Figure 7). Therefore, adiponectin decreased levels of pro-inflammatory cytokines but increased level of IL-10 in macrophage and PBMC. Melatonin and adiponectin might have synergistic effect in these cytokine profiles. However, there was no significant cytokine change after sleep deprivation. This could be due to the fact that we could only check serum cytokine levels in survived mice. Dead mice might have higher pro-inflammatory cytokine levels than survived mice.

Our results showed that sleep deprivation group mice had more severe pathologic inflammation with worse survival rate, indicating that sleep is important for survival. Under the same condition, all melatonin injected mice survived during the experimental period (Figure 3). Although the precise mechanism on how melatonin helped the survival of mice is currently unknown, melatonin might be beneficial to overcome stressful conditions such as sleep deprivation for human.

Th17 cell lineage is important for pathogenesis of IBD. It produces important inflammatory cytokine IL-17. Our results also demonstrated that melatonin injection decreased IL-17 level. Th17 lineage-specific transcription factor RORα is a natural target of melatonin in T cells[31]. RORα, along with RORγ, can regulate Th17 cell differentiation[32], whereas down regulation of RORα expression is part of the typical Treg transcriptional signature[33]. A preliminary study has highlighted the *in vivo* inhibitory actions of melatonin on Treg cell generation in cancer patients[34]. It has been reported that *in vivo* administration of melatonin to mice subjected to experimental cancer has down regulation of CD4+CD25+ Treg cells and Foxp3 expression in tumor tissues[35]. These results suggest that melatonin may have adjuvant effect in inhibiting colitis associated cancer development in patients with IBD.

The levels of plasma melatonin in patients with ulcerative colitis (UC) have been reported to be significantly lower than those of healthy control[36]. They are higher in remission state than in active disease. Melatonin can also cause the disappearance of clinical symptoms of patients with UC. These symptoms are reported to have reappeared when melatonin consumption is stopped[37]. Adjuvant treatment with melatonin in UC patients is reported to keep remission for 12 mo with normal C- reactive protein ranges and high hemoglobin levels, suggesting that adjuvant melatonin therapy may help sustain remission of patients with UC[38]. However, more clinical trials should be performed to confirm the beneficial effect of melatonin on IBD, although basic studies strongly suggest such beneficial effect.

To the best of our knowledge, this is the first study to report that melatonin and sleep deprivation are related to adiponectin expression in colonic mucosa of DSS colitis. Results of this study were consistent with results of previous study about the genetic expression of adiponectin after melatonin and sleep deprivation.

In summary, we found that sleep deprivation aggravated inflammation and lowered survival rate. However, melatonin had a protective effect on inflammatory change and helped the survival of DSS-induced colitis of mice. In addition, melatonin modulated adiponectin expression in colitis mice with sleep deprivation. This study suggests that melatonin and adiponectin synergistically potentiate the anti-inflammatory effects in the colitis. Because severe colitis with sleep deprivation is a frequent condition of active IBD patients, melatonin is expected to be used for control of inflammation and sleep deprivation.

COMMENTS

Background

Melatonin is secreted by pineal gland. It functions as a regulator of circadian rhythms and an antioxidant. Melatonin levels in the gut are independent of pineal production. The gut contains at least 400 times more melatonin than the pineal gland, emphasizing the functional importance of melatonin in the gut. The melatonin in GI tract has anti-inflammatory effect in experimental models of colitis in many previous reports.

Research frontiers

This previous study also shows that mRNA level of adiponectin is down regulated by sleep deprivation but up-regulated by melatonin based on microarrays and real-time PCR analysis of mice colon tissues

Innovations and breakthroughs

This is the first study that melatonin and sleep deprivation are related to adiponectin expression in the colonic mucosa of murine colitis being performed by immuno-histochemical staining and Western blot analysis. So, the authors confirmed previous genetic change of adiponectin on microarrays and real-time PCR analysis.

Applications

The expression level of adiponectin in mucosa was decreased in colitis, with the lowest level observed in colitis combined with sleep deprivation. Melatonin injection significantly recovered the expression of adiponectin. This study

suggests that melatonin and adiponectin synergistically potentiate the anti-inflammatory effects in murine colitis.

Terminology

No specific terminology are used in this paper

Peer-review

Using the DSS-induced colitis mice model, the authors studied the adiponectin expression in colonic tissue of murine colitis and cytokine expression in response to melatonin treatments and sleep deprivation. The results showed that sleep deprivation exacerbated DSS colitis, which can be alleviated by melatonin injection. The expression level of adiponectin in mucosa was decreased in colitis, with the lowest level observed in colitis combined with sleep deprivation.

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ORIGINAL ARTICLE

Retrospective Study

Pretreatment AKR1B10 expression predicts the risk of hepatocellular carcinoma development after hepatitis C virus eradication

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Abstract

AIM

To clarify the association between aldo-keto reductase family 1 member B10 (AKR1B10) expression and hepatocarcinogenesis after hepatitis C virus eradication.

METHODS

In this study, we enrolled 303 chronic hepatitis C patients who had achieved sustained virological response (SVR) through interferon-based antiviral

therapy. Pretreatment AKR1B10 expression in the liver was immunohistochemically assessed and quantified as a percentage of positive staining area by using image-analysis software. A multivariate Cox analysis was used to estimate the hazard ratios (HRs) of AKR1B10 expression for hepatocellular carcinoma (HCC) development after achieving SVR. The cumulative incidences of HCC development were evaluated using Kaplan-Meier analysis and the log-rank test.

RESULTS

Of the 303 chronic hepatitis C patients, 153 (50.5%) showed scarce hepatic AKR1B10 expression, quantified as 0%, which was similar to the expression in control normal liver tissues. However, the remaining 150 patients (49.5%) exhibited various degrees of AKR1B10 expression in the liver, with a maximal AKR1B10 expression of 73%. During the median follow-up time of 3.6 years (range 1.0-10.0 years), 8/303 patients developed HCC. Multivariate analysis revealed that only high AKR1B10 expression ($\geqslant$ 8%) was an independent risk factor for HCC development (HR = 15.4, 95%CI: 1.8-132.5, P = 0.012). The 5-year cumulative incidences of HCC development were 13.7% and 0.5% in patients with high and low AKR1B10 expression, respectively (P < 0.001). During the follow-up period after viral eradication, patients expressing high levels of AKR1B10 expressed markedly higher levels of alanine aminotransferase and α-fetoprotein than did patients exhibiting low AKR1B10 expression.

CONCLUSION

Chronic hepatitis C patients expressing high levels of hepatic AKR1B10 had an increased risk of HCC development even after SVR.

Key words: Human AKR1B10 protein; Hepatocellular carcinoma; Chronic hepatitis C; Immunohistochemistry; Risk factor; Sustained virological response

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Core tip: Expression of a cancer-related oxidoreductase, aldo-keto reductase family 1 member B10 (AKR1B10) was upregulated in the liver in patients with chronic hepatitis C (CHC). High AKR1B10 expression was associated in a statistically significant manner with the risk of hepatocellular carcinoma (HCC) development even after sustained virological response (SVR) was achieved through interferon-based antiviral therapy. Pretreatment AKR1B10 expression of 8% was associated with a > 15-fold-increased risk of HCC development. Thus, AKR1B10 is not only a cancer biomarker but also a novel predictive marker for assessing the risk of HCC development in CHC patients who achieved SVR.

Murata A, Genda T, Ichida T, Amano N, Sato S, Tsuzura H, Sato S, Narita Y, Kanemitsu Y, Shimada Y, Hirano K, Iijima K, Wada R, Nagahara A, Watanabe S. Pretreatment AKR1B10 expression predicts the risk of hepatocellular carcinoma development after hepatitis C virus eradication. *World J Gastroenterol* 2016; 22(33): 7569-7578 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i33/7569.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i33.7569

INTRODUCTION

Persistent hepatitis C virus (HCV) infection is one of the major causes of chronic liver disease leading to the development of hepatocellular carcinoma (HCC), which is the fifth most common cancer and the third most common cause of cancer-related death worldwide[1]. HCV is responsible for 27%-75% of the HCC cases in Europe and United States and > 80% of the cases in Japan[2,3]. Notably, HCV-positive patients present a 20-fold higher risk of developing HCC than do HCV-negative patients[4], which indicates a major carcinogenic role for persistent HCV infection. Given this association, chronic hepatitis C patients are frequently treated with interferon-based antiviral therapy, because the treatment not only eradicates HCV but also reduces the rate of HCC development. Interferon therapy most effectively lowers the risk of developing HCC in patients who achieve a sustained virological response (SVR)[5-7], and the recent emergence of direct-acting antiviral drugs (DAAs) against HCV has drastically increased the SVR rate of antiviral therapy[8,9]. However, the risk of HCC development persists after interferon therapy even in patients who achieve SVR[10]. Because assessment of the risk of developing HCC is clinically important in the management of patients with chronic hepatitis C, the requirement of predictors for HCC development in patients who achieve SVR is now increasing.

Aldo-keto reductase family 1 member B10 (AKR1B10), a cancer-related oxidoreductase, was originally identified as a gene whose expression was upregulated in human HCC but was low in normal liver tissues[11,12]. Recently, AKR1B10 upregulation was observed in several studies in certain chronic liver diseases such as chronic hepatitis B and C and steatohepatitis[13-16], which are widely recognized to represent a precancerous condition of HCC. AKR1B10 was upregulated in a stepwise manner from the surrounding liver tissues, which showed chronic hepatitis or cirrhosis, to HCC[17], and AKR1B10 upregulation was also demonstrated to be associated in a statistically significant manner with the risk of HCC development in chronic hepatitis B and C[13,15]. Furthermore, the results of *in vitro* and *in vivo* experiments demonstrated the involvement of AKR1B10 in cancer-cell proliferation[18,19].

The aforementioned findings collectively support the view that AKR1B10 upregulation is involved in the early stages of hepatocarcinogenesis. We further hypothesized that in patients in whom AKR1B10 is upregulated in the liver, the carcinogenic process has already progressed, and that these patients face a high

risk of HCC even after successful viral eradication. If this is the case, then AKR1B10 expression could serve as a useful predictive marker for HCC development in chronic hepatitis C patients who achieve SVR. Thus, in this study, our aim was to clarify the association between pretreatment AKR1B10 expression and HCC development after SVR in patients with chronic hepatitis C.

MATERIALS AND METHODS

Patients

Between March 2004 and August 2014, a total of 605 patients with chronic HCV infection underwent interferon-based antiviral therapy at Juntendo University Shizuoka Hospital. Of the 605 patients, 401 achieved SVR, and these patients were considered for enrollment in this retrospective study. Chronic HCV infection was diagnosed based on continuous positivity for serum HCV RNA detected using reverse-transcription PCR. Exclusion criteria for this study were the following: (1) absence of liver biopsy within 6 mo before treatment; (2) positivity for hepatitis B surface antigen or HIV; (3) evidence of other chronic liver diseases (autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, and Wilson's disease); (4) presence of HCC or any suspicious lesions detected through ultrasonography, dynamic computed tomography, or magnetic resonance imaging at enrollment; (5) history of previous treatment for HCC and liver transplantation; (6) a follow-up period of < 1.0 year after the end of treatment (EOT); and (7) development of HCC at < 1.0 year after the EOT, because HCC developed within 1.0 year might have existed before treatment. Based on these criteria, a total of 303 patients were finally enrolled in this study. Control normal liver tissues presenting no aberrant histological features were obtained from surgically resected specimens from 8 patients with liver metastasis from colorectal cancer.

This study was approved by the Ethics Committee of Juntendo University Shizuoka Hospital and performed in accordance with the Helsinki Declaration (as revised in Brazil, 2013). Written informed consent was obtained from all patients.

Laboratory investigations and liver histology

HCV was genotyped by performing PCR with the HCV Genotype Primer Kit (Institute of Immunology Co., Ltd., Tokyo, Japan) and classified into genotype 1, genotype 2, or other genotypes according to Simmonds' classification system. Serum HCV viral load was determined with a Cobas Amplicor HCV monitor v2.0 by using the 10-fold-dilution method (Roche Diagnostics, Branchburg, NJ, United States). Patients who were negative for serum HCV RNA at 24 wk after the EOT were defined as having achieved SVR. The following laboratory data were collected immediately before treatment, at 24 wk after the EOT, and at every follow-up visit after SVR: complete blood count and levels of albumin, alanine aminotransferase (ALT), γ-glutamyl transpeptidase (GGT), α-fetoprotein (AFP), and des-γ-carboxy prothrombin. Histological staging and grading were evaluated by a pathologist, who was blinded to the patients' clinical information, according to the Metavir classification system[20].

Patient follow-up

Each patient was examined for serum tumor markers and HCC by performing ultrasonography at least once every 6 mo after SVR. The absence of serum HCV RNA was annually reconfirmed. HCC was diagnosed predominantly through imaging studies, including dynamic computed tomography and magnetic resonance imaging. When the hepatic nodule did not show typical imaging features, diagnosis was confirmed by means of fine-needle aspiration biopsy followed by histological examination. Patient follow-up ended on March 31, 2016.

AKR1B10 immunohistochemistry

Immunohistochemical analysis of AKR1B10 was performed as described previously with certain modifications[14,21]. Briefly, deparaffinized and rehydrated sections were processed by performing heat-induced antigen retrieval in 0.1 mol/L citrate buffer at pH 6.0. After blocking endogenous peroxidase activity, sections were incubated with a mouse monoclonal antibody against AKR1B10 (1:100, Ab 57547; Abcam, Cambridge, United Kingdom) at room temperature, and then with a biotinylated secondary antibody (Ventana iVIEW DAB Universal Kit; Ventana Medical Systems Inc., Tucson, AZ, United States). Staining was visualized using 3,3'-diaminobenzidine tetrahydrochloride, and sections were counterstained with hematoxylin and eosin. AKR1B10 immunostaining was identified based on positive cytoplasmic staining and was quantified as the average percentage of AKR1B10-positive areas in 2 independent fields of hepatic parenchyma at 100 × magnification by using Lumina Vision 2.4 Bio-imaging software (Mitani Corporation, Tokyo, Japan). The average percentage of AKR1B10-positive areas was rounded to the integer by discarding fractions that were < 1%. Previous studies have established that the levels of AKR1B10 immunoreactivity and its mRNA levels are well correlated[14,15].

Statistical analyses

All statistical analyses were performed using PASW Statistics 18 (IBM SPSS, Chicago, IL, United States). The Mann-Whitney *U* test was used for continuous variables and the corrected χ^2 method was used for categorical variables. Univariate and multivariate Cox proportional hazard models were used to evaluate factors that were significantly associated with HCC

Table 1 Baseline characteristics of patients enrolled in the study

Characteristics	All patients (n = 303)	With HCC development (n = 8)	Without HCC development (n = 295)	P value
Age, yr	57 (20-85)	62 (49-71)	57 (20-85)	0.198[2]
Males	182 (60.0)	6 (75.0)	176 (59.7)	0.484[3]
BMI (kg/m^2)	23.3 (15.3-39.5)	23.8 (20.2-26.5)	23.4 (15.3-39.5)	0.860[2]
Habitual drinker	75 (24.8)	2 (25.0)	73 (24.7)	1.000[3]
Diabetes mellitus[1]	25 (8.3)	3 (37.5)	22 (7.5)	0.019[3]
HCV-RNA (logIU/mL)[1]	6.2 (1.2-7.6)	5.7 (5.0-6.7)	6.2 (1.2-7.6)	0.246[2]
HCV genotype 1	152 (50.2)	6 (75.0)	146 (49.5)	0.283[3]
Stage of fibrosis F3-F4	47 (15.5)	6 (75.0)	41 (13.9)	< 0.001[3]
Grade of inflammation A2-A3	208 (68.6)	8 (100.0)	200 (67.8)	0.060[3]
Albumin (g/dL)	4.2 (3.3-4.8)	3.9 (3.3-4.4)	4.2 (3.3-4.7)	0.032[2]
ALT (IU/L)	52 (11-699)	146 (31-209)	52 (11-699)	0.017[2]
Platelet count (× 10^4/μL)	17.5 (5.6-39.6)	10.3 (7.9-19.3)	17.6 (5.6-31.9)	0.006[2]
GGT (IU/L)	37 (9-517)	66 (28-161)	36 (9-517)	0.053[2]
AFP (ng/mL)[1]	5 (1-1380)	11 (5-870)	5 (1-1380)	0.002[2]
PI use	43 (14.2)	1 (12.5)	42 (14.2)	1.000[2]

[1]Data not available for all patients; [2]Mann Whitney-*U* test; [3]χ^2 test. Data are expressed as medians (range) or numbers (%). *P* values are for comparisons between patients with and without HCC development. AFP: α-fetoprotein; ALT: Alanine aminotransferase; BMI: Body mass index; GGT: γ-glutamyl transpeptidase; HCV: Hepatitis C virus; PI: Protease inhibitor.

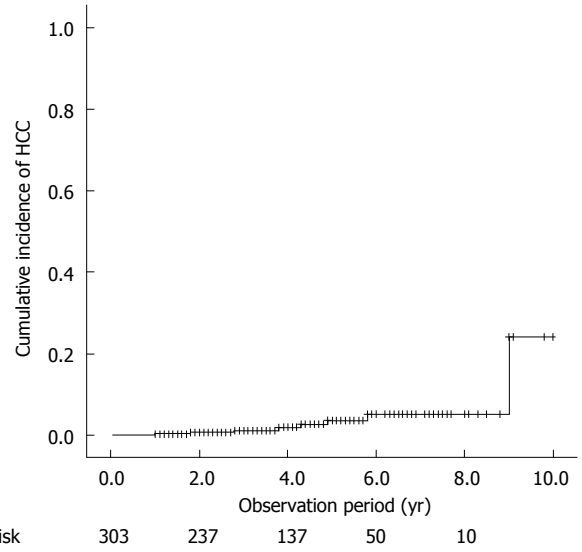

Figure 1 Cumulative incidence of hepatocellular carcinoma development after sustained virological response. HCC: Hepatocellular carcinoma.

development. The Kaplan-Meier method was used to analyze the cumulative incidence of HCC development, and differences were tested using the log-rank test. The hazard ratio (HR) and 95%CI were calculated. P < 0.05 was considered statistically significant.

RESULTS

Patients' characteristics and HCC development after SVR

The demographic, biochemical, and pathological characteristics of the 303 patients enrolled in this study are summarized in Table 1. Of the 303 patients, 260 received pegylated interferon plus ribavirin combination therapy for 24-72 wk, and 43 patients received protease inhibitor treatment (22 telaprevir, 10 simeprevir, and 11 faldaprevir) together with pegylated interferon plus ribavirin for 24 wk. During a median follow-up of 3.6 years (range, 1.0-10.0 years), 8 patients (2.6%) developed HCC. The estimated cumulative incidences of HCC were 1.2% and 3.7% at 3 and 5 years, respectively (Figure 1). As compared with patients who did not develop HCC, the patients who developed HCC more frequently presented the complication of diabetes mellitus (P = 0.019) and exhibited a higher degree of hepatic fibrosis (P < 0.001), lower albumin levels (P = 0.032), higher ALT levels (P = 0.017), lower platelet counts (P = 0.006), and higher AFP levels (P = 0.002) (Table 1).

Baseline AKR1B10 expression in the liver

Figure 2 shows representative immunohistochemical staining of AKR1B10 in liver tissues. In normal liver tissues, distinct positive staining for AKR1B10 was observed in bile-duct epithelia, but AKR1B10 immunoreactivity was either undetectable in the hepatic parenchyma, or faint immunoreactivity was observed in a few hepatocytes (Figure 2A and B). In some of the patients with chronic hepatitis C, AKR1B10 immunoreactivity in the liver was similar to that in control normal liver tissues: positive in bile-duct epithelia and negative in the hepatic parenchyma (Figure 2C and D). However, other patients showed prominent nucleocytoplasmic AKR1B10 immunoreactivity in scattered or clustered hepatocytes in the hepatic parenchyma (Figure 2E and F). Quantification of the AKR1B10-positive areas in the hepatic parenchyma revealed that none of the normal liver tissues showed AKR1B10 expression. Similarly, 153 patients (50.5%) presented scarce AKR1B10 expression, and their AKR1B10 positive staining area was quantified as 0%. By contrast, the remaining 150 patients (49.5%) presented various degrees of AKR1B10 expression in the liver parenchyma, with the maximal AKR1B10 expression area reaching 73% (Figure 3). The median

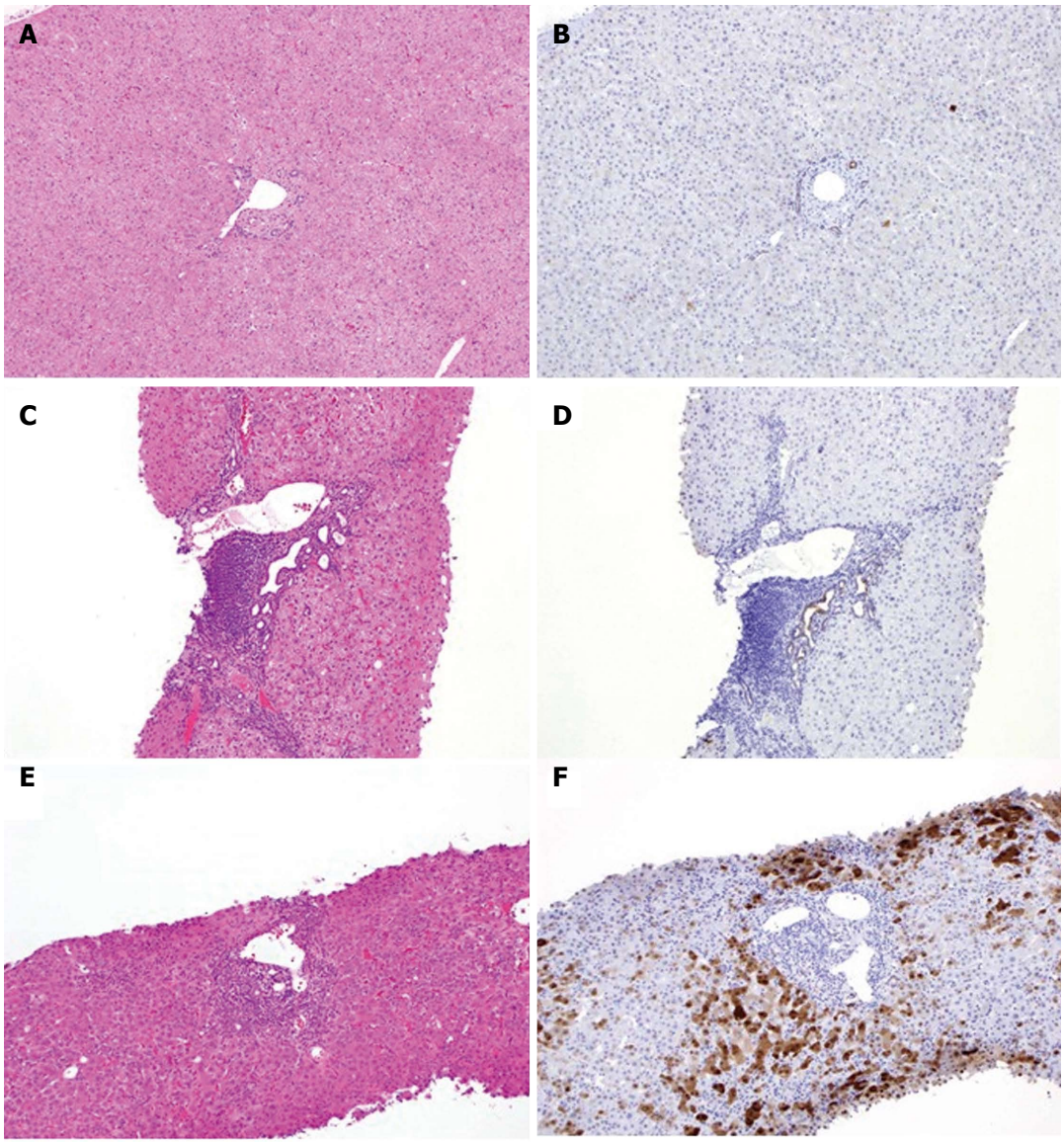

Figure 2 Representative AKR1B10 immunohistochemical staining of specimens. Normal liver tissue (A, B) and tissue from patients with chronic hepatitis C (C-F). Hematoxylin and eosin staining (A, C, E) and AKR1B10 immunostaining (B, D, F). Positive control, bile-duct epithelium; original magnification × 40.

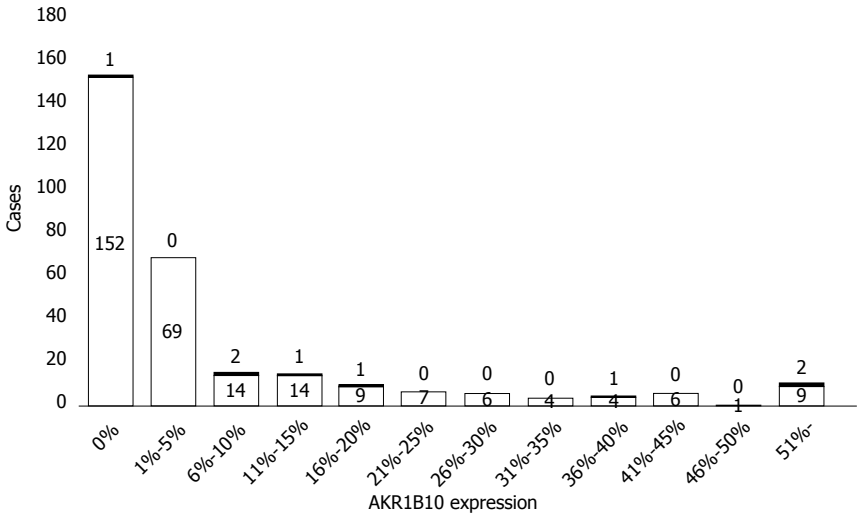

Figure 3 Distribution of AKR1B10 expression levels in the study cohort. Filled and blank patterns indicate patients with and without hepatocellular carcinoma development, respectively.

Table 2 Univariate and multivariate analyses for factors associated with hepatocellular carcinoma development

Variables	HR (95%CI)	*P* value
Univariate analysis		
Age (by each year)	1.04 (0.31-1.11)	0.347
Male sex	1.61 (0.32-8.04)	0.564
BMI (by each kg/m^2)	1.00 (0.80-1.25)	0.982
Habitual drinker	1.26 (0.25-6.27)	0.775
Diabetes mellitus	5.58 (1.19-26.21)	0.030
HCV-RNA (by each logIU/mL)	0.87 (0.44-1.72)	0.696
HCV genotype 1	2.90 (0.58-14.35)	0.195
Stage of fibrosis	3.59 (1.68-7.68)	0.001
Grade of inflammation	4.32 (1.02-18.40)	0.048
Albumin (by each g/dL)	0.03 (0.00-0.29)	0.003
ALT (by each IU/L)	1.00 (1.00-1.01)	0.196
Platelet count (by each 10^4/mL)	0.75 (0.63-0.90)	0.002
GGT (by each IU/L)	1.00 (1.00-1.01)	0.452
AFP (by each ng/mL)	1.00 (1.00-1.00)	0.033
PI use	2.93 (0.31-27.70)	0.347
AKR1B10 (by each %)	1.06 (1.03-1.10)	< 0.001
Multivariate analysis		
AKR1B10 (by each %)	1.04 (1.03-1.10)	0.001

AFP: α-fetoprotein; AKR1B10: Aldo-keto reductase family1 member B10; ALT: Alanine aminotransferase; BMI: Body mass index; GGT: γ-glutamyl transpeptidase; HCV: Hepatitis C virus; PI: Protease inhibitor.

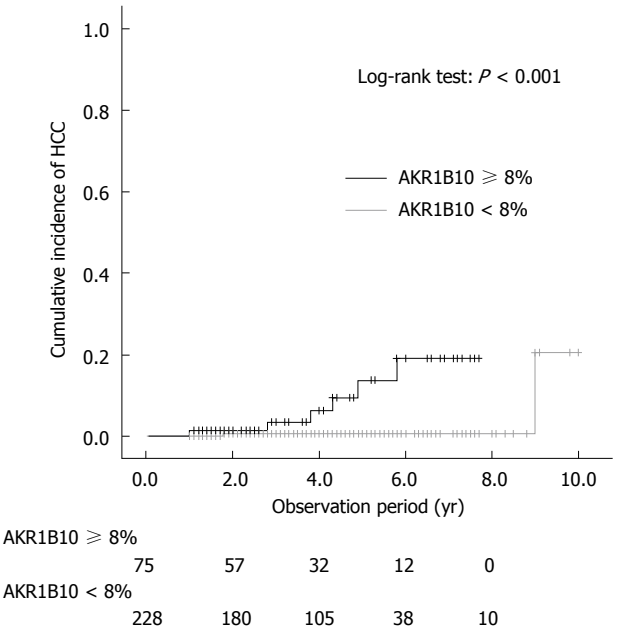

Figure 4 Cumulative incidence of hepatocellular carcinoma development after sustained virological response, shown according to AKR1B10 expression level.

AKR1B10-positive area in patients who developed HCC and did not develop HCC was 15% (range, 0%-60%) and 0% (range, 0%-73%), respectively, and this difference was statistically significant (P = 0.002).

Baseline AKR1B10 expression and risk of HCC development after SVR

The results of univariate Cox logistic regression analysis identified 6 baseline variables that were significantly associated with HCC development after SVR: complication of diabetes mellitus, advanced fibrosis stage, low serum albumin levels and platelet counts, elevated serum AFP, and increased AKR1B10 expression. Multivariate Cox logistic regression analysis revealed that only high AKR1B10 expression was an independent risk factor for HCC development after SVR (Table 2). The area under the receiver operator characteristics curve analysis further revealed that an AKR1B10 expression level of 8% was the cutoff value for HCC development. The sensitivity and specificity for AKR1B10 expression of ⩾ 8% were 0.750 and 0.766, respectively. The positive and negative predictive values were 0.080 and 0.991, respectively. The results of multivariate Cox logistic regression analysis indicated that the adjusted HR of high AKR1B10 expression (⩾ 8%) for HCC development was 15.4 (95%CI: 1.8-132.5, P = 0.012). Kaplan-Meier plot analysis revealed that the 3- and 5-year cumulative incidence rates of HCC development in patients with high AKR1B10 expression were 3.4% and 13.7%, respectively, whereas those in patients with low AKR1B10 expression (< 8%) were 0.5% and 0.5%, respectively (P < 0.001; Figure 4). Only 2 patients expressing low levels of AKR1B10 developed HCC: one patient showed an AKR1B10 expression level of 7%, and developed HCC at 1.8 years, whereas the other did not show AKR1B10 expression (0%), and developed HCC at 9.0 years (Table 3).

Changes in biochemical-test results after SVR and baseline AKR1B10 expression

Serum aminotransferase levels are sensitive indicators of necroinflammatory activity in the liver, and an elevation of serum AFP levels without HCC is also related to liver-cell damage. To investigate sustained liver-cell damage after HCV eradication, we evaluated whether serum ALT and AFP levels were altered after SVR, and whether baseline AKR1B10 expression was associated with such changes. At 6 mo after the EOT, the ALT levels were markedly decreased (median: 52 IU/L at baseline, 16 IU/L at 6 mo, P = 0.001), and 264 (87.1%) patients achieved ALT normalization (defined as ALT ⩽ 30 IU/mL). AFP levels also showed a notable decrease (median: 5 ng/mL at baseline, 3 ng/mL at 6 mo, P < 0.001), and 260 (85.8%) patients achieved an AFP level of ⩽ 5 ng/mL. As compared with the low-AKR1B10 group (n = 228), the high-AKR1B10 group (n = 75) showed considerably higher levels of ALT (median: 15 IU/L *vs* 22 IU/L, P < 0.001) and AFP (median: 3 ng/mL *vs* 4 ng/mL, P < 0.001) at 24 wk after the EOT (Figure 5).

DISCUSSION

In this study, the incidence of HCC development was 3.7% at 5 years after SVR. This low incidence

Table 3 Characteristics of the 8 patients with hepatocellular carcinoma development

	Age (yr)	Sex	Interval (yr)	F	A	Albumin (g/dL)	ALT (IU/L)	Platelet count ($\times 10^4$/mL)	AKR1B10
1	62	F	1.0	2	2	4.0	199	10.3	60%
2	69	F	1.8	3	2	3.3	60	9.9	7%
3	61	M	2.8	2	2	4.4	135	10.3	13%
4	65	M	3.8	4	3	3.4	88	9.0	52%
5	49	M	4.3	3	2	3.8	209	7.9	38%
6	56	M	4.9	3	2	4.2	156	19.3	9%
7	57	M	5.8	3	2	4.2	157	14.7	16%
8	71	M	9.0	3	2	3.7	31	17.9	0%

AKR1B10: Aldo-keto reductase family1 member B10; ALT: Alanine aminotransferase; HCC: Hepatocellular carcinoma.

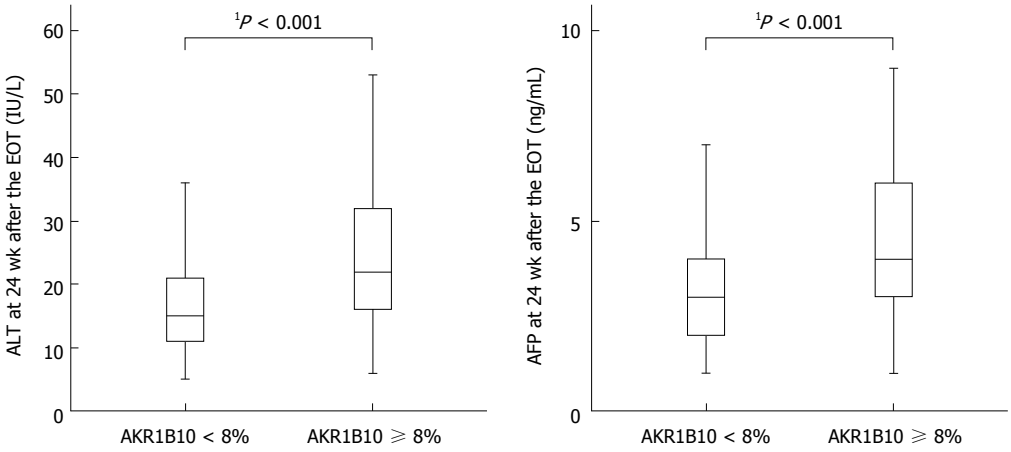

Figure 5 Relationships between baseline AKR1B10 expression levels and alanine aminotransferase and α-fetoprotein levels after sustained virological response. [1]Mann-Whitney *U* test. EOT: End of treatment.

is comparable to that presented in recent reports (1.2%-5.8%)[22-25], and this is considered to confirm the relationship between SVR achievement and a reduced risk of subsequent HCC[26]. Approval of various DAAs, including protease inhibitors, has enabled most patients receiving the therapy to achieve SVR[8,9], and the safety of the all-oral combination therapy of DAAs could increase the number of patients receiving antiviral therapy[27,28]. Consequently, the number of SVR patients is now increasing drastically, and thus predictors of HCC development after SVR are becoming increasingly important.

To date, several factors have been reported to predict the risk of HCC development in patients with chronic hepatitis C, such as older age, male gender, alcohol intake, and hepatic fibrosis[2]. Before DAA became available, Genotype 1 infection was refractory to treatment, and may have been correlated with risk of HCC. The presence of advanced hepatic fibrosis prior to treatment is recognized as a significant risk factor for HCC development after achieving SVR, however, not all patients with advanced hepatic fibrosis develop HCC[29]. In the present study, our main finding is that high AKR1B10 expression in the liver is an independent predictor for HCC development even after achieving SVR. The adjusted HR demonstrated that a pretreatment AKR1B10 expression of ⩾ 8% was associated with a > 15-fold-increased risk of HCC development after achieving SVR. Although the positive predictive value of AKR1B10 expression of ⩾ 8% was only 0.080, the negative predictive value was extremely high: 0.991; this result suggests that the risk of HCC development was extremely low in patients showing an AKR1B10 expression of < 8%. Based on the assessment of baseline AKR1B10 expression, very-low-risk patients could be selected from the growing number of SVR patients, and thus an inefficient surveillance examination for HCC could be avoided.

The previous study also demonstrated that high AKR1B10 expression was a significant predictor of HCC development in patients with chronic hepatitis C. Interestingly, the ROC analysis-determined AKR1B10 cut-off value in the present study (8%) was higher than that in the previous report (6%)[14]. A fundamental difference between these studies was that the previous study included 42% non-SVR patients in the study cohort while the present study included only SVR patients. The difference in the AKR1B10 cut-off values might indicate the impact of SVR achievement on the subsequent changes in AKR1B10 expression. However, confirming this would be difficult since liver biopsy samples from patients who have completed treatment are rarely available

AKR1B10 emerged as a cancer biomarker because

it is overexpressed in several cancers; however, the biological function of AKR1B10 and its potential involvement in carcinogenesis remain incompletely understood and are receiving increased attention. Because AKR1B10 is an efficient retinal reductase, the molecule is considered to inhibit retinoic acid signaling, which maintains epithelial cell differentiation[30,31]. Therefore, AKR1B10 upregulation has been hypothesized to play a pivotal role in promoting premature or neoplastic phenotypes in cancer cells[32,33]. In the case of human HCC, AKR1B10 upregulation was mainly observed in early-stage well-differentiated HCC and was considered to represent an early event in the hepatocarcinogenesis process[17,34]. Here, AKR1B10 upregulation occurred in patients with chronic hepatitis C, a preneoplastic condition of HCC, and reflected the risk of HCC development even after HCV eradication. Collectively, these data and those from previous studies suggest the involvement of AKR1B10 upregulation in the very early stages of hepatocarcinogenesis. In the patients in whom AKR1B10 was upregulated before treatment, the carcinogenic process might have already progressed, and in these patients, a high risk of HCC might remain even after successful viral eradication.

We also found that baseline AKR1B10 expression was related with ALT and AFP levels after SVR, both of which were identified as predictors of HCC development in several previous studies[35,36]. However, why ALT and AFP levels show sustained elevation even after HCV eradication is unknown. Intriguingly, baseline AKR1B10 expression was also associated with ALT levels in chronic hepatitis B patients who received successful antiviral therapy[13]. However, viral infection per se is unlikely to cause the sustained elevation of ALT and AFP after antiviral therapy: Recently, AKR1B10 expression was shown to be regulated by the transcription factor nuclear factor erythroid 2-related factor 2[37], which plays a pivotal role in the adaptive response to oxidative stress. Oxidative stress is a feature of steatohepatitis, and hepatic oxidative-stress markers have been correlated with the severity of hepatic necroinflammation[38-40]. Furthermore, AKR1B10 was identified as an upregulated gene in steatohepatits[16]. Given these findings, we suggest that oxidative stress might affect baseline AKR1B10 expression, sustain ALT and AFP elevation, and further HCC development after SVR.

The main limitations of this study were its monocentric aspect and retrospective nature. The number of cases of HCC development was very small because the incidence of HCC development after SVR was generally low. A future multicenter prospective analysis will be required to validate the association between AKR1B10 expression and the risk of HCC development in patients with chronic hepatitis C who achieve HCV eradication.

In conclusion, AKR1B10 upregulation is a major risk factor for HCC development in chronic hepatitis C patients who achieve SVR. Our findings not only identify AKR1B10 as a novel predictive marker of HCC, but also provide a new insight regarding AKR1B10 involvement in the molecular mechanism of hepatocarcinogenesis and suggest that AKR1B10 could serve as a novel therapeutic target for HCC prevention.

COMMENTS

Background

Persistent hepatitis C virus (HCV) infection is a major cause of chronic liver disease leading to hepatocellular carcinoma (HCC) development. Patients with chronic hepatitis C are frequently treated with interferon-based antiviral therapy, which most effectively lowers the risk of developing HCC in patients who achieve sustained virological response (SVR), and the use of direct-acting antiviral drugs against HCV has drastically increased the SVR rate of the therapy. However, the risk of HCC development persists after interferon therapy even in patients who achieve SVR. Therefore, the identification of predictors of HCC development after SVR is becoming increasingly important.

Research frontiers

The efficiency of surveillance examinations for HCC can be enhanced if we can identify reliable predictors of HCC development after HCV eradication.

Innovations and breakthroughs

In almost half of the chronic hepatitis C patients enrolled in this study, aldo-keto reductase family 1 member B10 (AKR1B10) was expressed in the liver, and high expression of AKR1B10 was found to be an independent risk factor for HCC development after SVR. Moreover, baseline AKR1B10 expression was correlated with the levels of two liver-damage markers after SVR.

Applications

The results of this study identify AKR1B10 as a novel predictive marker of HCC development. Furthermore, the findings suggest that AKR1B10 functions in the early stages of hepatocarcinogenesis, which raises the possibility that AKR1B10 could serve as a therapeutic target for HCC prevention.

Peer-review

Authors investigated the association between AKR1B10 expression and hepatocarcinogenesis after HCV eradication. They studied 303 chronic hepatitis c patients who had achieved SVR. This manuscript contains some interesting topics for prediction of HCC development after SVR.

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ORIGINAL ARTICLE

Retrospective Study

Clinical characteristics of drug-induced liver injury and primary biliary cirrhosis

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Author contributions: Zheng CQ designed the research; Yang J, Yu YL, Jin Y and Zhang Y performed the research; Yu YL and Zhang Y provided new agents and analytic tools; and Yang J wrote the paper.

Institutional review board statement: This study is a retrospective study of the clinical data of patients. The content of the study is not related to the ethical issues.

Informed consent statement: The authors of this paper guarantee that all study participants or their legal guardian provided informed written consent about personal and medical data collection prior to study enrolment.

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Abstract

AIM

To summarize and compare the clinical characteristics of drug-induced liver injury (DILI) and primary biliary cirrhosis (PBC).

METHODS

A total of 124 patients with DILI and 116 patients with PBC treated at Shengjing Hospital Affiliated to China Medical University from 2005 to 2013 were included. Demographic data (sex and age), biochemical indexes (total protein, albumin, alanine aminotransferase, aspartate aminotransferase, total bilirubin, direct bilirubin, indirect bilirubin, alkaline phosphatase, and gamma glutamyltransferase), immunological indexes [immunoglobulin (Ig) A, IgG, IgM, antinuclear antibody, anti-smooth muscle antibody, anti-mitochondrial antibody, and anti-mitochondrial antibodies] and pathological findings were compared in PBC patients, untyped DILI patients and patients with different types of DILI (hepatocellular type, cholestatic type and mixed type).

RESULTS

There were significant differences in age and gender distribution between DILI patients and PBC patients. Biochemical indexes (except ALB), immunological indexes, positive rates of autoantibodies (except SMA), and number of cases of patients with different ANA titers (except the group at a titer of 1:10000)

significantly differed between DILI patients and PBC patients. Biochemical indexes, immunological indexes, and positive rate of autoantibodies were not quite similar in different types of DILI. PBC was histologically characterized mainly by edematous degeneration of hepatocytes (n = 30), inflammatory cell infiltration around bile ducts (n = 29), and atypical hyperplasia of small bile ducts (n = 28). DILI manifested mainly as fatty degeneration of hepatocytes (n = 15) and spotty necrosis or loss of hepatocytes (n = 14).

CONCLUSION

Although DILI and PBC share some similar laboratory tests (biochemical and immunological indexes) and pathological findings, they also show some distinct characteristics, which are helpful to the differential diagnosis of the two diseases.

Key words: Drug-induced liver injury; Primary biliary cirrhosis; Autoantibodies; Immunoglobulin; Differential diagnosis; Pathological findings

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Core tip: This is a retrospective study to distinguish differential diagnosis of drug-induced liver injury (DILI) and primary biliary cirrhosis (PBC). There are many similarities between the clinical manifestations and biochemical tests of the two diseases. DILI and PBC also show some distinct characteristics, which are helpful to the differential diagnosis of the two diseases.

Yang J, Yu YL, Jin Y, Zhang Y, Zheng CQ. Clinical characteristics of drug-induced liver injury and primary biliary cirrhosis. *World J Gastroenterol* 2016; 22(33): 7579-7586 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i33/7579.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i33.7579

INTRODUCTION

Drug-induced liver injury (DILI) as an important cause of acute liver failure and chronic liver injury has increasingly been recognized. With the continuous development of new drugs and the increase in the type of drugs, there have been more and more clinical reports on DILI. However, since DILI has diverse clinical manifestations and lack a diagnostic gold standard, misdiagnosis and missed diagnosis often occur. Primary biliary cirrhosis (PBC) is an autoimmune liver disease. The constant improvement of diagnostic techniques has led to an increasing number of detected PBC cases. Both DILI and PBC may develop symptoms such as jaundice, fatigue, anorexia, and upper abdominal discomfort. PBC often has a chronic course, and it is diagnosed based mainly on the presence of liver function abnormalities and a variety of autoantibodies; however, a small portion of PBC patients may have an acute onset and show negative autoantibodies. In addition, DILI patients may be seropositive for some autoantibodies, and 20%-25% of DILI cases belong to the cholestatic type. Therefore, it is somewhat difficult to distinguish between DILI and PBC in some cases. In this study, we retrospectively analyzed the clinical data for 124 patients with DILI and 116 patients with PBC treated at Shengjing Hospital Affiliated to China Medical University from 2005 to 2013, with an aim to provide some meaningful evidence for improving the diagnosis and differential diagnosis of the two diseases.

MATERIALS AND METHODS

Patients

A total of 124 patients with DILI and 116 patients with PBC treated at Shengjing Hospital Affiliated to China Medical University from 2005 to 2013 were included. PBC was diagnosed according to the diagnostic criteria recommended by the 2009 European Association for the Study of the Liver (EASL) Clinical Practice Guidelines for management of cholestatic liver diseases[1]: (1) alkaline phosphatase (ALP) > 2 times the upper limit of normal (ULN) or gamma glutamyl transferase (GGT) > 5 times the ULN; (2) positivity for serum antimitochondrial antibody (AMA) or AMA-M2; and (3) liver biopsy showing characteristic small bile duct injury (non-suppurative cholangitis and intrahepatic small bile duct damage). When two of the above three items were met, PBC was diagnosed. Patients with viral hepatitis, autoimmune hepatitis, primary sclerosing cholangitis, drug-induced hepatitis, alcoholic liver disease and other diseases that can cause liver damage as well as patients with incomplete clinical information were excluded. DILI was diagnosed according to the RUCAM consensus developed in 1993[2,3], and patients with an RUCAM score $\geqslant$ 6 were included in this study. According to the criteria developed by the Council for International Organizations of Medical Sciences in 1989[4] and the criteria revised in 2005 by the United States Food and Drug Administration drug hepatotoxicity steering committee[5], DILI was clinically divided into (1) hepatocellular type: alanine aminotransferase (ALT) $\geqslant$ 3 × ULN and ALT/ALP $\geqslant$ 5; (2) cholestatic type: ALP $\geqslant$ 2 × ULN and ALT/ALP $\leqslant$ 2; (3) mixed type: ALT $\geqslant$ 3 × ULN, ALP $\geqslant$ 2 × ULN, and ALT/ALP > 2 but < 5.

Methods

The following clinical data were collected: (1) demographic data including gender and age; (2) biochemical indexes including total protein (TP), albumin (ALB), ALT, aspartate aminotransferase (AST), total bilirubin (TBIL), direct bilirubin (DBIL), indirect bilirubin (IBIL), ALP and GGT; (3) immunological indexes including immunoglobulin (Ig) A, IgG, IgM, antinuclear antibody (ANA), anti-smooth muscle antibody (SMA), AMA, and AMA subtypes M2, M4 and

Table 1 Comparison of biochemical indexes between drug-induced liver injury and primary biliary cirrhosis patients

	TP (g/L)	ALB (g/L)	AST (U/L)	ALT (U/L)	ALP (U/L)	GGT (U/L)	TBIL (μmol/L)	DBIL (μmol/L)	IBIL (μmol/L)
DILI (n = 124)	66.2 ± 7.2	38.8 ± 5.6	391.0 (148.0-731.0)	503.0 (263-904)	179.0 (121.0-294.0)	200 (108.0-373.0)	31.5 (14.88-109.2)	49.3 (15.9-139.6)	16.0 (10.6-37.2)
PBC (n = 116)	74.9 ± 9.4	37.6 ± 5.9	113.5 (74.8-190.5)	113.0 (69.0-193.3)	264.0 (166.3-443.3)	311.5 (148.0-660.3)	78.6 (25.2-189.0)	16.3 (5.78-60.6)	10.9 (7.38-21.6)

DILI: Drug-induced liver injury; PBC: Primary biliary cirrhosis; TP: Total protein; ALB: Albumin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TBIL: Total bilirubin; GGT: Gamma glutamyl transferase; DBIL: Direct bilirubin; IBIL: Indirect bilirubin.

Table 2 Comparison of immunoglobulins between untyped drug-induced liver injury and primary biliary cirrhosis patients

	IgG (g/L)	IgM (g/L)	IgA (g/L)
DILI (n = 124)	13.1 ± 3.7	1.4 ± 0.8	2.6 ± 1.2
PBC (n = 116)	18.8 ± 7.7	4.4 ± 2.9	3.4 ± 1.5

DILI: Drug-induced liver injury; PBC: Primary biliary cirrhosis.

M9; (3) liver biopsy (48 PBC patients and 19 DILI patients underwent B-mode ultrasound guided liver biopsy to analyze the pathological features).

Statistical analysis

All statistical analyses were performed using SPSS19.0 software. Continuous data are expressed the mean ± SD. Data following a normal distribution were compared using the t-test, while those not following a normal distribution are described as medians. Categorical data are expressed as percentages and compared using the χ^2 test. P-values < 0.05 were considered statistically significant.

RESULTS

Demographic data

In both the DILI and PBC groups, the percentage of females was higher than that of males (72.6% *vs* 27.4% and 91.4% *vs* 8.6%, respectively). The mean age of onset was 48.43 ± 14.3 years for DILI patients, and 56.89 ± 9.87 years for PBC patients. There were significant differences in gender distribution and mean age between the two groups (P < 0.05).

Biochemical indexes

Both DILI and PBC patients showed varying degrees of elevations of ALT, AST, GGT, ALP, TBIL, DBIL and IBIL. AST and ALT were significantly higher in the DILI group than in the PBC group (P < 0.05), while ALP and GGT were significantly higher in the PBC group (P < 0.05). ALB showed no significant difference between the two groups (Table 1).

Immunoglobulins

PBC patients showed elevations of IgG, IgM and IgA, with IgM having the most remarkable elevation. In contrast, DILI patients showed no elevation of IgG, IgM or IgA. There were significant differences in IgG, IgM and IgA levels between the two groups (P < 0.05) (Table 2).

Autoantibodies

Of all the DILI patients (n = 124), 59 (47.5%) were positive for autoantibodies, most of which had a low titer. There were 55 (44.3%) patients positive for ANA, including 12 (9.67%) having a titer > 1:100, 43 (34.6%) having a titer of 1:100, 9 (7.25%) having a titer of l:320, 2 (1.61%) having a titer of l:1000 and 1 (0.80%) having a titer of 1:3200; 4 (3.22%) positive for SMA; 4 (3.22%) positive for AMA; 3 (2.42%) positive for AMA-M2; 1 (0.80%) positive for AMA-M4; and 3 (2.42%) positive for AMA-M9. Except that one patient was moderately positive for SMA, the other patients only showed weak positivity for SMA, AMA-M2, AMA-M4 or AMA-M9. Of all the PBC patients (n = 116), 106 (91.3%) were positive for autoantibodies, most of which had a high titer. There were 98 (84.4%) patients positive for ANA, including 78 (67.2%) having a titer > 1:100, 20 (17.2%) having a titer of 1:100, 27 (23.2%) having a titer of 1:320, 30 (25.8%) having a titer of 1:1000, 19 (16.3%) having a titer of 1:3200, and 2 (1.72%) having a titer of 1:10000; 2 (3.45%) positive for SMA; 98 (84.4%) positive for AMA; 98 (84.4%) positive for AMA-M2; 34 (29.3%) positive for AMA-M4; and 19 (16.3%) positive for AMA-M9. With regards to the degree of positivity, there were 80 cases of moderate positivity and 18 cases of weak positivity for AMA; 19 cases of strong positivity, 69 cases of moderate positivity and 10 cases of weak positivity for AMA-M2; 12 cases of moderate positivity and 22 cases of weak positivity for AMA-M4; 5 cases of moderate positivity and 14 cases of weak positivity for AMA-M9; and all cases of weak positivity for SMA2. Except for SMA, the percentage of cases positive for other autoantibodies differed significantly between DILI and PBC patients (P < 0.05). The number of cases positive for ANA at all titers were significantly different between DILI and PBC patients (P < 0.05) except ANA at a titer of 1:10000 (Tables 3 and 4).

Biochemical indexes, immunoglobulins and autoantibodies in PBC and typed DILI patients

Of all the 124 cases of DILI, 90 belonged to the hepatocellular type, 20 belonged to the cholestatic

Table 3 Comparison of autoantibodies between untyped drug-induced liver injury and primary biliary cirrhosis patients *n* (%)

	ANA	AMA	SMA	AMA-M2	AMA-M4	AMA-M9
DILI (n = 124)	55 (44.3)	4 (3.22)	4 (3.22)	3 (2.42)	1 (0.80)	3 (2.42)
PBC (n = 116)	98 (84.4)	98 (84.4)	2 (3.45)	98 (84.4)	34 (29.3)	19 (16.3)
χ^2	41.761	161.932	0.554	165.598	39.091	14.027
P value	< 0.0001	< 0.0001	0.685	< 0.0001	< 0.0001	< 0.0001

DILI: Drug-induced liver injury; PBC: Primary biliary cirrhosis; ANA: Antinuclear antibody; AMA: Antimitochondrial antibody; SMA: Anti-smooth muscle antibody.

Table 4 Comparison of antinuclear antibody at different titers between untyped drug-induced liver injury and primary biliary cirrhosis patients *n* (%)

	1:100	1:320	1:1000	1:3200	1:10000
DILI (n = 124)	43 (34.6)	9 (7.25)	2 (1.61)	1 (0.80)	0
PBC (n = 116)	20 (17.2)	27 (23.2)	30 (25.8)	19 (16.3)	2 (1.72)
χ^2	9.412	12.060	30.498	19.027	2.156
P value	0.0002	0.001	< 0.0001	< 0.0001	0.233

DILI: Drug-induced liver injury; PBC: Primary biliary cirrhosis.

Table 5 Comparison of biochemical indexes between typed drug-induced liver injury and primary biliary cirrhosis patients (median)

	TP (g/L)	ALB (g/L)	AST (U/L)	ALT (U/L)	ALP (U/L)	GGT (U/L)	TBIL (μmol/L)	DBIL (μmol/L)	IBIL (μmol/L)
Hepatocellular type DILI (n = 90)	66.8 ± 8.0[a]	39.2 ± 6.2	442.5 (174.3-941.5)[a]	574.0 (205.6-1263.3)[a,c,e]	145.5 (86.3-217.5)[a,c,e]	179.0 (87.0-366.1)[a]	74.30 (32.4-160.7)	46.2 (17.8-96.6)	16.2 (11.4-19.5)
Cholestatic type DILI (n = 20)	63.7 ± 7.2[a]	39.1 ± 5.8	135.0 (82.6-185.8)	154.0 (49.2-253.8)	320.6 (196.6-454.2)	208.50 (110.5-426.8)	126.90 (55.3-266.7)	99.0 (38.8-214.3)	26.2 (13.3-35.2)
Mixed type DILI (n = 14)	70.8 ± 8.6	37.6 ± 4.4	654.1 (317.2-1389.1)[a,c]	844.0 (551.8-1945.8)[a,c]	328.75 (258.4-477.2)	260.50 (142.8-418.5)	111.10 (41.3-234.9)[a]	95.2 (56.7-266.8)[a]	21.9 (14.6-33.2)[a]
PBC (n = 116)	74.9 ± 9.4	37.6 ± 6.0	113.5 (74.8-190.5)	113.0(69.0-193.3)	264.0 (166.3-443.3)	311.5 (148.0-660.3)	78.6 (25.2-189.0)	16.3 (5.8-60.6)	10.9 (7.4-21.6)

[a]P < 0.05 *vs* the PBC group; [c]P < 0.05 *vs* the cholestatic type DILI group; [e]P < 0.05 *vs* the mixed type DILI group. DILI: Drug-induced liver injury; PBC: Primary biliary cirrhosis; TP: Total protein; ALB: Albumin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TBIL: Total bilirubin; GGT: Gamma glutamyl transferase; DBIL: Direct bilirubin; IBIL: Indirect bilirubin; ALP: Alkaline phosphatase.

Table 6 Comparison of immunoglobulins between typed drug-induced liver injury and primary biliary cirrhosis patients (median)

	IgG (g/L)	IgM (g/L)	IgA (g/L)
Hepatocellular type DILI (n = 90)	12.6 ± 5.2[a]	1.1 ± 0.5[a]	2.3 ± 1.0[a]
Cholestatic type DILI (n = 20)	13.2 ± 5.7[a]	1.3 ± 0.6[a]	2.3 ± 1.2
Mixed type DILI (n = 14)	11.9 ± 4.7[a]	1.1 ± 0.6[a]	2.1 ± 1.4
PBC (n = 116)	18.8 ± 7.7	4.4 ± 2.9	3.4 ± 1.5

[a]P < 0.05 *vs* the PBC group. DILI: Drug-induced liver injury; PBC: Primary biliary cirrhosis.

type, and 14 to the mixed type. AST, ALT, GGT, TBIL, DBIL, IBIL, IgM, IgA and IgG differed significantly between the hepatocellular type DILI, cholestatic type DILI, mixed type DILI, and PBC groups, although ALB showed no significant difference. AST, ALT, GGT, TBIL, DBIL, and IBIL were elevated in all types of DILI and PBC. AST and ALT were elevated more significantly in mixed type DILI than in cholestatic type DILI and PBC (P < 0.05). AST was elevated more significantly in hepatocellular type DILI than in mixed type DILI, cholestatic type DILI and PBC (P < 0.05). GGT rose remarkably in the PBC group, which was significantly higher than that in the hepatocellular type DILI group (P < 0.05). TBIL, DBIL and IBIL significantly rose in the cholestatic type group. Although ALP showed no remarkable elevation in the hepatocellular type group, it was elevated in the other three groups and showed no significant difference among the three groups. IgM, IgA and IgG were elevated in the PBC group, with IgM having the most prominent rise. IgM, IgA and IgG in the PBC group were significantly different from those in the other three groups (P < 0.05) (Tables 5 and 6). The positive rates of the majorities of autoantibodies differed significantly between the hepatocellular type DILI, cholestatic type DILI, mixed type DILI, and PBC groups. Except for SMA and ANA at a titer of 1:10000 (P = 0.797, 0.506), the positive rates of other autoantibodies differed significantly between the hepatocellular type DILI and PBC groups. Except for AMA-M9, SMA and ANA at a titer of 1:100 (P = 0.306, 0.382, 0.531, 0.306), the positive rates

Table 7 Comparison of autoantibodies between typed drug-induced liver injury and primary biliary cirrhosis patients *n* (%)

	Autoantibodies	ANA	AMA	SMA	AMA-M2	AMA-M4	AMA-M9
Hepatocellular type DILI (*n* = 90)	43 (47.7)[a]	41 (45.5)[a]	2 (2.22)[a]	2 (2.22)	2 (2.22)[a]	0[a]	1 (1.11)[a]
Cholestatic type DILI (*n* = 20)	10 (50.0)[a]	8 (40.0)[a]	2 (10.0)[a]	1 (5.00)	1 (5.00)[a]	0[a]	1 (5.00)
Mixed type DILI (*n* = 14)	6 (42.8)[a]	6 (42.8)[a]	0[a]	1 (7.14)	0[a]	1 (7.14)	1 (7.14)
PBC (*n* = 116)	106 (91.3)	98 (84.4)	98 (84.4)	2 (1.72)	98 (84.4)	34 (29.3)	19 (16.3)

[a]*P* < 0.05 *vs* the PBC group. DILI: Drug-induced liver injury; PBC: Primary biliary cirrhosis; ANA: Antinuclear antibody; AMA: Antimitochondrial antibody; SMA: Anti-smooth muscle antibody.

Table 8 Comparison of ANA at different titers between typed drug-induced liver injury and primary biliary cirrhosis patients *n* (%)

	1:100	1:320	1:1000	1:3200	1:10000
Hepatocellular type DILI (*n* = 90)	34 (37.7)[a]	7 (7.77)[a]	1 (1.11)[a]	0[a]	0
Cholestatic type DILI (*n* = 20)	5 (25.0)	2 (10.0)[a]	0[a]	1 (5.00)	0
Mixed type DILI (*n* = 14)	4 (28.5)	1 (7.14)	1 (7.14)	0 (0.00)	0
PBC (*n* = 116)	20 (17.2)	27 (23.2)	30 (25.8)	19 (16.3)	2 (1.72)

[a]*P* < 0.05 *vs* the PBC group. Since the number of cases with ANA at a titer of 1:10000 was 0 in the hepatocellular type DILI, cholestatic type DILI, and mixed type DILI groups, comparisons between them cannot be done. DILI: Drug-induced liver injury; PBC: Primary biliary cirrhosis.

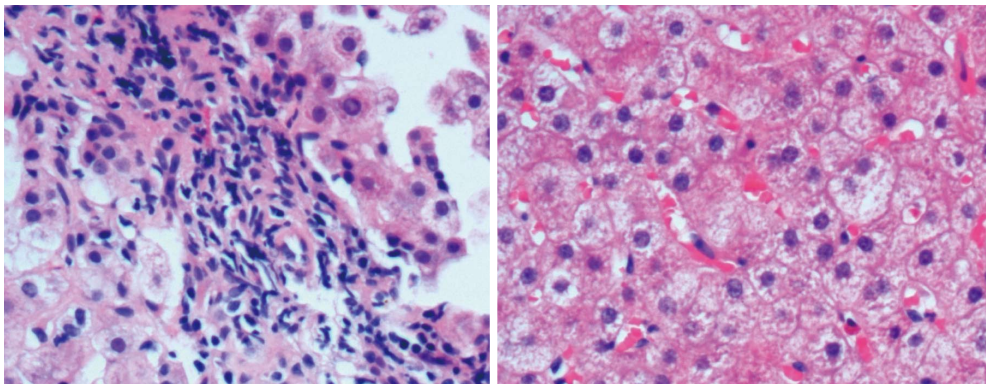

Figure 1 Liver histology of primary biliary cirrhosis (HE staining, × 200).

of other autoantibodies differed significantly between the cholestatic type DILI and PBC groups. When comparing the mixed type DILI and PBC groups, AMA-M4, AMA-M9 and SMA (*P* = 0.111, 0.694, 0.292) as well as ANA at different titers showed no significant differences. The positive rates of autoantibodies did not differ significantly between various types of DILI (Tables 7 and 8).

Pathological examination

Forty-eight PBC patients and 19 DILI patients underwent liver biopsy. Histologically, PBC manifested mainly as edematous degeneration of hepatocytes (*n* = 30) with cell necrosis (mainly spotty and patchy necrosis), inflammatory cell infiltration around the bile ducts (*n* = 29; the main infiltrating cell type was lymphocytes, with plasma cells, eosinophils, and neutrophils visible), atypical hyperplasia of small bile ducts (*n* = 28), decreased number or disappearance of interlobular bile ducts (*n* = 16), granulomatous changes (*n* = 11), cholestasis (*n* = 8), and fibrous hyperplasia and expansion around bile ducts (*n* = 14). DILI manifested mainly as fatty degeneration of hepatocytes (*n* = 15), spotty necrosis or loss of hepatocytes (*n* = 14), infiltration of mixed types of inflammatory cells (mainly eosinophils and neutrophils) around hepatocytes (*n* = 16), epithelioid granuloma (*n* = 1), hepatocellular cholestasis (*n* = 9), and cholangiolitic cholestasis (*n* = 3) (Figures 1 and 2).

DISCUSSION

DILI is an iatrogenic form of liver injury caused directly by drugs or their metabolites or hypersensitivity to them in the process of drug therapy, frequently occurring about 5-90 d after medication. In some Western countries, DILI is an important cause of acute liver failure[6,7]. The diagnosis of DILI is a process of exclusion, in which medication history, onset time after medication, duration, risk factors for adverse drug reactions, drugs used, factors that can result in exclusions, laboratory findings, previously reported liver damage associated with the drug use, and response to re-medication should be

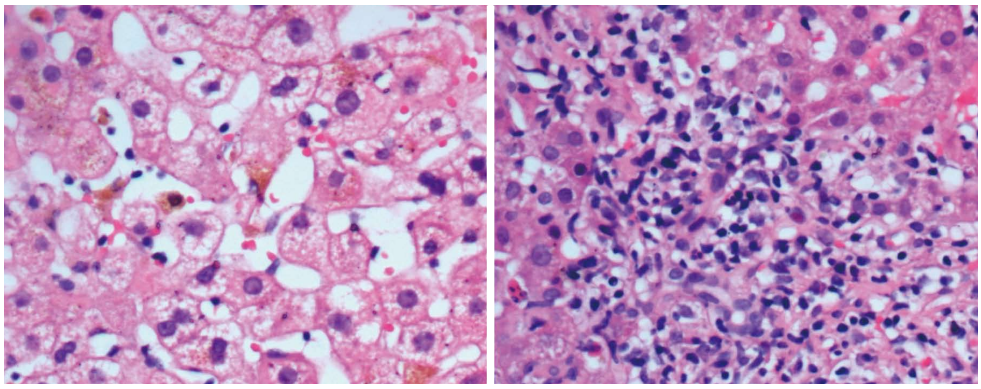

Figure 2 Liver histology of drug-induced liver injury (HE staining, × 200).

assessed to identify the possible causality[2]. PBC is a chronic, progressive, immune-mediated cholestatic disease that occurs mainly in middle-aged women. Characterized by AMA and (or) AMA-M2 positivity, PBC manifests mainly as non-suppurative damage to small intrahepatic bile ducts, portal area expansion, inflammatory responses, and serious intrahepatic cholestasis. Although the course of PBC is chronic, it can eventually lead to liver fibrosis and cirrhosis[8]. Risk factors for DILI include gender, age, race, underlying liver disease, alcohol consumption, combined drug use, and genetic susceptibility. Women are susceptible to DILI[9,10]. In the present study, it was found that the proportion of women was 72.6% in DILI patients, and 91.4% in PBC patients. The onset age was 48.43 ± 14.3 years for DILI patients, and 56.89 ± 9.87 years for PBC patients. These findings are similar to the mean onset age of DILI (approximately 45 years) and PBC (approximately 50 years) reported in the literature, and in line with the characteristic of woman susceptibility to DILI and PBC (approximately 90%)[11,12]. Our results showed that PBC had a more significant preponderance of females, and most patients had an onset age of 40 years or older and younger patients were rare. In contrast, the onset age of DILI had a large span: the youngest patient was 18 years old and the oldest was 81 years old. Young DILI patients were not rare.

Biochemical tests showed that AST and ALT levels were significantly higher in untyped DILI patients than in patients with PBC, while ALP and GGT levels were significantly higher in PBC patients than in patients with DILI. This is because DILI is often the hepatocellular type, which is characterized by significantly increased aminotransferase levels and hepatocyte necrosis, while PBC is characterized by severe cholestasis and bile duct damage. Based on AST and ALP levels, DILI is divided into the hepatocellular type, cholestatic type and mixed type. Our results showed that the proportion of the hepatocellular type was the highest (72.5%), followed by the cholestatic type (16.1%) and the mixed type (11.2%). This finding is consistent with the DILI typing results of some studies, which also showed that hepatocellular type is the most common type of DILI. In the present study, the mixed type had more severe liver injury than the other types, the hepatocellular type and mixed group had more significant AST and ALT elevations than cholestatic type DILI and PBC, bilirubin was significantly higher in the mixed group than in the other three groups, and GGT and ALP in the mixed type and cholestatic type DILI groups were not significantly different from those in the PBC group, but significantly differed from those in the hepatocellular type group. Some studies reported that mixed type DILI is in essence within the category of cholestatic type DILI, because they can change into each other in the course of the disease, and have a greater chance of chronicity than the hepatocellular type[13]. Some drugs (amoxicillin-clavulanic acid, erythromycin estolate, chlorpromazine, carbamazepine, trimethoprim-sulfamethoxazole, oral contraceptives, *etc*.) can activate the immune mechanism of the liver, and if not promptly withdrawn, they can lead to chronic liver injury, such as non-suppurative cholangitis, which is similar to PBC[6]. This study found that mixed type DILI and PBC had significant differences in elevated transaminases and bilirubin, while cholestatic type DILI and PBC had no significant differences in all biochemical indexes except total protein. Given that DILI patients may also test positive for autoantibodies and there have been reports that DILI can progress into PBC and AIH (autoimmune hepatitis)-PBC overlap syndrome[14] or PBC was misdiagnosed as DILI[15], clinicians should pay more attention to the differential diagnosis of DILI from PBC in clinical practice.

With regards to immunological indexes, IgG, IgM and IgA showed no significant elevations in untyped DILI and all three types of DILI, while these immunoglobulins were significantly elevated in the PBC group, with IgM having the most prominent elevation. This conforms to the characteristic of PBC that IgM has an obvious rise, which is helpful in differentiating PBC and DILI. Drug induced hepatotoxicity is generally divided into two categories: predictable and non-predictable. Predictable DILI is often due to direct toxic

effects of drug themselves or their metabolites on liver cells. Non-predictable DILI is caused by specific or non-specific immune responses induced by drugs[16,17], which result in the recognition of specific drug related antigens and make certain autoantibodies such as anti-liver-kidney microsomal antibody, anti-parietal cell antibody, anti-liver cytosol antibody type 1, SMA and ANA become positive[18]. Most scholars believe that the immune mechanism of pathogenesis of PBC involves the breakdown of the body's immune tolerance and the loss of ability to tolerate auto-antigens. As a result, the immune system continuously attacks intrahepatic bile ducts, thereby resulting in cholangitis and cholestasis. AMA and (or) AMA-M2 antibodies are the most typical examples[19]. In contrast, autoantibodies in DILI may not be a cause of liver damage, but may be the result of liver damage[18]. In most cases of DILI, autoantibodies disappeared or showed a reduction in titer in the process of liver repair, which is a different feature from autoantibodies in PBC. The present study showed that ANA positivity was most common in DILI patients, and a few cases tested positive for AMA, SMA, AMA-M2, AMA-M4, AMA-M9, although most of the cases were weakly positive. In contrast, PBC patients were positive mainly for ANA, AMA, and AMA-M2, and the majority of cases showed strong positivity and had high titer antibodies. The positive rates of all autoantibodies except ANA at a titer of 1:10000 exhibited significant differences between uptyped DILI and PBC. There were no significant differences in all autoantibodies between different types of DILI, and DILI of various types showed no significant differences from PBC in the positive rates of SMA and ANA at a titer of 1:10000. Mixed type and cholestatic type DILI were more similar to PBC in the positive rates of autoantibodies, and this may be because there were fewer cases of mixed type and cholestatic type DILI. In this study, the majority of DILI patients who tested positive for some autoantibodies underwent liver biopsy to achieve a clear diagnosis on the basis of medication history. For patients who were negative for PBC specific autoantibodies, liver biopsy was required to obtain a definite diagnosis. This suggests that liver biopsy is helpful to differentiate between DILI and PBC. For DILI patients who are positive for autoantibodies, regular monitoring of autoantibodies and biochemical indexes is necessary to detect the possibility of progressing to autoimmune liver disease.

Pathological analysis showed that both DILI and PBC had varying degrees of hepatocyte necrosis, edema, inflammatory cell infiltration and cholestasis, but DILI mainly manifested as eosinophil and neutrophil infiltration, cholangiolitic and hepatocellular cholestasis, while PBC was characterized by lymphocyte infiltration around bile ducts and cholestasis, destruction, progressive reduction and disappearance, and fibrosis of small bile ducts. Pathologically, DILI was characterized mainly by epithelioid granuloma, eosinophil infiltration around hepatocytes, and hepatocyte necrosis or loss. DILI with cholangiolitic cholestasis rarely progressed to vanishing bile duct syndrome. The features that PBC patients often test positive for specific autoantibodies, the liver function of DILI patients frequently recovers after the cessation of drugs, and very few DILI cases are complicated with other autoimmune diseases such as Hashimoto's thyroiditis and Sjogren's syndrome[20] provide powerful evidence for differentiating DILI and PBC.

With the increase in the type of drugs, more and more DILI cases have increasingly been caused. The improvement in PBC diagnostic standards, advances in diagnostic technology and wide use of liver biochemistry tests in China have resulted in increasing annual detection rates of DILI. However, there have been no diagnostic gold standards for DILI so far. Since DILI often exhibits similar clinical manifestations and biochemical findings to PBC, and both DILI and PBC patients may be positive for some autoantibodies and only show mild early symptoms or biochemical abnormalities in physical examination, the differential diagnosis of PBC and DILI is difficult in some cases. Given that there have currently been few studies on the differential diagnosis of PBC and DILI, this study analyzed the clinical data for PBC patients and typed and untyped DILI patients to summarize and compare their clinical characteristics, with an aim to provides useful information for the diagnosis and differential diagnosis of the two diseases. However, the total number of DILI patients and the number of pathological samples were not large enough, and the sample size for different types of DILI was small. Future larger-sample studies will be required to address this issue.

COMMENTS

Background

With the continuous improvement of diagnostic techniques, the incidence of primary biliary cirrhosis (PBC) as a kind of autoimmune liver disease, is also increasing. PBC and drug-induced liver injury (DILI) may appear similar clinical manifestations, yellow dye, fatigue, anorexia, abdominal discomfort, *etc*. PBC mostly shows a chronic course of disease. In diagnosis, PBC is mainly based on the abnormal liver function and many kinds of autoantibodies, but few patient may have negative autoantibodies. Some DILI patients may be antibody positive, and about 20%-25% of DILI cases belong to cholestatic liver disease. So it is difficult to distinguish between PBC and DILI.

Research frontiers

There have been few reports on the diagnosis and differential diagnosis of PBC and DILI. From the current report, DILI and PBC share some similar laboratory tests (biochemical and immunological indexes) and pathological findings. The result of this study contribute to the differential diagnosis of the two diseases.

Innovations and breakthroughs

Detection of biochemical indexes and immunology indexes in DILI (before and after typing) and PBC was very useful. By comparison, differences in both biochemical indexes (TP, AST, ALT, ALP, TBIL, DBIL, GGT, and IBIL) and immunological indexes (IgA, IgG, IgM, ANA, and AMA) were statistically significant.

Applications

This study suggests that detection of biochemical indexes (TP, AST, ALT, ALP, TBIL, DBIL, GGT, and IBIL) and immunological indexes is useful for diagnosing DILI and PBC.

Terminology

DILI is an iatrogenic form of liver injury caused directly by drugs or their metabolites or hypersensitivity to them in the process of drug therapy, frequently occurring about 5-90 d after medication. PBC is a chronic, progressive, immune-mediated cholestatic disease that occurs mainly in middle-aged women. Characterized by AMA and (or) AMA-M2 positivity, PBC manifests mainly as non-suppurative damage to small intrahepatic bile ducts, portal area expansion, inflammatory responses, and serious intrahepatic cholestasis.

Peer-review

In this manuscript, the authors analyzed the clinical characteristics of DILI and PBC. Although DILI and PBC share some similar laboratory tests (biochemical and immunological indexes) and pathological findings, they also show some distinct characteristics, which are helpful to the differential diagnosis of the two diseases. Over all, this study is well designed and the manuscript is well written.

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ORIGINAL ARTICLE

Observational Study

Detection of *Helicobacter pylori* resistance to clarithromycin and fluoroquinolones in Brazil: A national survey

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Abstract

AIM

To evaluate bacterial resistance to clarithromycin and fluoroquinolones in Brazil using molecular methods.

METHODS

The primary antibiotic resistance rates of *Helicobacter pylori* (*H. pylori*) were determined from November 2012 to March 2015 in the Southern, South-Eastern, Northern, North-Eastern, and Central-Western regions of Brazil. Four hundred ninety *H. pylori* patients [66% female, mean age 43 years (range: 18-79)] who had never been previously treated for this infection were enrolled. All patients underwent gastroscopy with antrum and corpus biopsies and molecular testing using GenoType HelicoDR (Hain Life Science, Germany). This test was performed to detect the presence of *H. pylori* and to identify point mutations in the genes responsible for clarithromycin and fluoroquinolone resistance. The molecular procedure was divided into three steps: DNA extraction from the biopsies, multiplex amplification, and reverse hybridization.

RESULTS

Clarithromycin resistance was found in 83 (16.9%) patients, and fluoroquinolone resistance was found in 66 (13.5%) patients. There was no statistical difference in resistance to either clarithromycin or fluoroquinolones (P = 0.55 and P = 0.06, respectively) among the different regions of Brazil. Dual resistance to clarithromycin and fluoroquinolones was found in 4.3% (21/490) of patients. The A2147G mutation was present in 90.4% (75/83), A2146G in 16.9% (14/83) and A2146C in 3.6% (3/83) of clarithromycin-resistant patients. In 10.8% (9/83) of clarithromycin-resistant samples, more than 01 mutation in the 23S rRNA gene was noticed. In fluoroquinolone-resistant samples, 37.9% (25/66) showed mutations not specified by the GenoType HelicoDR test. D91N mutation was observed in 34.8% (23/66), D91G in 18.1% (12/66), N87K in 16.6% (11/66) and D91Y in 13.6% (9/66) of cases. Among fluoroquinolone-resistant samples, 37.9% (25/66) showed mutations not specified by the GenoType HelicoDR test.

CONCLUSION

The *H. pylori* clarithromycin resistance rate in Brazil is at the borderline (15%-20%) for applying the standard triple therapy. The fluoroquinolone resistance rate (13.5%) is equally concerning.

Key words: *Helicobacter pylori*; Microbial drug resistance; Clarithromycin; Fluoroquinolones; Molecular diagnostic techniques

Core tip: Antibiotic resistance is the main cause of failure in the treatment of *Helicobacter pylori* (*H. pylori*) infection. Using molecular methods, this study investigated bacterial resistance to clarithromycin and fluoroquinolones in 490 adult patients recruited from five regions in Brazil. These patients had never been previously treated for *H. pylori* infection. Clarithromycin and fluoroquinolone resistance was found in 16.9% and 13.5% of patients, respectively. Resistance to both drugs was found in 4.3% of patients. The mean primary *H. pylori* clarithromycin resistance rate in Brazil is at the borderline for applying the standard triple therapy, and the primary fluoroquinolone resistance rate is concerning.

Sanches BS, Martins GM, Lima K, Cota B, Moretzsohn LD, Ribeiro LT, Breyer HP, Maguilnik I, Maia AB, Rezende-Filho J, Meira AC, Pinto H, Alves E, Mascarenhas R, Passos R, de Souza JD, Trindade OR, Coelho LG. Detection of *Helicobacter pylori* resistance to clarithromycin and fluoroquinolones in Brazil: A national survey. *World J Gastroenterol* 2016; 22(33): 7587-7594 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i33/7587.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i33.7587

INTRODUCTION

Helicobacter pylori (*H. pylori*) is the main etiologic agent of peptic ulcer and is recognized as the most important risk factor for adenocarcinoma and lymphoma of the mucosa-associated lymphoid tissue (MALT)[1]. Triple therapy, in which a combination of two antibiotics (amoxicillin and clarithromycin) and a proton pump inhibitor (PPI) is administered for 7 to 14 d, has been demonstrated to be an effective *H. pylori* infection treatment in different meta-analyses and has been recommended in national and international consensus meetings[2-5]. This regimen has, however, exhibited decreased effectiveness in recent years, with eradication rates lower than 80%, as reported in different studies[6,7].

Although factors including the lack of compliance, lifestyle habits such as smoking, Cag-negative strains, CYP2C19 genetic polymorphisms, altered immunity, and elevated bacterial load may all contribute to therapy failure, the main factor that causes therapy failure is bacterial resistance, especially to clarithromycin, metronidazole, and fluoroquinolones[8,9]. Similar to other bacterial species (*e.g.*, *Mycobacterium tuberculosis*), *H. pylori* acquires antibiotic resistance by chromosomal mutations, not by acquiring plasmids[10]. Although drug efflux proteins can contribute to the natural insensitivity to antibiotics and emerging antibiotic resistance, the main mechanism that contributes to *H. pylori* resistance

is vertically transmitted point mutations in the DNA[9-12].

Clarithromycin interacts with the peptidyl transferase in domain V of the 23S rRNA subunit, an interaction that suppresses bacterial ribosome activity and inhibits protein synthesis[9]. Point mutations at positions 2146 and 2147, formerly known as 2142 and 2143 (the numeration is from genome sequencing of GenBank NC000921 - J99 and NC000915 - HP 26695)[13], of the 23S rRNA gene have been shown to lead to a modification in ribosome conformation, which consequently reduces clarithromycin affinity and leads to bacterial resistance to the drug[9]. Three major point mutations in the 23S rRNA gene have been described to be responsible for over 90% of clarithromycin resistance cases observed in occidental countries[7]. These are A2146C (point mutation at position 2146 by substitution of adenine for cytosine), A2146G (point mutation at position 2146 by substitution of adenine for guanine), and A2147G (point mutation at position 2147 by substitution of adenine for guanine). Quinolone resistance on the other hand develops following point mutations in the DNA-gyrase enzyme involved in bacterial DNA replication[9]. *H. pylori* DNA gyrase comprises two subunits (gyrA and gyrB), and the mutations are found in a specific region of the *gyrA* gene called the quinolone resistance-determining region. Eleven mutations have been described, and these occur in codons 86, 87, 88, and 91[9]. The most frequently encountered mutations occur in codons 87 and 91[9,14,15], and these have been shown to be present in 80% to 100% of antibiotic resistance cases[16-18].

H. pylori antimicrobial resistance can be investigated in the laboratory by phenotypic and genotypic methods[7]. Bacterial culture and determination of the minimum inhibitory concentration (MIC) of the antibiotic are characteristics of the phenotypic method. In addition to providing a definite diagnosis of the infection, the phenotypic method also allows for drug sensitivity to be determined. Bacteria are, however, rarely cultured in medical practices due to the special care required in the transport of samples, the fastidious growth nature of *H. pylori*, and the need for appropriate culture media, which are most often unavailable[7].

Genotypic methods such as PCR, on the other hand, are increasingly used for bacterial detection and identification of point mutations[9]. European studies have recently validated a new molecular test that combines PCR and hybridization, allowing for the rapid detection of bacterial resistance to both clarithromycin and fluoroquinolones[13,19]. This novel GenoType HelicoDR (Hain Life Science, Germany) test involves a DNA strip coated with different specific primers (probes) designed to hybridize with the wild-type allele or reveal mutant sequences[13]. This method is faster than the phenotypic method and can be performed directly from endoscopic gastric biopsies, making the prior culture of the microorganism unnecessary[9].

Taking into consideration that primary resistance to *H. pylori* is a major factor for treatment failure in first-line *H. pylori* regimens, the Maastricht IV *H. pylori* Consensus Report recommends that the (PPI) clarithromycin-containing triple therapy, without prior susceptibility testing, should be abandoned when the clarithromycin resistance rate in the region is over 15%-20%[4]. Fluoroquinolones should be reserved for cases of retreatment or employed in high primary clarithromycin resistance areas[4]. However, the success of quinolones also decreases when there is bacterial resistance. It is important for clinicians to know the local prevalences of *H. pylori* resistance to clarithromycin and fluoroquinolones so that they can select the most appropriate *H. pylori* regime in first- and second-line eradication treatments. Therefore, the aim of this study was to assess the prevalence of primary *H. pylori* resistance to clarithromycin and fluoroquinolones in a large Brazilian population using the molecular GenoType HelicoDR test on gastric biopsy specimens.

MATERIALS AND METHODS

Patients

A multicenter study including the Southern (Porto Alegre, RS, Brazil), South-Eastern (Belo Horizonte, MG, Brazil), Northern (Manaus, AM, Brazil), North-Eastern (Salvador, BA and Maceio, AL, Brazil), and Central-Western (Goiania, GO, Brazil) regions of Brazil was designed. The recruitment of participants was proportional to the populations of the regions. A common protocol was adopted after approval by the Human Research Ethical Committee at all participant centers, and written consent was obtained prior to entering the study. From November 2012 through March 2015, enrolled patients never previously treated for *H. pylori* infection were submitted to diagnostic endoscopy and tested for *H. pylori* infection due to abdominal symptoms. All participants tested positive for *H. pylori* by a previously validated rapid serological test (Abon Biopharm, Hangzhou, China) performed immediately before the endoscopy[20].

Endoscopy and gastric biopsies

Four gastric biopsies (two from the antrum and two from the corpus) were taken during the endoscopy and were immediately immersed in micro-tubes containing RNAlater® (Ambion, Cat. # AM7020, United States), a solution that promotes immediate RNA stabilization and protection, thereby minimizing the need to immediately process the tissue samples. The samples were kept in a refrigerator at 4 ℃ before they were sent to the central laboratory of the study in Belo Horizonte where they were weighed and then frozen at -80 ℃ until the molecular tests were performed.

Molecular diagnostic technique

The molecular test GenoType HelicoDR was designed to identify the mutations A2146C, A2146G, and A2147G

Table 1 Distribution of patients by sampling region, gender, and age

Brazil - Region	Distribution by gender (% of females)	Distribution by age (average; minimum and maximum)
Northern (n = 36)	22 F; 14 M (61.1%)	41.4 (21-71)
North-Eastern (n = 138)	89 F; 49 M (64.5%)	37.7 (18-75)
Central-Western (n = 26)	13 F; 13 M (50.0%)	41.3 (21-65)
South-Eastern (n = 217)	145 F; 72 M (66.8%)	40.6 (19-76)
Southern (n = 73)	45 F; 28 M (61.6%)	58.0 (23-79)
Total (n = 490)	314 F; 176 M (64.1%)	42.4 (18-79)

F: Female; M: Male.

Table 2 Prevalence of *H. pylori* resistance to clarithromycin and fluoroquinolones in each region

	Northern (n = 36)	North-Eastern (n = 138)	Central-Western (n = 26)	South-Eastern (n = 217)	Southern (n = 73)	P value	Total (n = 490)
Clarithromycin resistance	16.6%	14.5%	19.2%	17.5%	19.1%	0.55	16.9% (95%CI: 13.7%-20.6%)
Fluoroquinolones resistance	2.7%	13.7%	15.4%	13.8%	16.4%	0.06	13.5% (95%CI: 10.6%-16.8%)

in the 23S rRNA gene and N87K, D91N, D91G, and D91Y in the gyrA gene. The test was divided into three steps: DNA extraction from the biopsy samples using the validated QIAmp DNA Mini Kit (Qiagen, Benelux, The Netherlands), multiplex amplification with biotinylated primers, and reverse hybridization using a specific incubator according to the manufacturer's instructions. The hybridization was performed on strips prepared at the Hain Lifescience factory that were coated with different specific oligonucleotides (DNA probes) using DNA strip technology.

Results regarding *H. pylori* detection and susceptibility to clarithromycin and fluoroquinolones were obtained by analyzing the positive and negative bands on the DNA strips. The most frequent mutations involved in resistance to the two antibiotics were also evaluated.

Statistical analysis

Descriptive statistics techniques, including central tendency and variability measures, were employed. The association between resistance/susceptibility to antimicrobials and the gender of the patients was evaluated using Fisher's Exact Test, and the association between resistance and age was evaluate, using Student's t-test. Resistance rates between regions were compared using Fisher's exact test. The odds ratios and 95% confidence intervals (CI) were used as risk estimates, and statistical significance was recognized at $P < 0.05$. The 22.0 version of IBM SPSS Statistics was used for the statistical calculations.

RESULTS

Five hundred nineteen patients were initially recruited for this study. Twenty-nine of them were subsequently excluded due to either the absence of *H. pylori* in the hybridization method (21 cases: 3 from the North-Eastern, 8 from the South-Eastern, 5 from the Northern, and 5 from the Central-Western regions) or the absence of a *gyrA* band (8 cases: 5 from the Central-Western, 1 from the Northern, 1 from the North-Eastern, and 1 from the Southern regions). The distribution of the remaining 490 patients according to gender, region, and age is shown in Table 1.

Clarithromycin and fluoroquinolone resistance were observed in 83 (16.9%; 95%CI: 13.7%-20.6%) and 66 (13.5%; 95%CI: 10.6%-16.8%) patients, respectively. Among the different centers, the rates of resistance ranged from 14.5% to 19.2% for clarithromycin and from 2.7% to 16.4% for fluoroquinolones. The differences were however not statistically significant for either the resistance ratios or *H. pylori* susceptibility (P = 0.55 and P = 0.06, respectively). Table 2 shows the results of general antibiotics resistance in each evaluated region.

More than one hybridization band (characterizing heterogeneous strains) was observed in the genes of 124 patients (25.3%): 11 patients for both the 23S and *gyrA* genes, 61 patients for the 23S gene, and 74 patients for the *gyrA* gene.

Resistance to clarithromycin was statistically significantly higher in women than in men (OR = 2.3, 95%CI: 1.3-4.0, P = 0.003). No statistically significant differences in fluoroquinolones resistance were found in the distribution between genders (P = 0.073).

In relation to age, no statistically significant differences were identified between patients with strains sensitive and resistant to clarithromycin (P = 0.796) and to fluoroquinolones (P = 0.176).

Among the 83 clarithromycin-resistant samples, 74.7% (62/83) exhibited heterogeneity in the 23S rRNA gene, 57.8% (48/83) in the *gyrA* gene, and 13.2% (11/83) in both genes. Among the 66 fluoroquinolone-resistant samples, 69.7% (46/66) exhibited heterogeneity in the *gyrA* gene, 22.7% (15/66) in the 23S rRNA gene, and 10.6% (7/66) in both genes.

Table 3 Distribution of mutations in the 23S gene in each region

Brazil - Region	Northern	North-Eastern	Central-Western	South-Eastern	Southern	Total
MUT1[1]	-	3	2	8	1	14
MUT2[2]	-	1	-	1	1	3
MUT3[3]	6	18	5	32	14	75
MUT2 + MUT3	-	1	-	1	-	2
MUT1 + MUT3	-	1	2	3	-	6
MUT1 + MUT2 + MUT3	-	-	-	-	1	1
Total mutations	6	24	9	45	17	101

[1]Mutation A2146G; [2]Mutation A2146C; [3]Mutation A2147G.

Table 4 Distribution of mutations in the gyrA gene in each region

Region	Northern	North-Eastern	Central-Western	South-Eastern	Southern	Total
Codon 87 mutant						
No ident[1]	-	6	1	10	2	19
Gyr87 MUT[2]	1	4	-	4	2	11
Total	1	10	1	14	4	30
Codon 91 mutant						
No ident[1]	-	2	-	4	2	8
MUT1[3]	-	7	2	9	5	23
MUT2[4]	-	2	-	7	3	12
MUT3[5]	-	1	1	4	3	9
Total		12	3	24	13	52
Multiple mutations in codon 91						
MUT1 + MUT3	-	1	1	-	2	4
MUT1 + MUT2	-	-	-	3	1	4
MUT1 + MUT2 + MUT3		-	-	1		1
Total	-	1	1	4	3	9
Codons 87 + 91 mutants	-	2	-	3	2	7
Total mutations	1	22	4	38	17	82

[1]Mutant codon not specified; [2]Mutation at codon N87K, nucleotide AAA; [3]Mutation at codon D91N, nucleotide AAT; [4]Mutation at codon D91G, nucleotide GGT; [5]Mutation at codon D91Y, nucleotide TAT.

Heteroresistance (wild-type band and mutant band in the same studied codon) was identified in 73.5% (61/83) of the clarithromycin-resistant samples and in 51.5% (34/66) of the fluoroquinolone-resistant samples.

In 37.9% (25/66) of patients whose samples were resistant to fluoroquinolones, mutations that were not specified by the GenoType HelicoDR test were found. There was an absence of both wild-type and mutant bands in codon 87 for 19 patients, as well as in codon 91 for 8 patients. Among these, 2 patients also exhibited the absence of bands in codon 87. After analyzing the above data by sampling region, 3 samples were from the South, 8 were from the North-Eastern, 13 were from the Southeast, and 1 was from the Central-Western. Six patients exhibited both clarithromycin and fluoroquinolone resistance. However, only the *gyrA* gene was involved in the non-specified mutation.

The most common mutation in the 23S rRNA gene was A2147G, present in 90.4% (75/83) of clarithromycin-resistant patients. The second most common mutation in the gene was A2146G, present in 16.9% (14/83) of mutations, and the third most common mutation was A2146C, present in 3.6% (3/83) clarithromycin-resistant patients (Table 3). In 10.8% (9/83) of clarithromycin-resistant samples, more than one mutation in the 23S rRNA gene was found, which might indicate cases of co-infection or two different mutations in the same strain. Among fluoroquinolone-resistant samples, 37.9% (25/66) showed mutations not specified by the GenoType HelicoDR test. The D91N mutation was observed in 34.8% (23/66) of cases, D91G in 18.1% (12/66), N87K in 16.6% (11/66), and D91Y in 13.6% (9/66). In 24.2% (16/66) of the resistant cases, there was more than one mutation involved; 13.6% (9/66) of resistant cases had more than one mutation in codon 91, and 10.6% (7/66) of cases had mutations in both codons 87 and 91 (Table 4).

Resistance to both antimicrobials in the same sample was found in 4.3% (21/490) of the cases. On the other hand, 73.9% (362/490) of the samples did not show resistance to any of the drugs tested.

DISCUSSION

Despite the knowledge accumulated regarding *H. pylori* infection, the therapeutic arsenal remains limited to a few drugs, and the eradication rates of the classic

triple therapy have exhibited a downward trend in the Western world[6,7,21]. Following proper adherence to the treatment, *H. pylori* resistance to antimicrobials is the main factor associated with treatment failure[7]. For this reason, knowing the local profile of *H. pylori* resistance may help in the selection of antimicrobials to optimize treatment for eradication.

Because the phenotypic methods present logistical difficulties for use in routine daily practice, validated and accurate molecular methods have become valuable tools in the evaluation of resistance to antimicrobials[13,21,22]. In this study, we used the easy to perform GenoType HelicoDR test. The equipment for carrying out this test is usually already available in molecular biology laboratories[18]. Comparative studies carried out in different countries, including Brazil, reported agreement above 90% between the phenotypic and genotypic methods[13,19,22-24].

This is the first study conducted in Brazil in which *H. pylori* genotypic resistance to clarithromycin and fluoroquinolones was evaluated in different regions of the country. The rate of resistance to clarithromycin found in our study (16.9%) is high but is still acceptable for the empirical use of clarithromycin-based regimens. The resistance rate to fluoroquinolones (13.5%) is also a concern for its use in empirical second-line regimens for eradicating *H. pylori*. The finding that women had a higher prevalence of primary resistance to clarithromycin in our study corroborates the values reported in other studies[25,26]. There is speculation that cross-resistance caused by previous use of macrolides may be related to the higher *H. pylori* clarithromycin resistance rate among women, as women generally consume more antibiotics than men[27]. Considering the high rates of metronidazole resistance observed in Brazil[28,29] our findings suggest that the association of this agent with clarithromycin or fluoroquinolones could promote reduction in the *H. pylori* eradication rates.

Despite not being the objective of the study, considering the study design, sample size calculation, and convenience samples, the study found no statistically significant differences between the various collection centers regarding the resistance ratios and *H. pylori* susceptibility ($P > 0.05$) to clarithromycin and fluoroquinolones in the huge Brazilian territory. However, specially designed studies for this purpose are still required.

In Brazil, only unicentric studies have been conducted to evaluate the primary resistance to anti-*H. pylori* agents, with varying results. Primary resistance rates to clarithromycin have ranged from 8% to 16% in South-Eastern Brazil using phenotypic methods[28,29]. A study carried out in North-Eastern Brazil reported a primary resistance rate of 16.5% using phenotypic and genotypic methods[23]. The primary resistance rate to fluoroquinolones has ranged from 11% (genotypic method)[30] to 23% (phenotypic method) in South-Eastern Brazil[31]. The differences between the rates found in previous unicentric studies and in the present work may be attributed to differences in sample size, presence of heteroresistance[32], and overall differences in the history of previous antimicrobial consumption in the studied regions[33].

In clarithromycin-resistant samples, the A2147G mutation (90.4%) was prevalent, as was found in previous Brazilian studies[23,28] and in studies in other countries, such as France (83.5%)[13] and Belgium (80%)[19]. Among the 13.5% (66/490) fluoroquinolone-resistant samples studied, 37.9% (25/66) presented a mutant codon that could not be identified by the GenoType HelicoDR method, and the D91N mutation was found in 34.8% (23/66) of samples.

Although there are no molecular studies with information on the distribution profile of *gyrA* gene mutations in Brazil, a recent Colombian study identified the following as major mutations: N87I (47.2%), D91N (30.1%), and N87K (13.2%)[34]. It can be speculated that the 37.9% of fluoroquinolone-resistant samples that were not specified by the method used in this study may represent the N87I mutation, which was predominant in the Colombian study and is not detected in the GenoTtype HelicoDR test. The proportions of the D91N and N87K mutations found in this study are similar to those observed in Colombia. Such findings reinforce the need for studies to investigate the regional variations in the mutation pattern of the *gyrA* gene. In samples with *gyrA* heterogeneity (mixture of wild-type and/or mutant bands), a high resistance rate (43.2%) was found, and it is likely that this polymorphism may represent different stages in the development of mutations[35].

Our study also identified the occurrence of multiple mutations for fluoroquinolones and clarithromycin. We observed multiple mutations for fluoroquinolones in 21.9% of samples (codons 87 and 91 or more than one mutation in codon 91), which are essentially the result of a variety of point mutations in several loci of the *gyrA* gene. The presence of multiple mutations in the 23S rRNA gene for clarithromycin was found in 8.9% of the samples. Similar results were also reported in Belgium, where multiple mutations were found in 25% of samples for fluoroquinolones and 12% for clarithromycin[19]. Finally, simultaneous resistance to both antibiotics was unusual (4.3%) in our study, similar to what was reported in South Africa (2.5%)[22], Hong Kong (3.7%)[36], Italy (1.6%)[37], and Spain (4%)[38].

In conclusion, the mean primary *H. pylori* clarithromycin resistance rate in Brazil is situated at the borderline (15%-20%) for applying the standard triple therapy. The primary fluoroquinolone resistance rate is also of growing concern. The genotypic method used is available, fast, and offers acceptable transportation conditions, and it can be used for continuous surveillance for the proper selection of drugs for anti-*H. pylori* therapy in different regions.

COMMENTS

Background

The therapeutic regimen anti- *Helicobacter pylori* (*H. pylori*) has exhibited a decreased effectiveness in recent years. *H. pylori* resistance to antimicrobial is the main factor associated with treatment failure. It is important for clinicians to know the local prevalences of *H. pylori* resistance to clarithromycin and fluoroquinolones to select the most appropriate *H. pylori* regime in first- and second-line eradication treatments.

Research frontiers

The main mechanism that contributes to *H. pylori* resistance is vertically transmitted point mutations in the DNA. This study assessed the prevalence of primary *H. pylori* resistance to clarithromycin and fluoroquinolones in a large Brazilian population by using the molecular test.

Innovations and breakthroughs

The genotypic method is faster than the phenotypic method and can be performed directly from endoscopic gastric biopsies, making the prior culture of the microorganism unnecessary. The set-up for carrying out this test is usually already available in molecular biology laboratories.

Applications

The rate of resistance to clarithromycin discovered in our study (16.9%) indicates a high, but is still acceptable for the empirical use of clarithromycin-based regimens. Resistance to fluoroquinolone (13.5%) also reveals a concern for its use in empirical second-line regimens in eradicating *H. pylori*. The genotypic method used is achievable, fast, offers acceptable transportation conditions, and can be used as continuous surveillance for the proper selection of drugs for anti-*H. pylori* therapy in different regions.

Peer-review

H. pylori treatment failure is due to resistance of many isolates to antibiotics used for eradication. Due to this it is important to know the local profile of *H. pylori* resistance, which may help to select optimal treatment. The methodology is sound and clear and the manuscript is well written. On the basis of the results this genotyping method used in this study could be recommended for the proper selection for anti-*H. pylori* therapy. This study is valuable from the practical point of view. It increases the knowledge about local *H. pylori* resistance to clarithromycin and fluoroquinolones.

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ORIGINAL ARTICLE

Observational Study

Clinical management of acute liver failure: Results of an international multi-center survey

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Abstract

AIM

To assess the practice of caring for acute liver failure (ALF) patients in varying geographic locations and medical centers.

METHODS

Members of the European Acute Liver Failure Consortium completed an 88-item questionnaire detailing management of ALF. Responses from 22 transplantation centers in 11 countries were analyzed, treating between 300 and 500 ALF cases and performing over 100 liver transplants (LT) for ALF annually. The questions pertained to details of the institution and their clinical activity, standards of care, referral and admission, ward-based care versus intensive care unit (ICU) as well as questions regarding liver transplantation - including criteria, limitations, and perceived performance. Clinical data was also collected from 13 centres over a 3 mo period.

RESULTS

The interval between referral and admission of ALF patients to specialized units was usually less than 24 h and once admitted, treatment was provided by a multidisciplinary team. Principles of care of patients

with ALF were similar among centers, particularly in relation to recognition of severity and care of the more critically ill. Centers exhibited similarities in thresholds for ICU admission and management of severe hepatic encephalopathy. Over 80% of centers administered n-acetyl-cysteine to ICU patients for non-paracetamol-related ALF. There was significant divergence in the use of prophylactic antibiotics and anti-fungals, lactulose, nutritional support and imaging investigations in admitted patients and in the monitoring and treatment of intra-cranial pressure (ICP). ICP monitoring was employed in 12 centers, with the most common indications being papilledema and renal failure. Most patients listed for transplantation underwent surgery within an average waiting time of 1-2 d. Over a period of 3 mo clinical data from 85 ALF patients was collected. Overall patient survival at 90-d was 76%. Thirty six percent of patients underwent emergency LT, with a 90% post transplant survival to hospital discharge, 42% survived with medical management alone.

CONCLUSION

Alongside similarities in principles of care of ALF patients, major areas of divergence were present in key areas of diagnosis, monitoring, treatment and decision to transplant.

Key words: Acute liver failure; Liver transplantation; Intra-cranial pressure; Hepatic encephalopathy

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Core tip: Acute liver failure is rare, but carries high mortality and resource use. Standard of care and clinical practice varies between centers. In a survey conducted among members of the European-Acute-Liver-Failure consortium we have identified similarities in principles of care, including basic clinical management, recognition of severity and care of critically ill patients. Major areas of divergence were pre-intensive care unit (ICU) care and elements of ICU care. Further research is required regarding intra-cranial pressure monitoring and therapy, prophylactic antibiotics and anti-fungals, and liver support systems; we also identified a great need for improving prognostic evaluation for liver transplantation and refinement of transplantation criteria.

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INTRODUCTION

Acute liver failure (ALF) is a rare clinical syndrome resulting in rapid loss of hepatocyte function in a patient without preexisting liver disease[1]. ALF accounts for 5%-7% of liver transplantations annually and its incidence rate is < 10 cases per million population[2]. ALF carries high morbidity and mortality, which often exceeded 90% in the pre-transplant era[3]. The introduction of liver transplantation (LT), along with changing patterns of etiology, has markedly increased short-term survival[4], but the treatment of ALF remains challenging. The rarity of ALF and its unpredictable course make it a difficult entity to study and treat. Only a few small, randomized controlled trials dealing with standard treatment have been performed in patients with ALF. As a result, many interventions continue to be administered on an intuitive basis or are adopted from other critical care settings. Familiarity with current real-life clinical practice is necessary to establish an outline for future therapeutic studies. We performed a survey among the European Acute Liver Failure (EUROALF) consortium members relating to medical ward and intensive care unit (ICU) management of ALF patients, assessing similarities and differences in management of these patients. Our findings show large variations in management among centers and call for urgent standardization of care. Furthermore, these findings identify opportunities for future interventional clinical trials.

MATERIALS AND METHODS

The EUROALF study group is an international consortium of 22 medical centers in 11 countries established in 2010 (For details of the consortium see www.medscinet.net/euroalf). A shared registry of ALF cases was developed, containing over 400 cases to date. ALF cases entered in to the registry are defined as marked hepatic synthetic dysfunction in patients with no previously known liver disease.

In order to survey clinical practices in ALF, an 88-item questionnaire was sent to the EUROALF consortium members (Appendix A). The first set of questions pertained to details of the institution and extent of clinical activity. The following sections referred to standards of care, including issues of referral and admission and ward versus ICU based care (triggers for admission, drug therapy, imaging studies, coagulation studies, renal replacement therapy, nutritional support, utilization of blood products *etc.*). The last section referred to liver transplantation (criteria and limitations, and perceived performance). Requested answers were oriented (different items to choose from), quantitative (a 0-100% scale) or open-ended (choice of words by the participant) (Appendix B).

Data regarding the "real-life" experience of 13 centers in 7 countries treating patients with ALF was collected over a 3 mo period (January- March 2009). All centres have an established programme of LT and ALF management. ALF was classified as being present in patients without clinical, histological or radiological

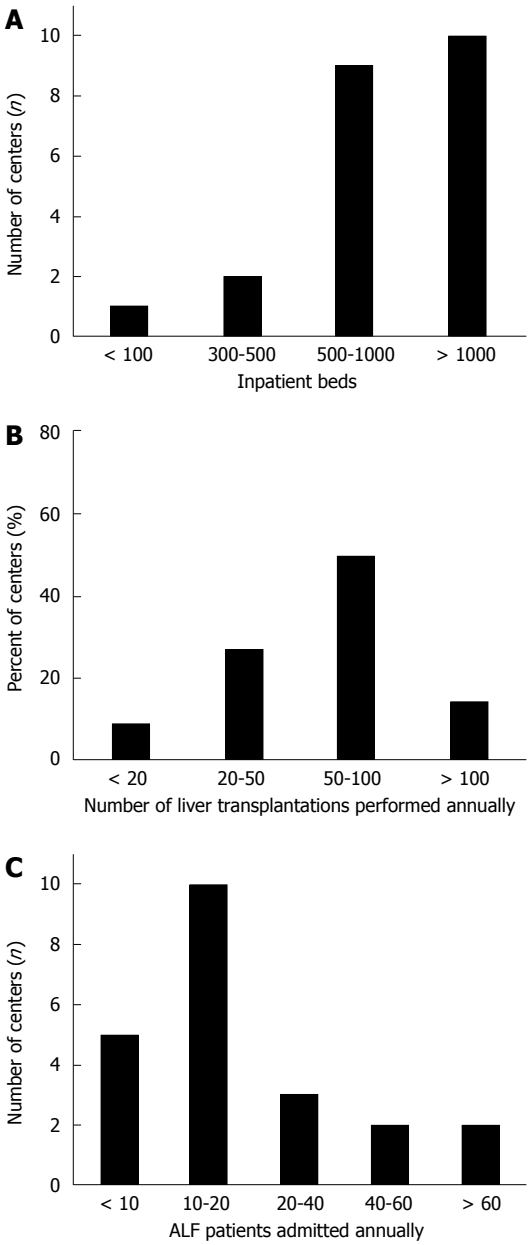

Figure 1 Demographic data. A: Number of inpatients beds per center; B: Number of liver transplantations performed annually per center; C: Number of ALF patients admitted annually per center.

evidence of chronic liver disease, an international normalized ratio (INR) of > 1.5 and any level of hepatic encephalopathy (HE). Data was collected concerning clinical presentation, management and outcome.

Statistical analysis

All data was summarized and reported as the number and percent of centers for each question. Comparison of variables between high- and low-volume centers was done using the Fischer exact test, while the paired comparison between the behaviors on the ward compared to the ICU in each center was done using the non-parametric McNemar analysis. All analyses were two-sided and $P < 0.05$ was considered statistically significant. The SPSS statistical package version 19.0 was used to perform all statistical analyses (SSPS Inc., Chicago, IL, United States).

RESULTS

The responses of 22 centers in 11 countries were analyzed (Appendix A). Nineteen centers (86.4%) had > 500 inpatients beds, with 10 (45.5%) having > 1000 beds (Figure 1A). Eleven centers performed between 50-100 elective and emergency LT annually, and 3 performed > 100 (Figure 1B).

Collectively, an estimated number of 300-500 ALF cases were treated annually. Ten centers (45%) admitted 10-20 patients with ALF annually, while 7 "high volume" centers (32%) admitted 20 to > 60 ALF patients annually. The remaining 23% centers treated < 10 ALF patients annually (with those admitting ≤ 20 cases annually classed as "low volume" centers) (Figure 1C). On average, > 100 LT of ALF patients are performed annually among consortium centers. However, most centers (77%, 17/22) perform < 10 LT, 4 perform 20-40 and only one center performed > 40 LT for ALF annually.

Practices involving referral, hospital placement and jurisdictions

Many patients were referred to the participating centers from other hospitals. The average time from referral to admission was less than one day in 16/22 (73%) of the centers, 1-2 d in 4 centers (18%) and 2-5 d in 2 (9%). In 96% (21/22) and 100% (22/22) of the participating centers, ALF patients in the medical ward or the ICU respectively, were interviewed and examined by a hepatology consultant or senior physician at least daily. Once in the ICU, or high dependency unit (HDU), patients were treated by a multidisciplinary team (consisting of a hepatologist, intensive care specialist and transplant surgeon) in 50% of the centers. In other centers, the ICU patients were managed by an intensive care specialist (32%), a hepatologist (9%) or co-treated by the two (9%).

The level of HE that prompted transfer to an ICU or HDU was grade 1 HE in 33%, grade 2 in most centers (43%), or grade 3 in 10%.

Practices of diagnostic studies

Imaging: Abdominal ultrasound (US) was used as the initial imaging modality in the majority of centers (95% and 91% on the ward or ICU respectively). Utilization of computed tomography (CT) imaging was more variable.

Liver biopsy: LBX was performed in < 25% of patients by the majority of centers. There was no difference in performing LBX between high and low volume centers or centers with high transplantation rates.

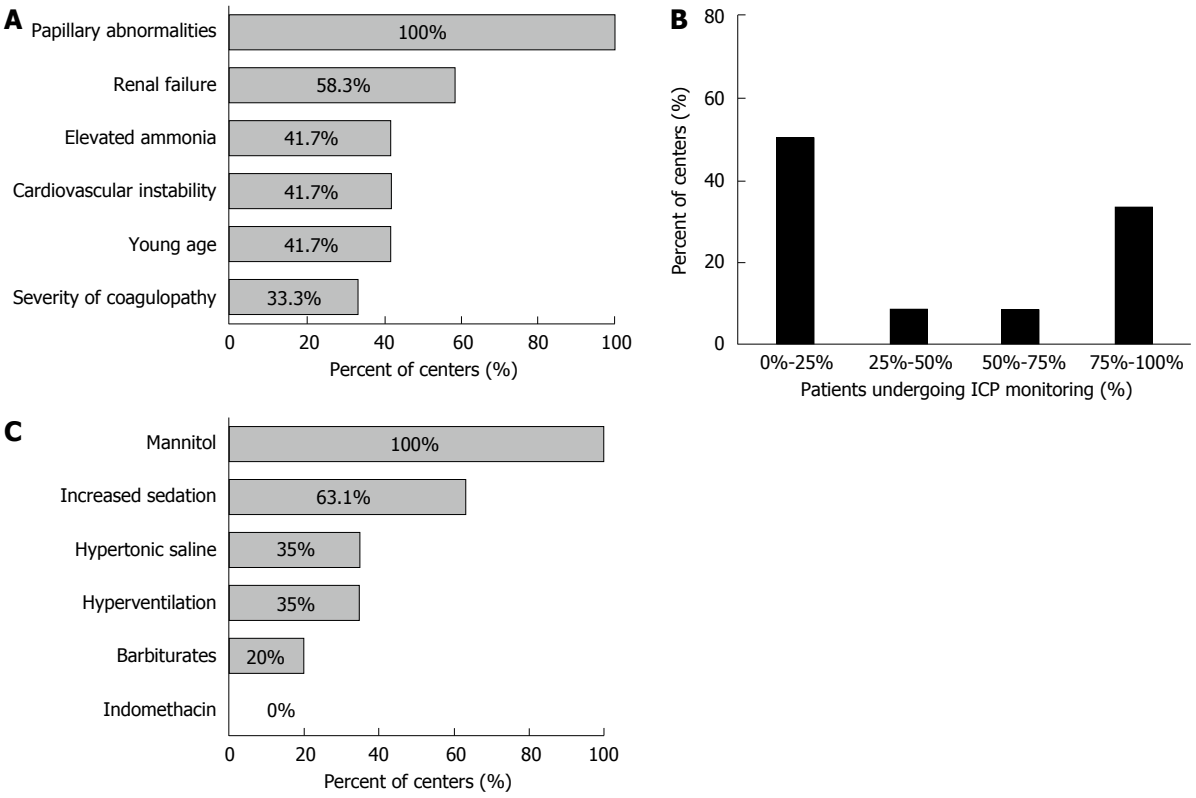

Figure 2 Practice involving intra-cranial pressure monitoring. A: Indications for intra-cranial pressure (ICP) monitoring in ALF patients; B: Percent of patients with HE ⩾ grade 3 undergoing ICP monitoring (among the centers using ICP monitoring); C: First line treatment interventions for raised ICP.

Practices involving patient monitoring

Intra-cranial pressure (ICP) monitoring: ICP monitoring was used in 55% of centers. The rate was higher in high volume centers (6/7, 86%) than low volume centers (6/15, 40%) (P = 0.074). The most common indications for ICP monitoring were papilledema (all centers) and renal failure (58%). Elevated ammonia (42%), cardiovascular instability (42%), young age (42%) and severity of coagulopathy (33%) were less commonly reported indications for ICP monitoring (Figure 2A).

Among the 12 centers reporting the use of ICP monitoring, 50% reported using it in < 25% of the patients with high grade HE, with no difference between high and low volume centers. However, 4 of the 12 centers performed ICP monitoring in > 75% of the patients with high grade HE (Figure 2B).

The ICP pressure that triggered treatment was 20-25 mmHg and 25-30 in 58% (7/12) and 33% (4/12) of centers respectively. Over 90% (11/12) of the centers targeted a specific cerebral perfusion pressure (CPP). The majority (54.5%, 6/11) used a CPP value of 50-60 mmHg, while 27% (3/11) used a CPP of 60-70 mmHg as their target.

Practices involving medical treatment

Antibiotics and Antifungals: Use of routine antibiotics prophylaxis was reported in < 50% of patients by 63% (12/19) of the centers. However, wide discrepancy existed as demonstrated in Figure 3A. High volume centers administered routine antibiotics on the ward significantly less than low volume centers (P = 0.001). Antibiotics were used in < 50% of the patients by 5 of the 7 high volume centers (71%) compared to 58% (7/12) of the low volume centers. Antibiotic use was more common in the ICU; 73% (16/22) of the centers reported that > 75% of the patients received antibiotics. There was no statistically significant difference between high and low volume centers regarding ICU antibiotic use (Figure 3B).

Systemic anti-fungals were given to < 25% of patients in 13/19 centers (68%) while on the ward. In the ICU a slightly higher rate was evident. Twelve centers (55%) administered anti-fungals to < 50% of patients and 10 (45%) treated > 50% of patients. We also observed a greater tendency to treat patients in the ICU with anti-fungals in the high volume centers, 85% (6/7) administering anti-fungals to the majority of their patients while only 27% (4/15) of low volume centers did so (P = 0.016) (Figure 3C and D).

N-acetylcysteine (NAC): Most centers administered NAC to the majority of their patients either on the ward (74%, 14/19) or the ICU (86%, 19/22), with 21% (4/19) and 36% (8/22) of the centers always doing so on the ward and ICU respectively. Seventy four percent (14/19) and 81% (18/22) of the centers administered NAC to patients with non-paracetamol ALF in the ward and ICU respectively.

ICP lowering medications**:** Mannitol (100%) and increased sedation (63%) were used as first-line

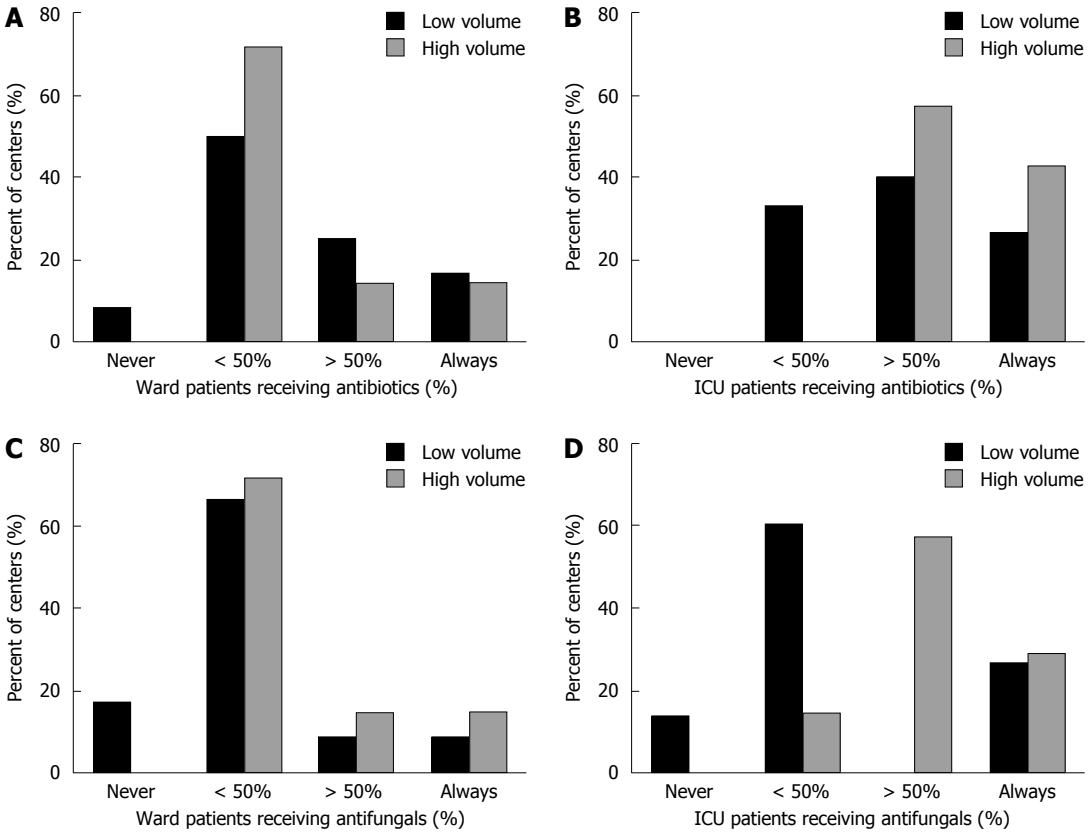

Figure 3 Use of prophylactic antibiotics and antifungals. A: Prophylactic antibiotics in ward based care, low versus high volume centers; B: Prophylactic antibiotics in ICU based care, low versus high volume centers; C: Prophylactic antifungals in ward based care, low versus high volume centers; D: Prophylactic antifungals in ICU based care, low versus high volume centers.

treatment interventions. Other options less commonly used were hypertonic saline (35%), hyperventilation (35%) and barbiturates (20%) (Figure 2C). High volume centers reported greater use of hypertonic saline (86%) compared to low volume centers (31%) (P = 0.057).

Blood products: Fresh frozen plasma (FFP) or platelets were not routinely used in the vast majority of centers (95% 18/19 and 84%, 16/19 respectively). The threshold for platelets administration was < 20 × 10^9/L in 80% (15/19) of the centers. In the centers performing ICP monitoring, 92% (11/12) administered FFP prior to the procedure, 75% (9/12) administered platelets and 42% (5/12) used cryoprecipitate. Use of recombinant activated factor VII was rare (8%, 1/12).

Nutrition support: Feeding modalities differed markedly in the ward and ICU. While patients in the ICU where either mostly (> 50% of patients) or always fed *via* nasogastric tube (NGT) (73%, 16/22 and 23%, 5/22 respectively), only a minority of ward patients were fed by NGT (79%, 15/19 used NGT feeding in < 50% of the patients and 10%, 2/19 never used it). Total parenteral nutrition was used in < 50% of the patients in 77%-79% centers. Branched chain amino acids were rarely used.

Utilization of other treatment modalities: Ventilation and sedation**:** The level of HE that precipitated mechanical ventilation was grade ⩾ 3 in 54% of the centers and ⩾ 2 in 36%. Propofol was the sedative agent of choice in almost all centers, 68% also administered opiates. Only 1 center reported using benzodiazepines. Muscle relaxants were rarely used.

Renal replacement therapy (RRT): Continuous hemofiltration or hemodialysis was the most utilized primary form of RRT (86%, 19/22), as opposed to Intermittent hemodialysis (9%, 2/22). Indications for use of RRT are shown in Figure 4.

Liver transplantation: The King's College criteria (KCC) (86%, 18/21) and Clichy criteria (33%, 7/21) were the most commonly used prognostic models to select patients for LT. The KCC and Clichy criteria were used as a single criteria system by 46% (10/22) and 4.5% (1/22) of the centers respectively. Thirty-eight percent (8/21) of the centers used a combination of the two criteria systems. The KCC were used in all the high volume centers.

There was no age limit for LT in 55% (12/22) centers, with 60 years of age being the limit in the remainder. The average waiting time for transplantation was 1-2 days in 55% (12/22) of the centers, 2-3 days in 27% (6/12) of the centers and > 3 d in 18% (4/22). None of the centers reported surgery occurring within 24 h of patient listing. The predicted survival rate of patients who fulfilled transplantation criteria but

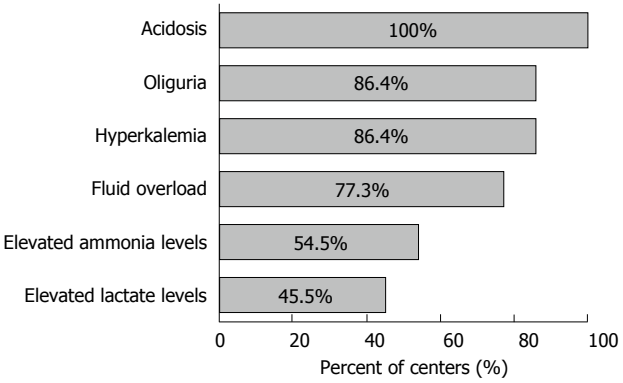

Figure 4 Indications for renal replacement therapy.

were not transplanted was < 50% in all participating centers, with 80% (18/21) reporting non-transplanted survival of < 25%.

Clinical presentation and outcome of ALF patients: A total of 85 patients were treated over a 3 mo period in 13 the centres that participated in our "real-life" data collection. Median age was 38 (29-52) and 54 (64%) were female. The etiologic mix varied by location with paracetamol-induced disease predominating in Denmark, Belgium and the United Kingdom. Overall 43 patients (51%) were diagnosed with paracetamol-induced disease. Other causes included: auto-immune (2 cases), hypoxic hepatitis (6), non-paracetamol drugs (7), viral disease (4), pregnancy-related (2), herbal remedies (1), malignant infiltration (1), Budd-Chiari syndrome (1) and unknown (18).

Severity of Illness: Coagulopathy: Median INR was 3.8 (2.1-6.5) at presentation and peak was 4.7 (2.9-7.3).

Encephalopathy: At presentation HE was mild [median grade 1 (0-2)], however high grade HE (grade 3-4) developed later in the clinical course in 51 (60%) of patients. Of these cases, 12 (24%) developed clinical evidence of ICH. Medical management: Seventy-six patients (89%) were admited to a critical care unit . During the course of illness 59 (69%) patients required intubation and mechanical ventilation, 54 (64%) vasopressor support and 48 (56%) RRT. Of the 30 patients who required one or fewer systemic support, 21 (70%) survived with medical management alone. However 40 of the 55 patients who required more than one organ system support either died or underwent LT and 15 (27%) survived with medical management alone.

Outcome: Overall 90-d survival was 65/85 (76%). Forty-two patients (49%) fulfilled transplantation selection criteria and of these 31 (74%) underwent emergency LT. Post transplant survival to hospital discharge was 28/31 (90%). Of the remaining eleven patients who fulfilled criteria but were not transplanted 7 died and 4 survived. Thirty-two (74%) of the 43 patients who did not fulfil transplant criteria survived with medical management alone. Eight (73%) of the 11 patients who died had hypoxic hepatitis as a cause of ALF and median age was 56 (49-79) years.

DISCUSSION

The results of our survey highlight important aspects regarding patterns of practice of ALF patients across various geographic locations. Whilst there were many similarities in the principles of care, particularly in relation to utilizing prognostic models and basic clinical management of the severely ill, major areas of divergence remain, including many aspects of ward and ICU care. These variations in care stem from a lack of high quality evidence-based data to guide the decision-making process, and uncertainty as to what constitutes best practice.

The data indicates that referral, hospitalization and jurisdiction practices were homogenous among EUROALF members. Almost 75% of the centers reported an average time from referral to admission of < 1 d. Given transportation logistics, space allocation and other bureaucratic barriers, it is unlikely that this time frame can be significantly shortened.

Indications for LBX in the management of patients with ALF are not well defined. Although it has been suggested to be of diagnostic and prognostic value, assisting clinical decision-making and timing of LT[5,6], in our survey it was not generally considered a requirement in clinical practice and was performed only in a small minority of patients.

While diagnostic procedures were relatively uniform in the consortium, management protocols varied more widely. NAC is routinely used in paracetamol induced hepatotoxicity[7,8]. Most centers administered NAC to the majority of patients early in the course of illness as part of ward and especially ICU based care. This is inspite of recent data suggesting that NAC is ineffective in critically ill patients[9], over 80% of the responders also routinely administered NAC to non-paracetamol ALF, in concordance with the findings of a recent RCT[10].

Patients with ALF are highly susceptible to infections. Bacterial infections have been documented in up to 80% of patients and fungal infections occur in a third[11]. Though early studies showed that prophylactic antimicrobial therapy decreased infections, a survival benefit was not demonstrated[12]. Currently, the use of prophylactic antibiotics and anti-fungals in patients with ALF is not generally recommended and instead, periodic surveillance for infection is advocated[13,14]. Whilst most centers did not routinely administer antibiotics to ALF patients in the wards, almost 75% of centers treated the majority of patients in the ICU (Figure 3B). Administration of anti-fungals in ward based care of ALF patients was limited, but marked

variation was found regarding their use in the ICU (Figure 3D). This divergence in care suggests that the prophylactic anti-infectious treatment is an area of uncertainty for treating clinicians.

Coagulopathy and thrombocytopenia are frequently seen with ALF[15]. The most frequently used coagulation parameter was the INR test followed by PT and fibrinogen levels. Interestingly, only 52% of the centers measured Factor V levels, as a means of assessing liver function and prediction of patient outcome[16]. Finally, the thromboelastogram (TEG) test, which assesses overall homeostasis[17], was rarely used.

In the absence of bleeding, correction of INR or thrombocytopenia is not justified and may obscure the use of INR as a prognostic marker[13]. Our results show that most centers did not routinely administer coagulation factors or platelets.

The issue of bleeding subsequent to invasive procedures constitutes a more significant dilemma. In clinical practice, the risk of bleeding following routine procedures such as insertion of central venous catheters or paracentesis and even for more invasive procedures such as trans-jugular LBX is considered small[5,18]. In contrast, the risk of intracranial hemorrhage following ICP monitor insertion is a major cause of concern in ALF patients with an incidence of fatal hemorrhage ranging from 1%-5%[19,20]. No guidelines exist regarding administration of coagulation factors prior to specific procedures in the presence of coagulopathy[13,14]. In our questionnaire, we addressed utilization of coagulation factors in general and specifically with regards to ICP monitor insertion. Platelets and FFP were the most common factors given in the majority of centers prior to performing LBX and inserting ICP monitors. Cryoprecipitate was used by < 50% of the centers while the use of recombinant activated factor VII was rare.

The role of HE as a prognostic marker was reflected in the decision to transfer patients from the medical ward to the ICU. Grade 2 HE served as the most commonly accepted indication for ICU admission. However, over a third of the participants admitted to the ICU patients with HE grade 1 or did so regardless of the patients' cognitive state. Most centers intubated patients with grade 3 HE and over a third reported intubating at grade 2 HE. These practices indicate that in clinical practice physicians prefer to treat patients earlier, perhaps recognizing the potential for abrupt deterioration. This approach is supported by our "real-life" results showing that although most patients presented with minor HE, 60% developed high grade HE later in the clinical course.

ICP monitoring use varied by center with only approximately 50% of the participating centers reported using it. The overall proportion of patients with HE of grade 3 or above that underwent ICP monitoring was low, even in centers that performed this procedure often. However, there was marked divergence, with a few centers reporting extensive use of this modality (Figure 2B). These centers were all considered high volume, admitting > 20 ALF patients annually and performing LT in > 50% of their patients. Our results reflect uncertainty regarding the specific indications and benefit of ICP monitoring (Figure 2A).

Mannitol is widely accepted as first-line therapy to decrease intracranial hypertension, followed by hypertonic saline and moderate hypothermia, but their use is supported only by limited evidence and doesn't appear to improve survival[13,14,21-24]. Barbiturates, Indomethacin and hyperventilation are considered short-term salvage therapies in refractory cases[13,14,25]. All centers reported using all these modalities (Figure 2C) without significant difference in treatment choices between centers that did or did not perform ICP monitoring.

Prognostic models to assess allocation to LT in ALF include the KCC[26], Clichy-Villejuif criteria[27,28], model for end stage liver disease, and the new Acute Liver Failure Study Group index[29]. All of which show good specificity but more limited sensitivity and accuracy[29-33]. We found that KCC criteria were the most commonly used, followed by the Clichy criteria, and were occasionally used in combination. The need for better prognostic models and biomarkers to accurately define indications for LT, were raised by many survey participants.

Most patients listed for LT in our survey underwent surgery. These transplantation rates appear to coincide with the current literature as 85% of the centers reported performing transplantation in > 50% of the patients; and 43% reported that over 75% of the patients underwent LT. The predicted survival rate of patients who fulfilled transplantation criteria but did not undergo transplantation was < 50% in all participating centers, with the majority of centers predicting a survival rate of < 25%.

"Real-life" data from our survey, showed 76% overall 90-day survival (65/85 patients). Although limited by a relatively small number of patients this figure demonstrates the improved survival of ALF patients over the years, as this number is higher than that previously reported in the United States and England[4,34]. Almost half of the patients fulfilled the criteria for emergency LT, 74% of them underwent LT. LT in this high risk group was associated with 90% survival. The death rate among the patients who fulfilled LT criteria but were not transplanted (63%) was considerably higher compared with those who were not LT candidates (25%) treated with medical management alone.

Although we did not directly address the role of liver-assist devices in our survey, many centers raised this issue as one deserving further investigation and definition, both as a potential bridge for LT and as a means of providing vital support in hope of functional recovery.

Our study is a survey addressing the clinical practice and management of ALF patients. One of the

limitations of our study is the number of participating centers. Although treating a large group of patients, standards of treatment may not be representative of all treatment centers in Europe. As a survey, the results represent the perceived views of the participants and are not backed by evidence. However, all the participants are senior hepatology consultants, and we believe that their answers portray current practices in their respective centers. Furthermore, this is an inherent premise of any survey. Furthermore, 13 centers reported "real-life" data, which provides validation to the questionnaire.

Over the past decades the outcomes of patients with ALF have improved considerably. However, it still remains a disease with high mortality. Management of ALF is challenging not only because of its severity and rapid progression but also due to the many uncertainties accompanying current clinical practice. Whilst many similarities were found in principles of care of patients with ALF across the centers participating in our study, there are still major areas of divergence, representing a need for further studies. Areas for potential research include use of ICP monitors and ICP therapy, prophylactic use of antibiotics and anti-fungals, as well as further investigation into the role of liver support systems and establishing an ALF care bundle. There is also a great need for improving prognostic evaluation for LT and for the refinement of transplantation criteria.

COMMENTS

Background

Acute liver failure (ALF) is a rare condition with high mortality and resource use, but limited evidence to support clinical practice. Little is known as to what constitutes standard of care and how practice varies between centers.

Research frontiers

The rarity of ALF and its unpredictable course make it a difficult entity to study and treat. Only a few studies dealing with standard treatment have been performed and therefore many interventions continue to be administered on an intuitive basis or are adopted from other clinical settings. Familiarity with current real-life clinical practice is necessary to establish an outline for clinical management and future therapeutic studies.

Innovations and breakthroughs

This study examined the practice of caring for ALF patients in varying geographic locations and medical centers, pointing out the similarities alongside major variations in clinical management.

Applications

The authors findings identify major areas of disagreement and opportunities for future interventional clinical trials.

Terminology

ALF is the sudden onset of severe liver cell dysfunction, in a patient without previously known liver disease, leading to coagulopathy and hepatic encephalopathy.

Peer-review

This study provides data regarding clinical practice of caring for ALF patients in various centres. It is of interest for other clinicians treating liver patients as well as researchers in this field.

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ORIGINAL ARTICLE

Prospective Study

Dried blood spots, valid screening for viral hepatitis and human immunodeficiency virus in real-life

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Author contributions: Mössner BK, Lillevang ST, Christensen PB and Holm DK designed the study; Mössner BK, Staugaard B, Jensen J and Christensen PB performed the experiments; Lillevang ST and Holm DK contributed new analytic tools/methods; Mössner BK analyzed the data; and Mössner BK, Christensen PB and Holm DK wrote the paper; all authors revised the final manuscript.

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Abstract

AIM

To detect chronic hepatitis B (CHB), chronic hepatitis C (CHC) and human immunodeficiency virus (HIV) infections in dried blood spot (DBS) and compare these samples to venous blood sampling in real-life.

METHODS

We included prospective patients with known viral infections from drug treatment centers, a prison and outpatient clinics and included blood donors as negative controls. Five drops of finger capillary blood were spotted on filter paper, and a venous blood sample was obtained. The samples were analyzed for HBsAg, anti-HBc, anti-HBs, anti-HCV, and anti-HIV levels as well as subjected to a combined nucleic acid test (NAT) for HBV DNA, HCV RNA and HIV RNA.

RESULTS
Samples from 404 subjects were screened (85 CHB, 116 CHC, 114 HIV and 99 blood donors). DBS had a sensitivity of > 96% and a specificity of > 98% for the detection of all three infections. NAT testing did not improve sensitivity, but correctly classified 95% of the anti-HCV-positive patients with chronic and past infections. Anti-HBc and anti-HBS showed low sensitivity in DBS (68% and 42%).

CONCLUSION
DBS sampling, combined with an automated analysis system, is a feasible screening method to diagnose chronic viral hepatitis and HIV infections outside of the health care system.

Key words: Dried blood spot; Real-life; Screening; Hepatitis B; Hepatitis C; Human immunodeficiency virus; People who inject drugs; Drug-users; Prisoners

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Core tip: This study shows that it is feasible to combine serology and nucleic acid screening for hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency virus (HIV) infections in one dried blood sample (DBS) collected in real life and analyzed using a modern laboratory platform. We observed a sensitivity and specificity of > 96% for HBV, HCV, and HIV and correctly classified 95% of all HCV patients into past *vs* chronic infections compared to simultaneously collected venous blood samples. The study confirms that DBSs are feasible samples in outreach clinics and confirms the high sensitivity and specificity of previous laboratory-based studies.

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INTRODUCTION

Chronic viral hepatitis B (CHB) and C (CHC) represent a major health burden worldwide, with approximately 350 million and 170 million people infected, respectively[1]. Persistent infection may progress to end-stage liver disease and hepatocellular carcinoma, and primary liver cancer is now the second leading cause of cancer death worldwide[2].

In Denmark, as in most western countries, drug users have the highest prevalence of chronic hepatitis C[3,4]. Still, a Danish register-based study showed that the majority of drug users(67%) have never been tested[5].

The existing literature indicates a wide range of barriers for testing that are related to both the individual patient (*e.g.*, lack of knowledge, not feeling sick, fear of invasive tests, and distrust of the medical system) and the healthcare provider system (*e.g.*, no access to basic medical care or a lack of specific medical care in substance user treatment centers)[6-11].

In Denmark, chronic Hepatitis B and HIV infections are highly prevalent among men who have sex with men, sex workers and immigrants from highly endemic countries. These groups also have difficulties accessing the existing health system and may have poor knowledge of the diseases and risk factors[12].

A recent Danish study has shown that the large majority of HIV patients are successfully managed in the existing health system; however, new interventions are needed to improve care for immigrants and drug users[13].

Easy access to serological testing for Hepatitis C and non-invasive methods to diagnose liver fibrosis have improved the screening rates among drug users. In the era of the new treatment regimens for hepatitis C with direct-acting antivirals (DAA) with few side effects and a short treatment duration, it has now become feasible to test and treat the drug-user population[14-16].

Dried blood samples on filter paper (DBS) were originally used to screen for neonatal phenylketonuria[17]. Since then, the method has been evaluated and used with increased frequency, *e.g.*, antibody testing for a wide range of infectious diseases, particularly in resource-limited settings[18-21].

Recently, it was shown that DBS can be combined with automatic analysis on a modern analysis platform (*e.g.*, Abbott Architect), allowing high throughput testing at a reasonable price[22,23]. However, in most of these studies, venous whole blood was used for the DBS samples instead of capillary blood, and the performance and feasibility of "real life" DBS testing in this set-up is, to our knowledge, not well described.

Objectives

The objective of this study was to determine and compare the sensitivity of DBS from capillary blood to whole plasma when the DBS sampling was obtained in real life settings and analyzed at a modern high throughput diagnostic laboratory using an automated analytic platform.

The study was conducted from September 2014 to September 2015. Combined NAT screening for HIV RNA, HBV DNA and HCV RNA was performed using the Procleix Ultrio Elite assay from Grifols (Grifols Diagnostic Solutions, Allschwil, Switzerland), and serological screening for HIV, HBV and HCV was performed using Abbott Architect assays (Abbott Diagnostics, Delkenheim, Germany) .

The study was conducted in the region of southern Denmark.

Patients had a known HBV, HCV or HIV infection, and attended either the Outreach Clinics in the Drug Treatment Center or state prison in Nyborg or the Outpatient Clinic at the Hospital to be eligible for inclusion. Patients were informed about the study at previously planned visits. If they were willing to participate, they were included with due consideration time.

Due to the relatively low prevalence of HIV and HBV infections among Danish drug users and prisoners, paired EDTA plasma samples/DBS were obtained from these patients at the Departments of Infectious Diseases at, Odense University Hospital and Lillebaelt Hospital, Kolding. Samples from Hepatitis C-infected patients were obtained from drug treatment centers and the prison. According to the power estimation, optimally, 100 patients with each infection should be included, corresponding to an overall 30% prevalence of the tested markers, which is comparable to the 30%-40% prevalence of HCV infection in the target drug-using population. In addition, paired EDTA plasma samples/DBS were obtained from 99 blood donors who were negative for all three viruses from the blood bank at Odense University Hospital.

The DBS and EDTA plasma samples were collected simultaneously in most cases, but up to 10 d was allowed between the two samples.

MATERIALS AND METHODS

All samples were analyzed at the Department of Clinical Immunology, Odense University Hospital. Five spots on a Whatman® 903 protein saver card (Sigma-Aldrich, Copenhagen, Denmark) were each covered with a large drop of capillary blood (approximately 75 μL) from the fingertip(s) of the patient/donor and subsequently dried at room temperature for at least 24 h and up to 5 d to prepare the DBS.

The approximate volume of 75 μL of whole blood in each dried blood spot was based on preliminary studies where different amounts of exact volumes of whole blood were added to the Whatman® 903 protein saver card. We found that 75 μL was the amount needed to fill a single spot on the Whatman filter paper. In the real-life study, the intention was to fill each of the 5 spots using their visual appearance, thereby approximating a volume of 75 microliters in each spot.

We compared the test results for all viruses in DBS samples that had dried for 24 h or for up to 7 d at room temperature using the NAT test to validate the stability and found no difference (data not shown). Each of the dried blood spots was eluted with 1000 μL of a buffer (PBS/0.05% Tween 20/0.08% sodium azide) overnight at room temperature on a shaker, followed by centrifugation of the eluate for two minutes at 10000 rpm. Except for the discriminatory NAT analysis, all laboratory analyses were performed immediately after centrifugation of the eluates. The average estimated DBS sample dilution was 1:23 compared to plasma.

The DBS eluates and the corresponding plasma samples were tested for the presence of anti-HIV, anti-HCV, hepatitis B surface antigen, anti-HBc and anti-HBs with the Architect HIV Ab/Ag, Architect HCV Ab, Architect HBsAg, Architect HBs Ab and Architect HBc Ab assays using the Architect system (Abbott Diagnostics, Delkenheim, Germany). In addition, all samples were tested with the Procleix Ultrio Elite assay using the Procleix Panther System (Grifols Diagnostic Solutions, Allschwil, Switzerland). The Ultrio Elite assay is a qualitative nucleic acid amplification test (NAT) for the simultaneous detection of HIV-1/2 RNA, HCV RNA and HBV DNA. The detection limits (at the 95% confidence level) for the Ultrio Elite assay are 18.0 IU/mL (HIV-1-RNA), 3.0 IU/mL (HCV-RNA) and 4.3 IU/mL (HBV-DNA), according to the package insert. For samples that were reactive in the initial NAT analysis, an additional discriminatory NAT was performed for HIV-1/2 RNA (Ultrio Elite dHIV), HCV RNA (Ultrio Elite dHCV), and HBV DNA (Ultrio Elite dHBV). The initially reactive samples were stored at -20 °C for 1-5 d before the discriminatory NAT was performed. All plasma samples were analyzed according to the manufacturer's instructions.

A quantitative nucleic acid amplification test was performed using the COBAS® AmpliPrep/COBAS® TaqMan® 48 system (Roche Diagnostics, Basel, Switzerland) to quantify the viral load in plasma samples that were positive for either HIV RNA, HCV RNA or HBV DNA in the Ultrio Elite assay. The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0, COBAS® AmpliPrep/COBAS® TaqMan® HCV Quantitative Test, v2.0 and COBAS® AmpliPrep/COBAS® TaqMan® HBV Test, v2.0 assays were used, and all the quantitative analyses were performed according to the manufacturer's instructions. The lower detection limit for the Cobas TaqMan assays described above was slightly higher than the NAT according to the package insert: 20 copies/mL (HIV), 15 IU/mL (HCV) and 20 IU/mL (HBV).

Statistical analysis

The DBS results were compared with plasma, which was used as the gold standard to assess the sensitivity, specificity, positive predictive value, negative predictive value and receiver operating characteristics curve (ROC-curve) of the DBS results. Descriptive statistics were shown as the means, medians and interquartile ranges, with the minimum and maximum values reported, as appropriate. A confidence interval of 95% was used. All data analysis was performed using STATA 14 IC software (Statacorp LP, College Station, TX).

Sample size and power estimation: With an expected prevalence of 33% (100 positive and 200 negative tests for all markers, except the HBs antibody),

Table 1 Descriptive summary and characteristics of viral infections

Viral infection	Total, *n*	Mono- + Co-infected, *n*	N with quantitative analysis	Median	IQR (25-75)
Blood donors	99	0	NA		
HBV	85	78 + 5 HIV + 2 HCV	78	144	19-2780
HCV	116	111 + 3 HIV + 2 HBV	105	225000	4300-1110000
HIV	114	106 + 5 HBV + 3 HCV	112	0	0-19

IQR: Interquartile range [expressed as IU/mL (HBV and HCV) and copies/mL (HIV)]. HBV: Hepatitis B virus; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus.

Table 2 Sensitivity and specificity of serological testing by Abbott Architect of dried blood spots compared to plasma samples

	Plasma, *n*		DBS, *n*		Sensitivity (%) (95%CI)		Specificity (%) (95%CI)		ROC	PPV	NPV
	Negative	Positive	Negative	Positive							
Anti-HCV	288	116	292	112	96.6	(91.4; 99.1)	100.0	(98.7; 100)	0.98	100.0%	98.6%
HBsAg	318	86	319	85	96.5	(90.1; 99.3)	99.4	(97.7; 99.9)	0.98	97.6%	99.1%
Anti-HBc	232	172	286	118	68.0	(60.5; 74.9)	99.6	(97.6; 100)	0.84	99.2%	80.8%
Anti-HBs	246	158	337	67	42.4	(34.6; 50.5)	100.0	(98.5; 100)	0.71	100.0%	73.0%
Anti-HIV	289	115	290	114	98.3	(93.9; 99.8)	99.7	(98.1; 100)	0.99	99.1%	99.3%

ROC: Area under the receiver operating curve; PPV: Positive predicting value; NPV: Negative predicting value.

an observed agreement of 95% between DBS and plasma samples would correspond to 5 false negative and 10 false positive results for each marker. More than 12 false negative and 20 false positive samples would lead to a rejection of the 95% agreement between the venous blood sample and DBS.

Ethical considerations: All patients signed written informed consent before inclusion in the study. The Danish Research Ethics Committee approved the study (Project-ID: S-20140128).

RESULTS

Corresponding plasma samples and DBS were obtained from the 404 subjects included in the study: 114 HIV-infected, 85 hepatitis B-infected, 116 hepatitis C-infected and 99 blood donors. The blood donors were all negative for the 3 infections (Table 1).

The overall performance of the serology markers for anti-HCV, anti-HIV and HBsAg had a sensitivity of > 96% and a specificity >99% for all three markers. In contrast, a lower sensitivity for anti-HBc and anti-HBs was observed (Table 2).

After combining all analyses, we correctly classified 98.0% (396/404) of patients for all 3 infections and 99.3% of all serological tests (1204/1212) using DBS, with 6 false negative (4 anti-HCV, 1 HBsAg and 1 anti-HIV) and 2 false positive (HBsAg) results. One patient with hepatitis B was HBsAg-negative in both plasma and DBS. Thus, the addition of NAT identified 1 (HBV) patient who would have been missed by serological testing alone. However, the overall advantage of adding NAT to the analysis was that 95% of Hepatitis C patients were correctly classified into ongoing *vs* past infection groups. For HBV and HIV, the classification of viremia by DBS was not reliable because a significant proportion (16% and 98%) of these patients had very low viral loads as result of antiviral treatment.

Hepatitis C

Among the 116 known HCV-infected patients, all were plasma anti-HCV-positive and 112 (96.6%) were also DBS anti-HCV-positive (Figure 1). The four DBS-negative patients were all HCV-RNA-negative in both plasma and DBS, indicating a past infection. Among the plasma anti-HCV-positive patients, 73.3% (85/116) were plasma NAT-positive, indicating an ongoing infection. Of these patients, 95.3% (81/85) were also DBS NAT-positive, and 100% (85/85) of the active infections were anti-HCV-positive in the DBS.

The four patients who were plasma HCV RNA-positive and DBS-negative had a viral load < 100 IU/mL (range 0-95), and two of these were on DAA treatment. One patient was plasma NAT-negative, but weakly DBS-positive, corresponding to a specificity of 99.5% (193 DBS/194 plasma) for the NAT.

Hepatitis B

Among the 85 known HBV-infected patients, 83 were both plasma- and DBS-positive for HBsAg, one was only positive in plasma and one was negative in both plasma and DBS (Figure 2). The patient who was HBsAg-positive in plasma and negative in DBS had a quantitative plasma HBsAg level below 175 IU/mL. This patient was under treatment for HBV infection and HBV DNA was positive in both plasma and DBS. The patient who was HBsAg-negative in both plasma and DBS was also under treatment for HBV. HBV DNA was positive in plasma, but below the LLOQ, anti-HBc and anti-HBs were positive in both plasma and DBS, suggesting seroconversion. All 71 untreated CHB

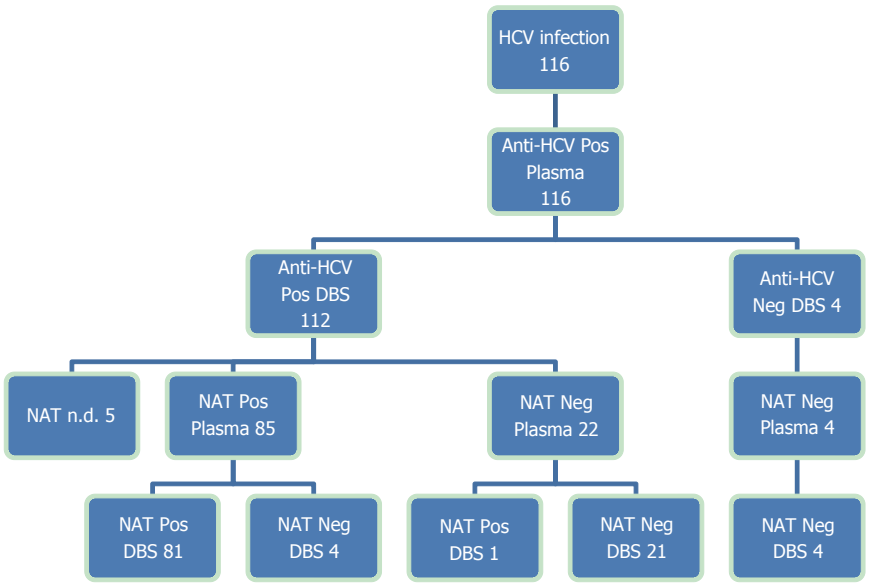

Figure 1 Flowchart for the test of hepatitis C patients.

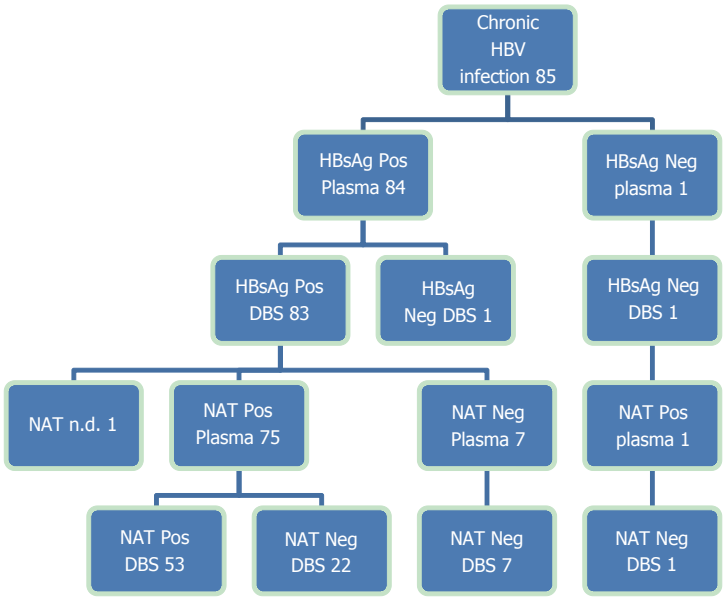

Figure 2 Flowchart for the test of hepatitis B patients.

patients were HBsAg-positive by DBS.

Two participants without known HBV infections were (weakly) positive for HBsAg in DBS and negative in plasma; one was an HCV-infected patient and one was a blood donor. Both were anti-HBs-positive in plasma and NAT-negative in both plasma and DBS. A duplicate retest of the two DBS samples showed that they were HBsAg-negative; thus, we concluded that the first DBS test was a false positive test.

A low sensitivity for anti-HBc (68%) and anti-HBs (42%) was observed. In 2/85 CHB patients, discrepant results for anti-HBc were observed, with positive plasma and negative DBS (CMIA-plasma 5.2-6.8 *vs* CMIA-DBS 0.3-0.4); both were HBsAg-positive and HBV DNA-negative.

Among the 91 anti-HBc-positive plasma samples from non-HBV infected patients, discrepant results were found in 53 samples in which the plasma was positive (CMIA median 6.19, range 1.1-10.1) and DBS was negative (CMIA median 0.295, range 0.06-0.99).

Among the 53 discrepant DBS-negative results, one was co-infected with HIV/ HCV, 31 were anti-HIV-positive, 19 were anti-HCV-positive, and 2 were negative for all other serological markers (blood donors).

In twelve out of 85 CHB patients, a discrepant result for the anti-HBs analysis was obtained. Anti-HBs was positive in plasma (1.08-9.44 mIU/mL), but negative in

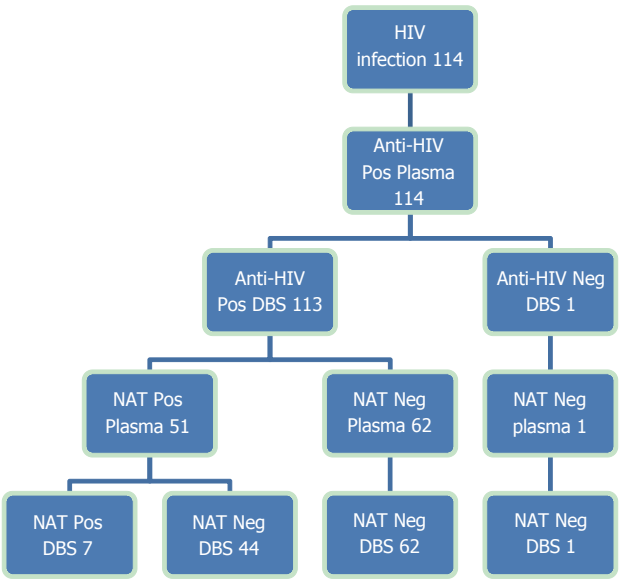

Figure 3 Flowchart for the test of human immunodeficiency virus patients. HIV: Human immunodeficiency virus; NAT: Nucleic acid test.

the DBS (0-0.3 mIU/mL). However, all twelve patients were HBsAg-positive in both plasma and DBS.

Among the 145 anti-HBs-positive plasma samples in non-HBV infected patients, discrepant results were observed in 79 patients in which the plasma was positive (median 9.9 mIU/mL, range 1-75) and the DBS was negative (median 0.01 mIU/mL, range 0-0.93). Among the 79 discrepant DBS-negative results, one was co-infected with HIV/ HCV, 43 were anti-HCV-positive, 17 were anti-HIV-positive, and 18 were negative for all other serological markers (blood donors). Vaccination status for Hepatitis B was not recorded in our study, but of the HBV-negative patients, 106 had protective anti-HBs titers (anti-HBs $\geqslant$ 10 mIU/mL) in plasma compared to 28 in the DBS.

HIV

Among the 114 patients with known HIV infections, all were anti-HIV-positive in plasma, and 113 (99.1%) were also positive in the DBS (Figure 3). The one HIV-positive patient who was not detected using the DBS received antiretroviral treatment (ART) and had undetectable levels of the HIV-RNA in both plasma and DBS.

Furthermore, one blood donor was weakly anti-HIV-positive in plasma (1.62/cut-off 1.0), but negative in the anti-HIV confirmatory test. The donor was also DBS anti-HIV-negative and NAT-negative in both plasma and DBS.

Among the 114 anti-HIV-positive patients, 112 were undergoing antiretroviral treatment. HIV-RNA was negative in both plasma and DBS from 54.9% of patients (62/113), 6.2% (7/113) were positive in both tests, and 38.9% (44/113) were HIV-RNA-positive in plasma and negative in DBS. Among these patients, 43 received ART and had the following HIV-RNA levels: 20 patients were below the LLD (lower limit of detection), 15 were below the LLOQ (lower limit of quantification) and 8 patients had quantifiable HIV-RNA levels (range 32-483 copies/mL).

DISCUSSION

The use of DBS with a high throughput automated platform to screen for viral Hepatitis B, C and HIV showed acceptable agreement between the plasma and DBS tests for anti-HCV, anti-HIV and HBsAg in real-life settings. However, the overall sensitivity of anti-HBc and anti-HBs was low. There are several possible explanations for this observation, including the amount of blood used for the DBS samples was small and the addition of the eluate-buffer further diluted the sample 23-fold. In a study by Lee *et al*[24], DBS was prepared from venous blood samples, and they noted that the assay titers obtained from the DBS samples were generally lower than the titers obtained from the plasma samples. The authors suggested that different cut-off values should be used to validate tests that were positive in the DBS because the small amount of blood in the DBS led to lower assay titers.

The low sensitivity for the serological markers anti-HBs and anti-HBc in DBS *vs* plasma observed in our study is in contrast to recent studies using automated platforms[22,23]. This observation may to some extent be caused by the different starting titers of the samples, resulting in different dilution factors by elution. The results are consistent with other studies, where low DBS sensitivities have been reported, ranging between 70% and 100%, with specificities from 94% to 100%[18,25,26]. In the study by Ross, almost perfect sensitivities and specificities for DBS were observed compared to serum samples; however, DBS were prepared by applying 100 μL of whole blood to generate DBS (Whatman filter paper), a procedure that most likely is not possible with DBS in real-world settings, where we estimated the average amount to be 75 μL. However, with this standardized method, allowances could be made for the dilution and a lower cut-off calculated for anti-HBs in DBS samples. When applying this cut-off, the sensitivity rose from 43% to 54% in our study, which was still not acceptable (data not shown).

We speculate that if an indicator for the amount of blood collected on the paper could be developed to ensure that enough blood is present for the analysis (*e.g.*, weight, hemoglobin, visual guidance, *etc.*), it would also allow the calculation of a quantitative anti-HBs level/mL of serum from DBS, enabling the method to be used in outreach vaccination trials.

A study by Villar showed that a lower sampling volume led to more discordant results and emphasized that even though the sampling is relatively easy, care must be taken to ensure an adequate sample size[27]. We recommend that the visual inspection should be used to confirm that the circles of the filter paper have

been appropriately filled with blood when using DBS for routine screening.

In our study population, only moderate agreement was observed for HBV DNA and HIV RNA by NAT, probably because a significant number of the included patients had very low viral loads as a result of antiviral therapy. Several studies have shown decreased sensitivity of DBS compared to plasma in samples with lower viral loads[20,28,29]. In our study, we estimated that the LLD was below 500 copies/mL for HIV-RNA, 100 IU/mL for HCV-RNA and 200 IU/mL for HBV-DNA by DBS testing. This result may be partially explained by the 23-fold dilution of the DBS compared to plasma during preparation. However, this dilution is unlikely to be a significant problem when using DBS as a screening method in an untreated population. In this setting, viral loads below DBS cut-offs are rare, and all these patients will be positive by serology testing.

Our study has several limitations. As it is a real-life study, we did not know the volume of blood applied to the DBS filter paper (and we did not record whether the filter circles were appropriately filled with blood). Therefore, we cannot rule out the possibility that the discrepant results were due to a low sampling volume. Furthermore, due to the low prevalence of HIV and HBV in our target population, a significant number of the HBV- and HIV-infected participants were recruited from the Department of Infectious Diseases and had low or undetectable viral loads as a result of antiviral treatment. However, as a screening tool, DBS will be used among treatment naïve patients, and in this population, DBS had 100% sensitivity, except for HBV patients in the latent phase, who will be detected by HBsAg.

As for the 4 HCV patients who were anti-HCV-positive but HCV RNA false negative in DBS, we suggest that patients who were categorized as having past HCV infection using DBS should have this result confirmed using a venous blood sample.

As the NAT did not improve DBS sensitivity, it would have been more cost-effective to perform DBS testing as a two-step analysis: first screening with anti-HCV, anti-HIV and HBsAg, and then performing a NAT of samples that were positive in serology. Depending on the prevalence of infection, this strategy could save 50%-95% of NATs and still identify > 95% of chronic infections. Some primary advantages of DBS are that screening of all three infections can be performed in one sample; the samples can be collected by on-site personnel, ensuring correct person identification; and the samples may be sent by regular mail to the laboratory. These advantages enable "on-site" testing without the need for specialized personnel, and therefore, the test is both cheaper and more effective than the current methods used to identify patients with chronic viral hepatitis or HIV infections.

In conclusion, the collection of DBS outside of the health care system, combined with an automated laboratory system for analysis, is a feasible method of screening for chronic viral hepatitis and HIV infections among drug users in prisons and other resource-limited settings.

In Danish guidelines, yearly screening in drug users is recommended; however, this test has been difficult to implement using venous blood sampling. Easy access to DBS testing enables large-scale implementation in difficult to reach populations. The identification of the infected patients is the first step to control these infections, and we suggest that national testing strategies should include DBS testing to improve the coverage of clinical care in these populations.

COMMENTS

Background

Easy access to serological testing for hepatitis C (HCV) and non-invasive methods to diagnose liver fibrosis have improved the screening rates among drug users. dried blood spot (DBS) testing enables large-scale implementation in difficult to reach populations. Recently, it has been shown that combining DBS with automatic analysis on a modern analysis platform allows high throughput testing at a reasonable price. However, in most of these studies, venous whole blood was used for the DBS instead of capillary blood, and the performance and feasibility of "real-life" DBS testing using this set-up is, to our knowledge, not well described.

Research frontiers

Performance of DBS testing in real-life settings, as well as strengths and limitations.

Innovations and breakthroughs

Easy access to DBS testing enables large-scale implementation in difficult to reach populations. The identification of the infected patients is the first step to control these infections. In real-life settings, high sensitivity for detecting HCV, HBV or HIV infections was observed. However, the sensitivities for anti-HBs and anti-HBc were lower than in previous laboratory-based studies, and refinement/further studies are needed before implementing the DBS method as a screening method for vaccination status.

Applications

The collection of DBS samples outside of the health care system, combined with an automated laboratory system for analysis, is a feasible method for screening for chronic viral hepatitis and HIV infections in prison populations, drug users and other resource-limited settings.

Terminology

DBS; Dried blood spot is a method where blood samples are blotted and dried on filter paper, which enables on-site testing without the need for specialized personnel. The dried samples can be sent to the laboratory by mail.

Peer-review

Mössner *et al* investigates the sensitivity and specificity of a Dried Blood Spot (DBS) screening for HIV, HBV and HCV in patients in drug treatment centers. The authors find that DBS had a high sensitivity > 96% and a high specificity 98% for all three infections; however, the antiHBc and antiHBs showed low sensitivities in DBS (42% and 68%, respectively). Chronic infections such as HIV, HBV and HCV remains major sources of mortality and morbidity. However, due to their mild symptoms during early phases of infection, most of infected people are not aware of infection status. Developing a low-cost medically scalable diagnostic tool for testing chronic infections such as HIV, HBV and HCV, is of great importance and has substantial public health implications. Evaluating the feasibility using DBS as diagnostic tool is especially important in a resource limited settings. Thus, this manuscript addresses an important

question, and the results are promising in many ways. It is well written. The methodology and analysis are well documented and the results are analyzed rigorously.

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SYSTEMATIC REVIEWS

Mechanistic insights of rapid liver regeneration after associating liver partition and portal vein ligation for stage hepatectomy

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Abstract

AIM

To highlight the potential mechanisms of regeneration in the Associating Liver Partition and Portal vein ligation for Stage hepatectomy models (clinical and experimental) that could unlock the myth behind the extraordinary capability of the liver for regeneration, which would help in designing new therapeutic options for the regenerative drive in difficult setup, such as chronic liver diseases. Associating Liver Partition and Portal vein ligation for Stage hepatectomy has been recently advocated to induce rapid future liver remnant hypertrophy that significantly shortens the time for the second stage hepatectomy. The introduction of Associating Liver Partition and Portal vein ligation for Stage hepatectomy in the surgical armamentarium of therapeutic tools for liver surgeons represented a real breakthrough in the history of liver surgery.

METHODS

A comprehensive literature review of Associating Liver Partition and Portal vein ligation for Stage hepatectomy and its utility in liver regeneration is performed.

RESULTS

Liver regeneration after Associating Liver Partition and Portal vein ligation for Stage hepatectomy is a

combination of portal flow changes and parenchymal transection that generate a systematic response inducing hepatocyte proliferation and remodeling.

CONCLUSION

Associating Liver Partition and Portal vein ligation for Stage hepatectomy represents a real breakthrough in the history of liver surgery because it offers rapid liver regeneration potential that facilitate resection of liver tumors that were previously though unresectable. The jury is still out though in terms of safety, efficacy and oncological outcomes. As far as Associating Liver Partition and Portal vein ligation for Stage hepatectomy -induced liver regeneration is concerned, further research on the field should focus on the role of non-parenchymal cells in liver regeneration as well as on the effect of Associating Liver Partition and Portal vein ligation for Stage hepatectomy in liver regeneration in the setup of parenchymal liver disease.

Key words: Liver regeneration; Associating liver partition with portal vein ligation for staged hepatectomy; Portal vein embolization; Portal vein ligation; Liver transection

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Core tip: It seems that liver regeneration after associating liver partition with portal vein ligation for staged hepatectomy (ALPPS) is a combination of portal flow changes and parenchymal transection that generate a systematic response inducing hepatocyte proliferation and remodeling. Further research on the field should focus on the role of non-parenchymal cells as well as on the effect of ALPPS in liver regeneration in the setup of parenchymal liver disease.

Moris D, Vernadakis S, Papalampros A, Vailas M, Dimitrokallis N, Petrou A, Dimitroulis D. Mechanistic insights of rapid liver regeneration after associating liver partition and portal vein ligation for stage hepatectomy. *World J Gastroenterol* 2016; 22(33): 7613-7624 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i33/7613.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i33.7613

INTRODUCTION

Hepatectomy still stands of the first-line treatment modality for malignant liver tumors (primary and metastatic). Postoperative liver failure, though, still consists of the main mortality cause after extended hepatectomy despite the recent advances in surgical techniques due to insufficient future liver remnant (FLR)[1-6].

It is generally agreed that FLR must be around 25% of the liver volume to achieve normal liver function in patients with a healthy liver[1-5]. This leaves only 10% to 20% of patients with primary or metastatic liver disease suitable for surgery at presentation. In patients with drug-induced (chemotherapy) injury or cirrhosis, an FLR of at least 40% is required. In resectable cases, extended hepatectomy offers clear resection margins, that in turn, stands for the major determining factor for long-term survival[1,7,8].

Liver parenchyma is thought to demonstrate unique regenerative capacity but actual mechanisms of the regeneration still remain unclear. More than one strategies have been proposed to induce parenchymal hypertrophy including portal vein embolization (PVE) or ligation (PVL), but the failure rates reach 40% due to tumor progression during the hypertrophic stimulus period (4-8 wk). The regenerative potential associated with these techniques is different from conventional hepatectomy without clear superiority among these techniques (associating PVE or PVL) in terms of hypertrophy of the FLR[9].

Associating liver partition and portal vein ligation for stage hepatectomy (ALPPS) is thought to induce rapid FLR hypertrophy, that in turn, decreases the time for the second stage hepatectomy[10,11]. The introduction of ALPPS in the armamentarium of liver surgeons is, without any doubt, an innovation in liver surgery. It is the last surgical successor of Pychlmayr's work[12], who first introduced in situ split in liver transplantations. Schnitzbauer *et al*[10] first described ALPPS, demonstrating an FLR increase of 74% in a short time frame. Unfortunately, the postoperative complication rates[10,13,14] are estimated around 33% to 64% compared with 16% after PVE[15] and much higher that 2-stage hepatectomy[16,17].

ALPPS is getting more familiar in surgical community due to its high variation of indications and modifications[18-20], it still needs further meticulous evaluation before its broader clinical application[21], especially as far as the underlying mechanisms behind the ALPPS-induced accelerated liver regeneration is concerned.

We aim to highlight the potential mechanisms of regeneration in the ALPPS models (clinical and experimental) that could shed some light to the uncharted regenerative capacity of liver parenchyma after ALPPS.

MATERIALS AND METHODS

The MEDLINE/PubMed database was searched for publications with the medical subject heading "ALPPS" and keywords "liver regeneration", "PVL", and "PVE". Three independent reviewers (D.M, S.V and A.P) performed the literature search, the study selection and the data extraction. All the references from the identified articles were searched for relevant information. The end date of the literature search was set to April 2016. We focused on articles of any design or scientific method and purpose.

RESULTS

Basic principles of liver regeneration

The unique capacity of liver regeneration was described by ancient Greeks who first described the liver regeneration concept in the myth of Prometheus. Having stolen the secret of fire from the gods of Olympus, Prometheus drew down on himself the anger of Zeus, the ruler of gods and men. Zeus punished Prometheus by chaining him to Mount Caucasus where he was tormented by an eagle. The eagle preyed on Prometheus' liver, which was renewed as fast as it was devoured. An experimental model came to confirm the myth, as rodents undergone two-thirds partial hepatectomy (PHx) demonstrated rapid liver enlargement till the restoration of original liver mass, after which the regenerative process seizes[22].

It seems that hypertrophy is based on the proliferation of mature functioning cells in FLR[22-25] and it does not require the recruitment of liver progenitor cells[22-25]. More specifically, adult hepatocytes despite the fact they normally do not undergo cell division, they can proliferate in response to injury[22]. In other words, hepatocytes stay in G0 phase of the cell cycle[23,25] but when a stimulus such as hepatectomy or liver injury occurs, almost all (95%) hepatic cells re-enter the cell cycle inducing DNA synthesis (S phase-12h to 24h). DNA synthesis takes later place in the non-parenchymal cells (NPCs)[22,23,25]. Complete restoration of human liver mass requires less than 2 cycles of replication in all cells[22]. Of interest, the peak in DNA synthesis in rodents occurs later (36 h after PHx)[22]. DNA synthesis begins from periportal area towards the central vein. Most of the increase in liver mass occurs during the first 3 d after PHx and parenchymal restoration is complete in 5-7 d[26].

Role of growth factors in liver regeneration

Hepatocyte proliferation is also induced by potential growth factors as hepatic growth factor (HGF), tumor growth factor-alpha (TGF-α) and the anti-proliferative factor TGF-β[27]. However, it is not well-established if any of these factors play a vital role in liver regeneration itself. HGF induces DNA synthesis in hepatocytes but also alters its morphology. It demonstrates pleiotropic effects on various signaling or downstream pathways, including phosphatidylinositol 3-kinase (PI3K), extracellular signal-regulated kinases (ERK), S6 kinase and AKT[28]. In *in vitro* setting it was found that the effect of HGF is mediated *via* up-regulation of TGF-α[29].

Vascular-endothelial growth factor (VEGF) counteracts with liver sinusoids that leads to an increase in HGF production by NPCs. This effect is dependent on endothelial cells with unknown mechanism[30].

Involvement of transcription factors in hepatocyte proliferation

Transcription factors including nuclear factor (NF)-κB, signal transducer and activator of transcription (STAT)-3 and AP1[24] are activated in FLR immediately after PHx[22,31] and intracellular-signalling pathways that involve mitogen-activated protein kinase (MAPK) such as pERKs, Jun amino-terminal kinase (JNK) and receptor tyrosine kinases, are also rapidly activated[23,32-34].

Cytokine effects on liver regeneration

NF-κB and STAT-3 transcription factors are activated by cytokines after PHx that led to the consumption that cytokines might regulate the regenerative response[22,31]. Experimental mouse studies demonstrated that after PHx, normal liver regeneration requires IL-6[35,36]. IL-6, though, it was not enough to generate this process, as parenchymal regeneration is only delayed in the absence of IL-6[35].

At the same time, IL-6 is involved in several processes, including hepatoprotection, the acute-phase response and mitogenesis. Binding of IL-6 to its receptor IL-6R, stimulates the tyrosine-kinase activity of the associated Janus-kinase-family (JAK) member[37]. Activated JAK then phosphorylates STAT-3[37]. After PHx, liver regeneration is impaired in IL-6$^{-/-}$ mouse livers with pathognomonic signs that of liver necrosis, reduced hepatocyte-DNA synthesis and discrete G1-phase abnormalities, including decreased STAT-3 activation[35]. The defect is limited to hepatocytes as the DNA-synthesis response seems normal in IL-6$^{-/-}$ NPCs[35].

Similarly, using TNF-receptor-1 (TNF-R1)$^{-/-}$ mice[38], it was found that TNF-α is also compulsory for normal proliferation after PHx *via* the induction of IL-6. The absence of TNF-α, though, does not affect liver regeneration[39]. Kupffer cells seem to produce most of the IL-6 in the liver[40] and TNF-α induces IL-6 production by enhancing NF-κB, which in turn induced the expression of IL-6.

Figure 1 illustrates all proposed mechanisms of liver regeneration after injury.

Effect of Portal Flow on liver regeneration

As we have already analyzed, after injury, cytokine, growth factors and hormonal expression, induce the beginning and the termination of regeneration[25,41,42]. Parenchymal hypertrophy is also mediated by hemodynamic changes[43-45] and particularly by alterations in portal flow[46]. Portal vein obstruction by PVE or PVL redirects portal vein flow toward specific hepatic segments and is able to pre-operatively increase the volume of FLR. The increase in portal flow to the FLR after PVL or PVE stimulates liver regeneration.

Wilms *et al*[47] found that recanalization of segmental portal neo-collaterals with occluded portal flow after PVE and PVL are one of the reasons for failure of adequate hypertrophy after technically successful PVE[44,48-50]. Similarly, van Lienden *et al*[51] investigated intrahepatic vascular changes in patients undergoing PVL and PVE in correlation with hypertrophy and function of the left liver lobe. All patients in PVL group developed intrahepatic

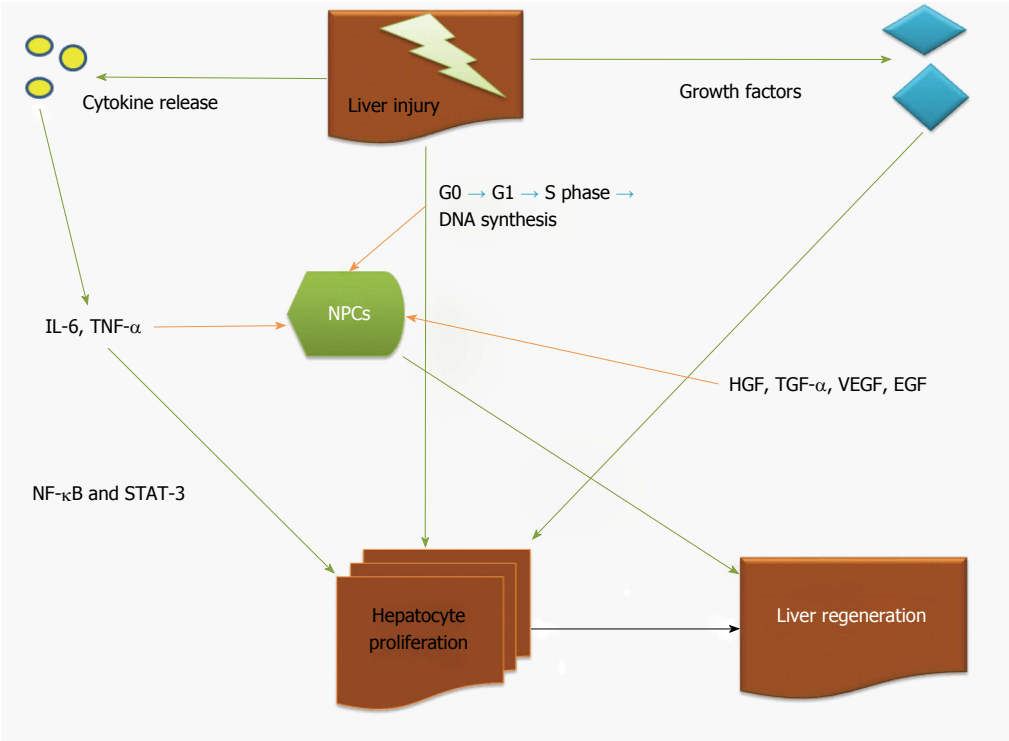

Figure 1 Mechanisms of liver regeneration after liver injury. Green arrow indicates strong induction, orange arrow indicates secondary induction. NPCs: Non-parenchymal cells; IL-6: Interleukin-6; HGF: Hepatic growth factor; TGF-α: Tumor growth factor-alpha; EGF: Epidermal growth factor; VEGF: Vascular-endothelial growth factor; NF-κB: Nuclear factor-κB; STAT-3: Signal transducer and activator of transcription.

portoportal collaterals through which the ligated portal branches are reperfused within 3 wk and one patient (7.1%) in the PVE group[51]. The median increase of FRL volume after PVE was 41.6 % (range: 10%-305%), and after PVL was only 8.1% (range 0%-102%) (P = 0.179). There were no differences in FRL function between both groups[51]. The absence of collaterals and recanalization may explain the greater hypertrophy found in ALPPS, an issue with very high clinical impact[48-50].

ALPPS succeeds in preventing neo-collateral formation across the ligated and non-ligated liver lobes with additional hepatic parenchyma transaction step. Parenchymal transection in stage I ALPPS also creates a traumatic stimulus, which may also contribute to the hypertrophy[49]. PVL/PVE alone often fail to generate adequate hypertrophy because of distal porto-portal collateral formation[52] and proximal portal vein occlusion causes portal cavernoma and recirculation of blood from the non-occluded liver to the occluded one[53]. Dhar *et al*[54] demonstrated portal pressure increase in the ALPPS group compared with the ligation group, that in turn enhances shear stress and provokes early regeneration in the ALPPS group. Shindoh *et al*[55] demonstrated a variant of PVE involving the segment IV that achieves similar to ALPPS parenchymal regeneration. PVE though is superior in terms of FLR regeneration compared with liver resection alone[56].

Proliferation of hepatocytes after ALPPS

The Ki-67 protein is a well-established cellular marker for proliferation that is present in active cells, but is absent in resting cells (G0)[57]. In ALPPS setting (experimental and clinical), the increase of Ki-67 expression after the procedure is a common finding.

In experimental setting, Schlegel *et al*[42] found a rapid liver parenchymal increase (100% in the first 24h) after stage I ALPPS that was combined with a significantly higher Ki-67 expression compared with simple transection, PVL and PHx group[42]. These results indicate that increased number of hepatocytes enter the cell cycle after ALPPS than any other intervention[42].

Furthermore, Dhar *et al*[54] demonstrated a significant FLR increase combined with increased periportal hepatocyte proliferation (Ki-67 index) in the ALPPS group compared with the PVL group at 24 h (P = 0.002) and 48 h (P = 0.031)[54].

In the same frame, Wei *et al*[58] evaluated the proliferation (1:50 monoclonal anti-BrdU antibody) of hepatocytes after ALPPS compared with non-ALPPS group (PVL, simple transection, PHx). The 2-fold increase of liver lobe weight was combined with an increased proliferation index (15.4% ± 0.9%) in ALPPS group compared to control (8.6% ± 2.9%, P = 0.009)[58].

García-Pérez *et al*[59] demonstrated in an experimental model of ALPPS that mitotic figures detected at 48 h were more prominent in ALPPS FLR compared with PVL (P < 0.0001). As far as proliferation potential is concerned, higher expression of Ki-67 was related with in the ALPPS group at 48 h (P < 0.001 compared

with PVL group)[59]. Finally, Shi *et al*[60] demonstrated in an experimental model that ALPPS was correlated with increased liver regeneration (liver weight to body weight ratio) and increased hepatocyte proliferation assessed by Ki-67 and proliferating cell nuclear antigen (PCNA) activity compared with PHx and PVL group.

In pediatric liver with hepatoblastoma, a rapid increase of FLR (46.1%) after stage Ⅰ ALPPS was noted with concomitant increased expression of Ki-67 in the left liver (proliferation index of right liver and left lateral segment-LLS to be 2% and 20% respectively)[61].

The proliferation after PVL can also affect tumor cells in the affected hemiliver. Kokudo *et al*[62] found increased Ki-67 expression of intrahepatic metastases in the embolized liver after PVE. This finding is similar in ALPPS despite a shorter interval between PVL and parenchymal resection. In small series, an increase in Ki-67 expression from 60% at stage Ⅰ ALPPS to 80% at stage Ⅱ was noted[49,63].

More specifically, Tanaka *et al*[49] evaluated the proliferation of tumor cells after step I ALPPS in patients with unresectable multiple liver metastases from colorectal cancer (CLM) and pancreatic neuroendocrine tumor. Control group for the comparison was chosen a group of patients with CLM initially considered unresectable with classical 2-stage hepatectomy. They demonstrated an increase of FLR in ALPPS group of 40%-50% which was less than the one observed in the classical 2-stage hepatectomy group (P < 0.01)[49], probably due to relatively large volume of liver parenchyma heavily pretreated by chemotherapy in ALPPS group, resulting in less capacity for regeneration. Additionally, Ki67 expression in tumor cells was lower in ALPPS group compared to classical 2-stage group (P = 0.09)[49]. This finding may support a potential oncologic benefit from ALPPS, with the short period between the 2 interventions helping to avoid risk of tumor progression.

Similarly, Matsuo *et al*[64] evaluated the proliferation potential in liver parenchyma after ALPPS or 2-stage hepatectomy in patients with colonic liver metastases. They used for this purpose a monoclonal antibody against the Ki-67 antigen (MIB-1, 1:100). The mean increase of FLR after stage Ⅰ ALPPS was 50% with concomitant increased MIB-1 labeling (expressed in 7.8% ± 4.9% of hepatocytes in the ALPPS group, compared to PVE group, 0.9% ± 0.7%, P = 0.01)[64].

Expression of cell cycle regulators after ALPPS

In ALPPS setting, both TNF-α and HGF can activate JNK and MAPK-ERK pathways and they can also induce the expression of cyclin D1[65]. In the regenerating liver, activation of cyclin D1 induces the progression of cell cycle through G1 and entry into S phase[66]. Cyclin-D1 expression is induced by IL-6 whereas the expression of the S-phase cyclins A and B1 is induced by insulin-like-growth-factor binding protein[22].

Shi *et al*[60] evaluated the effect of the different procedures (ALPPS, PVL and transection) on cell cycle regulators in experimental setting. Data extracted from immunochemical staining indicated that ALPPS stimulated cyclin D1 and cyclin E expression more significantly compared with other procedures and the maximum induction occurred on day 3 and day 2, respectively (P < 0.01 and 0.001 respectively)[60]. G1 Cdks as catalytic partner of cyclin E and cyclin D1 to facilitate cells entering S phase. They were also induced at 24 h after ALPPS, with a maximum induction on day 3 compared with sham group[60]. Cdk2-associated kinase activity was also increased during liver regeneration after ALPPS[60]. Collectively, these results demonstrated not only temporal increases in the cyclin E/Cdk2 complex, but also concomitant increases in Cdk2 kinase activity during hepatocyte proliferation after ALPPS.

Cytokine release and inflammation after ALPPS

In the experimental setting, Schlegel *et al*[42] demonstrated that ALPPS-plasma injection after PVL triggers comparable regeneration in terms of liver weight gain and regeneration, as original ALPPS which was not the case when plasma obtained from group of sham laparotomy did not present any additional regeneration when injected in PVL-treated livers. On the basis of these experiments, it seems that the rapid liver volume increase after ALPPS is triggered by systemic release of putative initiators. After meticulous analysis of the ALPPS plasma in PVL-treated mice, an early increase of plasma IL-6 levels compared to PVL alone was found[42]. Of interest though, transection alone induced similarly increased IL-6-expression at this early time point[42]. Genomic evaluation revealed significant up-regulation of IL-6-mRNA and TNF-α-mRNA 1 h after step Ⅰ of ALPPS or transection. These findings were confirmed in clinical setting, where increased gene expression of IL-6 and TNF-α was found in liver tissue and plasma 1 hour after step Ⅰ ALPPS or PVL alone[42]. All in all, the contribution of the IL-6-TNF-α-STAT3-pathway to the rapid liver hypertrophy after step I ALPPS is suggested in both clinical and experimental setting. Moreover, no changes in CD31 and VEGF expression was found in regenerating lobe during the first week after ALPPS or PVL[42].

In the same setting, Dhar *et al*[54] demonstrated that the cytokine-induced neutrophil chemoattractant -1 (CINC-1) had its highest levels in liver tissue with main difference in expression between the ALPPS and PVL group was in IL-6 expression at 24 h[54]. Finally, ALPPS group demonstrated increased VEGF and interferon gamma expression at 48 h[54]. In terms of inflammatory cells, ALPPS group demonstrated higher early infiltration of liver by inflammatory cells compared with PVL (P = 0.021)[54].

Shi *et al*[60] also investigated gene and cytokine involvement in liver regeneration after ALPPS. Results indicated that the levels of IL-6, NF-κB p65, STAT3,

Table 1 Experimental associating liver partition with portal vein ligation for staged hepatectomy models

Ref.	Year	Species	PVL	PHx in stage Ⅰ	PHx in stage Ⅱ	Loss of liver mass	Atrophy of ligated lobe on day 7	Future remnant liver lobe	Fold increase on day 7	Proliferation
Schlegel *et al*[42]	2014	Mouse	RML RL CL	LLL (30%)	Ligated lobes	85% totally (55% PVL + 30% PHx)	NA	LML (15%)	4-fold	Peaked on day 4
Yao *et al*[70]	2014	Rat	LLL LML RL CL	NA	NA	80% PVL	NA	RML (20%)	2.5-fold	Peaked on day 2
Almau Trenard *et al*[71]	2014	Rat	LLL LML RL CL	NA	NA	80% PVL	Reduction to 35.2%	RML (20%)	2-fold	NA
Dhar *et al*[54]	2015	Rat	LLL LML RL CL	NA	NA	80% PVL	NA	RML (20%)	2-fold	Peaked on day 2
Wei *et al*[58]	2015	Rat	LLL LML RL	CL (10%)	Ligated lobes	80% totally (70% PVL + 10% PHx)	Reduction to 48.2%	RML (20%)	2.53-fold	Peaked on day 1
García-Pérez *et al*[59]	2015	Rat	RSL RIL LLL RML	RML (20%)	Ligated lobes	80% totally (60% PVL + 20% PHx)	N/A	LML (22%)	2-fold	Peaked on day 2
Shi *et al*[60]	2015	Rat	RML RL CL	LLL (30%)	Ligated lobes	80% totally (50% PVL + 30% PHx)	Reduction to 34.9%	LML (20%)	2.3 fold	Peaked on day 2

ALPPS: Associating liver partition with portal vein ligation for staged hepatectomy; CL: Caudate lobe; LLL: Left lateral lobe; LML: Left median lobe; PHx: Partial hepatectomy; PVL: Portal vein ligation; RL: Right lobe; RSL: Right superior; RIL: Right inferior; RML: Right portion of the middle lobes.

Table 2 Comparison of the effect of different techniques on liver regeneration

Intervention	Cytokine release	Growth factor expression	DNA synthesis	Portal vein flow decrease	Hepatocyte hypertrophy induction	Increased NPC activity	Atrophy of the affected lobe	Ref.
PVL	++	++	++	++	++	++	++	[42,54,58-60,70,71]
PVE	+	+	+	+	+	++	+	[42,54, 58-60,70,71]
Parenchymal Transection	+++	+	+	0	+	+	+	[42,47-49,54,58-61,64,70-72]
ALPPS	+++	+++	+++	+++	+++	+	+++	[42,47-49,54,58-61,64,70-72]

TNF-α, EGF, HGF, ERK-1/2 and YAP were significantly increased in the ALPPS group compared with the other groups (PVL and transection alone), suggesting that these factors might play important roles in the fast liver regeneration after ALPPS[60]. As expected, the mRNA levels of these cytokines were also highly increased in the regenerating lobes 24 h, 3 d and 5 d after ALPPS[60].

García-Pérez *et al*[59] showed in an experimental model of ALPPS that HGF expression in FLR after PVL was increased compared with ALPPS at 24 h ($P < 0.05$)[59]. At 48h, ALPPS was associated with higher expression of HGF and TGF-α ($P < 0.05$) with similar relative expression of TGF-β in both groups[59]. Later, the HGF and TGF-β levels were still higher on ALPPS group ($P < 0.005$ and $P < 0.05$ respectively)[59]. Interestingly, hepatic transection caused an increase in the number of Kupffer cells in ALPPS group at 24 h ($P < 0.01$)[59].

Table 1 summarizes the data of the experimental models of ALPPS. Table 2 summarizes the mechanistic effect of each modality (PVE, PVL, simple transection and ALPPS) in liver regeneration. Figures 2 and 3 illustrate the potential mechanisms of liver regeneration after ALPPS as well as the cascade of events leading to liver volume restoration.

Histological changes after ALPPS

Matsuo *et al*[64] evaluated the special histologic characteristics of hepatocytes after ALPPS compared with liver tissue retrieved from patients undergoing PVE or staged hepatectomy PVE after chemotherapy. In the area of the FLR, glycogen-rich, vacant-cytoplasm bright-appearing hepatocytes were more frequent in ALPPS than in PVE. Both hepatocyte brightness and sinusoidal narrowing were observed more frequently

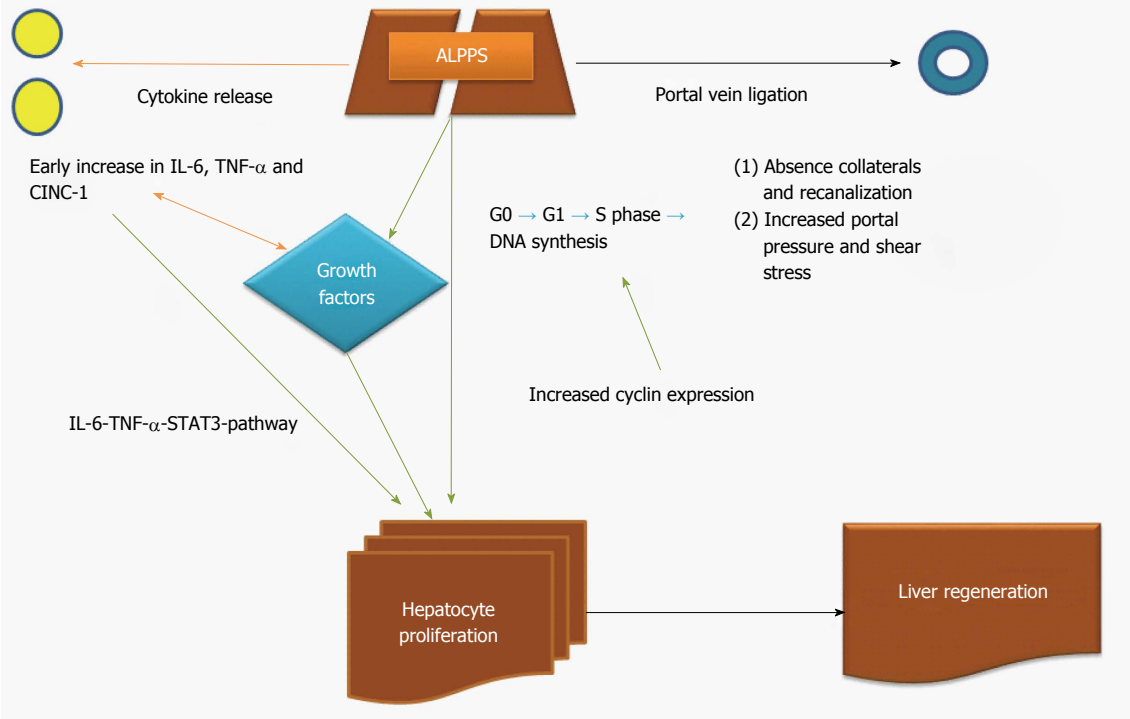

Figure 2 Mechanisms of liver regeneration after associating liver partition with portal vein ligation for staged hepatectomy. Green arrow indicates strong induction, orange arrow indicates secondary induction. IL-6: Interleukin-6; TGF-α: Tumor growth factor-alpha; STAT-3: Signal transducer and activator of transcription; CINC-1: Cytokine-induced neutrophil chemoattractant-1; ALPPS: Associating liver partition with portal vein ligation for staged hepatectomy.

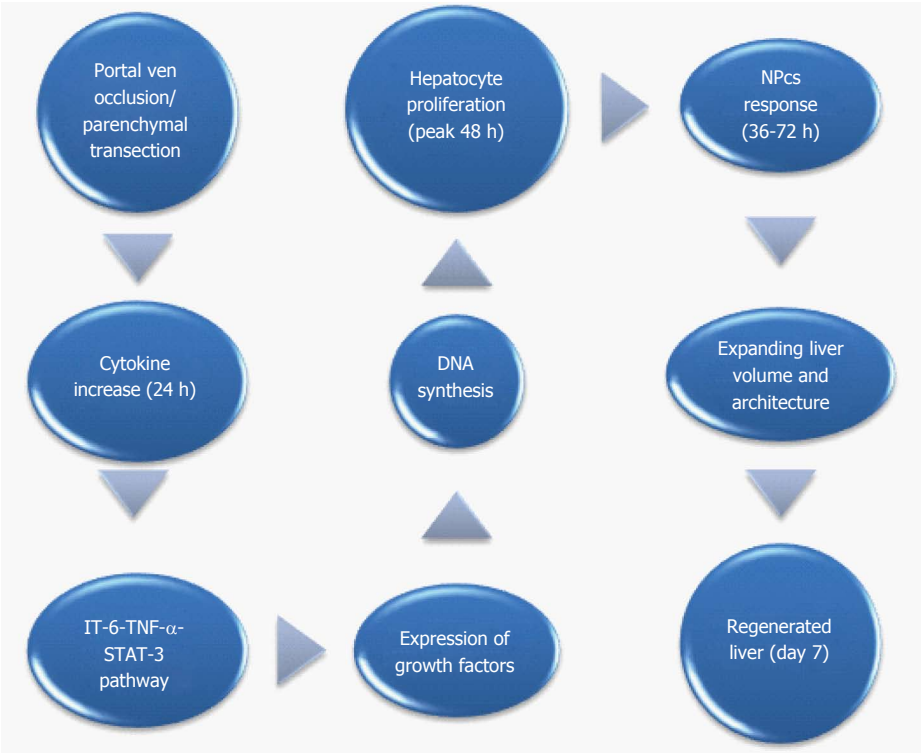

Figure 3 Cascade of mechanisms involved in liver regeneration after associating liver partition with portal vein ligation for staged hepatectomy. STAT-3: Signal transducer and activator of transcription; NPCs: Non-parenchymal cells; IL-6: Interleukin-6.

in ALPPS than in PVE (P = 0.025)[64]. Hepatocyte cell density was greater and hepatocyte size was smaller in the ALPPS group than in the PVE group (P < 0.01)[64]. In the ligated liver part, hepatocyte atrophy, degeneration or necrosis, sinusoidal dilation, fibrosis and congestion all were more frequent with ALPPS than with PVE (P = 0.001, P = 0.001, P = 0.002, P < 0.001, and P < 0.001, respectively)[64]. Cytoplasmic organelles such as mitochondria and endoplasmic reticulum were fewer than in the PVE group[64]. All these findings are indicative of cell immaturity in ALPPS setting.

In an experimental model of ALPPS, García-Pérez *et al*[59] demonstrated that the main histological features on the atrophic lobes were periportal congestion, sinusoid dilation and areas of necrotic or apoptotic hepatocytes.

DISCUSSION

The ALPPS technique has taken its place in liver surgery as alternative in those cases in which the FLR volume is inadequate for normal liver after parenchymal resection and shortens the gap between the first and second step avoiding the risk of tumor progression. This rising interest in ALPPS has, and will further, evaluate the efficacy of technical innovations to address the initial concern about high complication rates and long-term survival[67].

This analysis shed light to the potential mechanisms involved to liver regeneration after ALPPS by presenting well-established knowledge on liver regeneration per se and emerging knowledge on liver regeneration induction after ALPPS, PVL, PVE and simple parenchymal transection.

In a meta-analysis of portal vein obstruction as a stimulus to induce liver hypertophy, PVE with various methods of embolization induced a mean volumetric increment by 8%-27% over a period of 2-6 wk[15] and PVL achieved a FLR volumetric increment by 38%-53% over a period of 4-8 wk[68]. These differences in liver regeneration indicate the crucial role of portal vein occlusion in inducing the regeneration process. On the contrary, ALPPS procedure achieves a variable increase in the FLR ranging from 21%-200%. This could be partially attributable to the presence of underlying parenchymal liver diseases (cirrhosis and chronic liver diseases).

At the same time, besides enhancing extended liver hypertrophy, ALPPS procedure increases liver atrophy compared with PVL without transection. This could be mainly attributed to the inadequate portal blood supply that causes significant atrophy that stands for greater liver mass and volume loss, which stands of an important regenerative trigger[69].

Novel knowledge about the mechanisms of liver regeneration after ALPPS were established by many variants of ALPPS model. Seven different experiments of ALPPS were reported in the literature[9,42,70,71]. These models included the transection of the median lobe, but differed on the extent of the FLR, PVL the additional PHx. The mouse model of Schlegel *et al*[42] demonstrated a 55% PVL and a 30% PHx in the first stage ALPPS. The rat models[54,58-60,70,71] consisted of an 80% PVL *via* ligation of all but the right median lobe. Mouse model demonstrated a regenerative potential on the first postoperative day and reached the peak on postoperative day 4[22]. Yao *et al*[70], García-Pérez *et al*[59] and Dhar *et al*[54] observed a proliferation peak on day 2, which is usually observed after classic PVL. Despite the fact that liver mass reduction is similar to all models, Wei *et al*[58] demonstrated a peak of proliferation on first postoperative day, as observed after the classic 70% PHx. Subsequently, the increase in FLR in that study was slightly higher than other rat models. The differences in proliferative kinetics suggest that even small differences in the ratio between the extent of PVL and PHx may produce a substantial effect on the time course of intrahepatic size regulation. The main disadvantage of these models is that they evaluated liver regeneration after injury in healthy tissues, which is not representative to clinical practice. Future research should focus on liver regeneration after ALPPS in models of liver disease.

At the same time, several clinical settings evaluating the regenerative potential after ALPPS in patients with primary or secondary (colonic) metastatic disease[48,49,61,64,72]. The design of these studies included a group of patients with metastatic liver disease and a group of patients with unresectable tumors that underwent 2-stage hepatectomy. The common finding of these studies was that the ALPPS group demonstrated a higher hepatocyte proliferation potential (assessed by Ki-67).

As far as the mechanistics of liver regeneration after ALPPS is concerned, Schlegel *et al*[42] showed that parenchymal transection induces an inflammatory response in terms of growth factor and cytokines release contributing to regeneration. The response, though, is not organ-specific, induced by soluble initiators in the circulation. The observation of accelerated regeneration, when injecting plasma obtained from mice after step I ALPPS to animals undergoing PVL alone, strongly supports the existence of soluble growth factors. The similar effects on regeneration achieved by injuries to other organs further point out to existence of soluble mediators of liver regeneration, additionally suggesting that the origin of the circulating growth factors is not "liver-specific."

The detection of similar enhanced release of proinflammatory cytokines in samples obtained in ALPPS and PVL[42] patients is indicative of the crucial role of portal vein flow changes in inducing the regeneration process, especially early postoperatively. After the initial stage of injury due to portal vein flow changes, ALPPS group demonstrated dramatic increase of instigators of regeneration (IL-6 and TNF-α) compared with PVL alone but similar to

transection alone[42]. Surgical procedures of similar invasiveness (split liver in liver transplantation or living liver donation) were shown to induce comparable systemic inflammation and cytokine release as in the liver tissue early after step 1 of ALPPS[24,73,74]. This finding is indicative that ALPPS due to its dual nature (PVL+ transection) manages to keep high levels of expression of cytokines and growth factors that could explain the rapid liver regeneration potential of this technique. Liver transection with the PVL can induced programmed cell death followed by cytokine surge that facilitates accelerated liver regeneration.

Hepatocytes are the crucial player in liver regeneration after ALPPS since liver injury initiates high expression of cytokines, growth factors and cell cycle promoters, such as cyclins that lead quiescent hepatocytes to enter G1 and S phase of cell cycle and eventually proliferate (assessed by Ki-67 expression). This concept is similar to liver regeneration after injury of any kind. The leading pathway of the cascade above is IL-6- TNF-α-STAT-3 pathway. One interesting finding though is that after ALPPS, the majority of the hepatocytes that contribute to the regeneration are immature cells (narrowing of sinusoidal spaces, organelle distribution pattern) compared to the mature cells (numerous lipofuscin granules) that are involved in regeneration process after PVE[64,75]. Proliferation of hepatocytes begins from periportal area and is directed towards central vein area.

Among the wide range of cytokines examined in the literature, Dhar *et al*[54] suggested that CINC-1 had the strongest and early significant expression in the ALPPS liver when compared with the PVL liver, supporting a crucial role of CINC-1 in the ALPPS associated regeneration. Interestingly, Kaibori *et al*[76] noticed that CINC-1 induced by HGF produced enhanced proliferative and angiogenic activities through NFkB activation in the liver. The increased IL-6 level concords with the majority of animal models of ALPPS, where a significant increase in plasma IL-6 in the ALPPS group compared with the PVL group is demonstrated. Early activation of IL-6/STAT3 pathway in macrophages facilitates the generation of chemokines such as MIP-a and the migration of bone marrow derived MSCs to the liver during the restitution of liver mass[77].

The role of NPCs (Kupffer cells, endothelial cells) in liver regeneration after ALPPS is not elucidated. The majority of studies agree that its role is supportive rather than fundamental in terms of maintaining a cytokine-rich liver microenvironment (INF-γ, IL-6) that induces local inflammatory response to ALPPS. In different setting, Tanaka *et al*[78] found an increased number of Kupffer cells infiltration following PVE + PHx compared with PHx only group following endotoxin challenge in rats. Literature stands also equivocal towards VEGF, but it seems that throughout this regenerative process, not extensive angiogenesis takes place. All in all, liver parenchymal cells, as well as resident NPCs and infiltrated inflammatory cells seem to play crucial roles in the ALPPS-induced liver hypertrophy.

ALPPS represents a real breakthrough in the history of liver surgery because it offers rapid liver regeneration potential that facilitate resection of liver tumors that were previously though unresectable. The jury is still out though in terms of safety, efficacy and oncological outcomes. Liver regeneration after ALPPS is a combination of portal flow changes and parenchymal transection that generate a systematic response inducing hepatocyte proliferation and remodeling. Further research on the field should focus on the role of NPCs in liver regeneration as well as on the effect of ALPPS in liver regeneration in the setup of parenchymal liver disease.

COMMENTS

Background

Associating liver partition and portal vein ligation for stage hepatectomy (ALPPS) has been recently advocated to induce rapid future liver remnant hypertrophy that significantly shortens the time for the second stage hepatectomy

Research frontiers

Liver regeneration after ALPPS is a combination of portal flow changes and parenchymal transection that generate a systematic response inducing hepatocyte proliferation and remodeling. To date, no data are questions are risen about the safety, efficacy and oncological outcomes of ALPPS. In terms of liver regeneration, further research on the field should focus on the role of non-parenchymal cells in liver regeneration as well as on the effect of ALPPS in liver regeneration in the setup of parenchymal liver disease.

Innovations and breakthroughs

This is-the first to the authors knowledge-systematic review about the mechanisms of liver regeneration after ALPPS.

Applications

This article highlights the potential mechanisms of regeneration in the ALPPS models (clinical and experimental) that could unlock the myth behind the extraordinary capability of the liver for regeneration, which would help in designing new therapeutic options for the regenerative drive in difficult setup, such as chronic liver diseases.

Terminology

ALPPS operation is divided in two steps. The first consists of exploratory laparotomy, assessment of resectability with intraoperative ultrasound and positioning the tumor in relation with vessels. The liver is mobilized by dissecting the ligaments. The right liver lobe is completely mobilized from the cava vein. After the right portal vein branch is identified, it is divided. Right hepatic artery and right hepatic duct are identified and also kept. Finally, total parenchymal dissection at the right of the falciform ligament is performed. After in situ splitting, the right lobe is covered by a biomaterial and the abdomen is drained and closed. The second step of the procedure is completed by re-laparotomy. The right hepatic artery, right hepatic duct and the right hepatic vein are ligated. The liver resection is completed. The left lateral lobe is then fixed to the remnant falciform ligament.

Peer-review

The manuscript provides a wide vision of liver regeneration in the context of different therapeutic interventions combining results from experimental models and from the clinical management of patients suffering from parenchymal liver disorders. All issues discussed are relevant, provide molecular mechanisms that might well explain the clinical observations and leave still open questions

that will surely foster future investigation avenues.

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