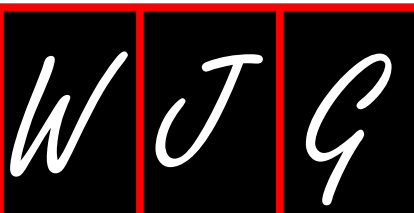


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Surveillance of colonic polyps: Are we getting it right?

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Abstract

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide. The identification of colonic polyps can reduce CRC mortality through earlier diagnosis of cancers and the removal of polyps: the

precursor lesion of CRC. Following the finding and removal of colonic polyps at an initial colonoscopy, some patients are at an increased risk of developing CRC in the future. This is the rationale for post-polypectomy surveillance colonoscopy. However, not all individuals found to have colonic adenomas have a risk of CRC higher than that of the general population. This review examines the literature on post-polypectomy surveillance including current international clinical guidelines. The potential benefits of surveillance procedures must be weighed against the burden of colonoscopy: resource use, the potential for patient discomfort, and the risk of complications. Therefore surveillance colonoscopy is best utilised in a selected group of individuals at a high risk of developing cancer. Further study is needed into the specific factors conferring higher risk as well as the efficacy of surveillance in mitigating this risk. Such evidence will better inform clinicians and patients of the relative benefits of colonoscopic surveillance for the individual. In addition, the decision to continue with surveillance must be informed by the changing profile of risks and benefits of further procedures with the patient's advancing age.

Key words: Adenoma; Polyp; Colonoscopy; Surveillance; Colorectal cancer

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Core tip: Increasing numbers of surveillance colonoscopies for previous colonic polyps are being performed. Each colonoscopy brings the burden of bowel preparation, potential discomfort, and risk of complications. Colonoscopy is a finite resource and must be recommended only with a strong indication. Individuals with non-advanced adenomas have no significantly increased risk of colorectal cancer (CRC) compared to the general population. Patients with an advanced adenoma, have a CRC risk similar to that of the general population after just one surveillance colonoscopy. This review examines the evidence behind

current surveillance guidelines and questions the rationale for surveillance in individuals with relatively low cancer risk.

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INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of death from cancer in the United Kingdom^[1] and United States^[2]. Over 41000 people in the United Kingdom are diagnosed with CRC annually and over 16000 people die of the disease.

Recognised risk factors for the development of CRC include advancing age, a personal or family history of CRC, longstanding inflammatory bowel disease affecting the colon, and specific conditions such as familial adenomatous polyposis (FAP), and hereditary non-polyposis colon cancer (HNPCC). This review focuses on an important risk factor for the development of CRC: a personal history of colorectal adenomas.

Some colonic polyps such as adenomatous and serrated polyps carry malignant potential, while others do not (hyperplastic, post-inflammatory, hamartomatous). This review will discuss only those polyps with malignant potential.

The majority of CRCs arise from colonic adenomas. Adenomas arise following aberrant proliferation of epithelial cells in the colon. These lesions may then progress to varying degrees in size and dysplasia^[3]. Adenomas represent the major precursor for CRC both in high-risk groups such as patients with a family history of familial adenomatous polyposis (FAP) or hereditary non-polyposis colorectal cancer (HNPCC), as well as in the general population. This concept is termed the "adenoma-carcinoma sequence"^[4-8].

However, 20%-30% of colorectal cancers arise through a different molecular pathway to the conventional adenoma-carcinoma sequence. These CIMP-positive cancers (CpG island methylator phenotype) are believed to arise from serrated polyps. Such lesions are over-represented among "interval cancers" (cancers diagnosed 6-36 mo after a colonoscopy)^[9]. Growing evidence points to the importance of recognising and managing serrated lesions in preventing CRC^[10].

The speed of progression along the pathway of proliferation and dysplasia is a key factor in determining clinical practice in patients found to have colonic adenomas. Progression from adenoma to invasive cancer can occur in 5 years or take more than 20 years^[11]. Additionally, progression along this

pathway is highly variable: one study estimates that only 0.25% of adenomas per year will progress to cancer^[12]; some stabilise and some regress^[11,13-15].

Adenoma prevalence in Western screening populations (age 50-75 years) can be as high as 40%, with advancing age and male sex associated with higher prevalence. However, lifetime risk of CRC is only 5.5% due to the highly variable progression of adenomas^[16-22]. Overall, projections of 10-year cumulative risk for progression from adenoma to carcinoma are less than 10%^[15,23].

RISK FACTORS

In recent years, an understanding of adenoma features predicting risk of progression to cancer has led to the term "advanced adenoma"^[15], referring to adenomas possessing at least one of three high risk characteristics: size of at least 10 mm, villous architecture of at least 25%, or high grade dysplasia^[24-26]. Overall, these lesions progress to cancer at an annual rate of up to 5%: significantly higher than the average rate for all adenomas^[12], and this risk increases with age to 25% at age 55 years and to 40% at age 80 years. Annual rates of progression from adenoma to carcinoma also vary depending on which of these advanced features is present. Size of at least 10 mm confers a 3% annual risk; villous architecture 17%, and high grade dysplasia 37%^[12].

As these figures illustrate, high grade dysplasia (HGD) confers high risk of progression to cancer. However, in keeping with the adenoma-carcinoma sequence described previously, high grade dysplasia is more likely to be found in larger lesions: as adenomas progress in size, so too dysplasia progresses^[27]. The number of adenomas possessing advanced features (HGD or > 25% villous architecture) increases with polyp size from approximately 1%-2% in diminutive adenomas (< 5 mm) to 7%-12% for small adenomas (5-9 mm) and 20%-30% for large adenomas (≥ 10 mm)^[24,28,29]. Advancing age of the patient also increases the likelihood of HGD within an adenoma, independent of polyp size and histological type^[30].

Most adenomas detected at colonoscopy (60%-75%) are smaller than 10 mm diameter^[31]. Larger adenomas of at least 10 mm in diameter are at higher risk of containing CRC and are also a risk factor for metachronous cancer development (*i.e.*, a cancer diagnosed at least 6 mo after the index procedure)^[24]. The absolute risk of metachronous advanced adenomas is close to 20% in patients whose largest baseline adenoma is 20 mm or more in size^[32].

The risk factor most closely correlating to CRC risk is the total number of adenomas, both at index procedure and cumulatively over the individual's lifetime. Patients with one or two small tubular adenomas removed do not have a significantly increased metachronous colorectal cancer risk^[33]. In contrast, the presence of

one or more advanced adenomas predicts a higher rate of both any and advanced metachronous adenomas^[25]. The risk of metachronous CRC increases with the number of advanced adenomas^[24]. Large polyp size (≥ 10 mm) and proximal location in the colon are independent predictors of further advanced neoplasia at follow-up^[34]. The risk for metachronous advanced adenomas increases progressively with the number of adenomas at baseline examination: patients with only 1 adenoma have a risk of 9% while those with 5 or more adenomas have a 24% risk.

BENEFIT OF COLONOSCOPY

Colonoscopic screening has been shown to be effective in reducing CRC incidence and mortality^[27,35-38].

This effect is *via* a number mechanisms. Firstly, the removal of pre-cancerous lesions, *i.e.*, adenomatous polyps, thereby interrupting the progression to carcinoma: preventing cancers. Secondly, detection of CRC at an earlier, pre-symptomatic stage with resultant increased likelihood of successful endoscopic or surgical treatment^[27,39-41].

The third mechanism, which may reduce CRC incidence and mortality, is surveillance colonoscopy. Risk stratification based upon index colonoscopy findings allows patients with polyps at higher risk of progression to cancer to be offered a further examination in the future^[19,20,42]. The evidence for the potential benefits of surveillance will be discussed in detail later.

Patients diagnosed with CRC at an earlier stage have significantly better prognosis than those diagnosed with more extensive disease. Of patients diagnosed with Dukes' A CRC, 93% will survive 5 years. Those diagnosed with modified Dukes' D cancer however, have a less than 7% chance of living a further 5 years.

Colonoscopy is considered to be the gold standard for adenoma detection and affords an opportunity for therapy, through polypectomy, as well as allowing histological diagnosis. Double-contrast barium enema and CT colonography (CTC) show poorer sensitivity compared to colonoscopy, particularly with respect to very small and flat polyps^[43,44]. An optimally performed double-contrast barium enema and FIT (faecal immunohistochemical test) detect only half of adenomas of 5 mm or larger that are detected by colonoscopy^[45].

LIMITATIONS OF COLONOSCOPY

However, there remain limitations to colonoscopic screening. Even colonoscopy does not allow detection of all adenomas. "Back-to-back" colonoscopies have indicated significant miss rates of 27% for small adenomas (< 5 mm) and 6% for adenomas of more than 10 mm diameter^[46]. Studies performing both CTC and colonoscopy estimate that the colonoscopy miss

rate for polyps over 10 mm in size may be as high as 12%^[47]. There are multiple factors likely to contribute to missed polyps at colonoscopy including quality of bowel preparation, and the training and experience of the colonoscopist. The time taken by colonoscopists during withdrawal of the colonoscope from the caecum is a powerful predictor of adenoma detection rate (ADR)^[48]. Higher rates of interval cancers are seen in association with low ADR at screening colonoscopy^[49,50].

The protection afforded by colonoscopy is significantly greater in respect of distal CRC as compared to lesions of the proximal colon. There are a number of factors postulated to explain this differential: poorer right-sided bowel preparation, incomplete colonoscopy, anatomical factors impeding visibility, and potentially different biology of right-sided lesions, especially via the serrated pathway^[35,51].

Incomplete resection of adenomatous tissue is believed to be a substantial contributor to interval cancers. Rates of incomplete resection for diminutive polyps are 29% for conventional biopsy and 17% for hot biopsy^[52,53]. Residual polyp tissue is more likely to remain after resection of sessile polyps and risk increases with polyp size. Rates of 17% for polyps of 10-20 mm and 7% for lesions of 5-9 mm have been quoted. There also appears to be a higher rate of incomplete resection for serrated lesions in comparison to conventional adenomas (31% and 7% respectively)^[54].

Missed lesions are likely to account for more than half of interval cancers diagnosed at 3 to 5 years after the index procedure^[55]. Therefore, the quality of the index and subsequent colonoscopies is paramount in maximising the potential benefit of surveillance procedures. Quality of colonoscopy is directly associated with rates of interval CRC^[50].

RATIONALE FOR SURVEILLANCE

The major CRC mortality risk reduction is achieved at index colonoscopy, *i.e.*, diagnosis of cancers at an earlier stage and removal of adenomas with the aim of reducing CRC incidence.

Individuals found to have colonic polyps are at increased risk of advanced neoplasia in the future^[11,23,56,57]. This risk may be due to a number of mechanisms: (1) Missed lesions at the initial colonoscopy; (2) Incomplete removal of adenomatous tissue at initial colonoscopy; and (3) The individual's propensity to colonic neoplasia (either lifestyle factors, an inherent imbalance of cell proliferation, or a combination of these)^[25,46,57-60].

In view of the increased risk of CRC, it seems logical that this group may benefit from closer monitoring than the general population. There are two reasons to consider surveillance colonoscopy in patients found to have adenomas at the index procedure. Firstly, as discussed above, there may be missed lesions, particularly small polyps, which may be identified at a subsequent procedure. Secondly, after a time interval,

Table 1 British Society of Gastroenterology guidelines 2010^[79], supported by the 2011 guidelines of The National Institute for Health and Care Excellence

Risk of colorectal cancer or advanced adenomas (≥ 1 cm as measured at endoscopy or high-grade dysplasia)

Patients with only one or two small (< 1 cm) adenomas are at low risk, and need no colonoscopic surveillance or 5-yearly until one negative examination then cease surveillance. Recommendation grade: B

Patients with three or four small adenomas or at least one adenoma ≥ 1 cm are at intermediate risk and should be screened 3-yearly until two consecutive examinations are negative. Recommendation grade: B

If either of the following is detected at any single examination (at baseline or follow-up): five or more adenomas, or three or more adenomas at least one of which is ≥ 1 cm, the patient is at high risk and an extra examination should be undertaken at 12 mo before returning to 3-yearly surveillance. Recommendation grade: B

Patients can be offered surveillance until age 75 yr and thereafter continue depending on relative cancer risk and comorbidity. Colonoscopy is likely to be less successful and more risky at older ages. Further, the average lead time for progression of an adenoma to cancer is 10 yr which is of the same order as the average life expectancy of an individual aged 75 yr or older, suggesting that most will not benefit from surveillance. Recommendation grade: B

These guidelines are based on accurate detection of adenomas, otherwise risk status will be underestimated. Patients with a failed colonoscopy, for whatever reason, should undergo repeat colonoscopy or an alternative complete colonic examination. Recommendation grade: B

The site of large sessile adenomas removed piecemeal should be re-examined at 2-3 mo. Small areas of residual polyp can then be treated endoscopically, with a further check for complete eradication in 2-3 mo. India ink tattooing aids recognition of the polypectomy site at follow-up. If extensive residual polyp is seen, surgical resection needs to be considered, or alternatively referral to a colonoscopist with special expertise in advanced polypectomy techniques. If there is complete healing of the polypectomy site, then there should be a colonoscopy at 1 yr, to check for missed synchronous polyps, before returning to 3 yearly surveillance. Recommendation grade: B

Table 2 American Gastroenterological Association 2012^[80]

| Findings at index procedure | Suggested surveillance interval | Strength of evidence |
|--|---------------------------------|----------------------|
| No polyps/small (< 10 mm) rectosigmoid hyperplastic | 10 yr | Moderate |
| 1-2 small (< 10 mm) tubular adenomas | 5-10 yr | Moderate |
| 3-10 tubular adenomas | 3 yr | Moderate |
| > 10 adenomas | < 3 yr | Moderate |
| One tubular adenoma ≥ 10 mm | 3 yr | High |
| One villous adenoma | 3 yr | Moderate |
| Adenoma with high grade dysplasia (HGD) | 3 yr | Moderate |
| Serrated lesions | | |
| Sessile serrated polyp (SSP) < 10 mm with no dysplasia | 5 yr | Low |
| SSP ≥ 10 mm OR with dysplasia OR serrated adenoma | 3 yr | Low |
| Serrated polyposis syndrome | 1 yr | Moderate |

new lesions may have developed.

Although the risk of developing further adenomas is known, no randomised study has directly assessed the effect of post-polypectomy surveillance on CRC incidence or mortality. The efficacy of surveillance has been assessed by retrospective epidemiological series indicating that patients not entered into a surveillance programme have three- to fourfold greater risk of CRC. However, the increased risk pertains to those found to have advanced adenomas at the index procedure. Individuals with non-advanced adenomas did not have significantly higher risk than the general population^[23,60].

It is established that individuals with previously identified adenomas have an increased risk of further adenomas at a follow-up examination. At 4 year interval, 35.5% of patients will again be found to have at least one adenoma, but only 8.6%-12% will have advanced neoplasia (either an advanced

adenoma or carcinoma) with 0.6% having carcinoma. Factors conferring higher risk of further adenomas at surveillance are age greater than 60 years, male sex, and the presence of more than one adenoma at the initial procedure. The finding of more than 2 adenomas at initial examination increases the risk of advanced neoplasia at follow-up examination^[32,61].

STRATIFICATION

Reported prevalence of adenomas ranges from 15%-40%, with advancing age and male sex associated with increasing prevalence. However, rates of adenoma detection may be as high as 50% in the general population when using modern "high definition" endoscopes^[62,63]. Therefore the number of patients who could potentially be offered surveillance colonoscopy is substantial.

To avoid unnecessary, or "low yield", surveillance colonoscopies, it is necessary to identify those individuals with increased risk of CRC. This can be achieved through a risk stratification approach, as adopted by all the major current clinical guidelines (Tables 1-3).

Current guidelines vary in their definition of each risk group. However, there is consensus that individuals with one or two adenomas possessing no advanced features are classified as "low risk". At the opposite end of the spectrum, it is agreed that finding high grade dysplasia or greater than 10 adenomas confers a "high risk".

Current guidelines' variability in recommendations is due to the lack of good quality evidence to support surveillance strategy.

United Kingdom guidelines do not take account of polyp architecture, while guidance in the United States and Europe classifies individuals with a villous adenoma as "high risk".

Table 3 European Society of Gastrointestinal Endoscopy 2013^[81]

| |
|--|
| The following recommendations for post-polypectomy endoscopic surveillance should be applied only after a high quality baseline colonoscopy with complete removal of all detected neoplastic lesions |
| In the low risk group (patients with 1-2 tubular adenomas < 10 mm with low grade dysplasia), the European Society of Gastrointestinal Endoscopy (ESGE) recommends participation in existing national screening programmes 10 yr after the index colonoscopy. If no screening programme is available, repetition of colonoscopy 10 yr after the index colonoscopy is recommended (strong recommendation, moderate quality evidence) |
| In the high risk group (patients with adenomas with villous architecture or high grade dysplasia or ≥ 10 mm in size, or ≥ 3 adenomas), the ESGE recommends surveillance colonoscopy 3 yr after the index colonoscopy (strong recommendation, moderate quality evidence). Patients with 10 or more adenomas should be referred for genetic counselling (strong recommendation, moderate quality evidence) |
| In the high risk group, if no high risk adenomas are detected at the first surveillance examination, the ESGE suggests a 5-yr interval before a second surveillance colonoscopy (weak recommendation, low quality evidence). If high risk adenomas are detected at first or subsequent surveillance examinations, a 3-yr repetition of surveillance colonoscopy is recommended (strong recommendation, low quality evidence) |
| The ESGE recommends that patients with serrated polyps < 10 mm in size with no dysplasia should be classified as low risk (weak recommendation, low quality evidence). The ESGE suggests that patients with large serrated polyps (≥ 10 mm) or those with dysplasia should be classified as high risk (weak recommendation, low quality evidence) |
| The ESGE recommends that the endoscopist is responsible for providing a written recommendation for the post-polypectomy surveillance schedule (strong recommendation, low quality evidence) |

In a comparison of current United Kingdom and United States guidelines, it was found that following United Kingdom guidelines would better identify a group of patients at high risk of advanced neoplasia: those with ≥ 5 small adenomas or ≥ 3 adenomas including at least one of ≥ 10 mm. These patients would be offered a surveillance interval of 3 years according to United States guidelines or 1 year according to United Kingdom guidance. At one year follow-up, this group had an 18.6% risk of advanced neoplasia^[64].

Conversely, patients with 1 or 2 small adenomas would be classified as low risk by United Kingdom guidelines regardless of histology. This group could be at relatively high risk if histology revealed advanced adenomas (HGD or villous architecture) and as such would be advised 3 year surveillance under United States guidelines. The same group of patients could have been offered no surveillance by following United Kingdom guidelines, but have a 7.1% absolute risk of advanced neoplasia at 1 year^[64].

Current guidelines take account of findings at both the index and first surveillance colonoscopy in determining the second surveillance interval. This approach would be supported by a recent study showing that high risk features identified at either the index or first surveillance procedure increase the risk of advanced neoplasia at second surveillance^[65].

SURVEILLANCE INTERVALS

High risk

The evidence to support the use of surveillance applies predominantly to the "high risk" group. The incidence of advanced neoplasia and carcinoma in these individuals is significantly increased at follow-up, and CRC mortality is reduced by their surveillance^[33,59,60].

Data from the United Kingdom screening programme shows that in high risk individuals (by United Kingdom guidelines), the overall yield for advanced neoplasia at first surveillance (at 12 mo) was 6.6%,

with a yield of 0.8% for CRC. These findings would support the current strategy of 12 mo surveillance in this group^[66]. The same study found that villous architecture and a right-sided adenoma at the index procedure were associated with an increased risk of finding advanced neoplasia at 1 year follow-up. Therefore within the high risk group, there are other factors which could be used to further inform the appropriate surveillance interval for an individual.

Current United States guidelines classify patients with > 10 adenomas as highest risk. However, as only 0.1% of screening patients fall into this category, its clinical utility is limited.

Low risk

Within the low risk group, it is known that the absolute risk of advanced neoplasia at follow-up is low. Current guidelines are based on evidence that this group carries no increased risk of CRC compared to the general population^[23,25]. A recent meta-analysis suggested individuals in the low risk group at the index procedure have a higher risk of advanced neoplasia at follow-up compared to those found to have no adenoma^[67]. However, the absolute risk in both groups remains very low.

On the basis that the low risk group carry a risk of CRC equivalent to the general population, the guidelines advise surveillance at the interval prescribed by the relevant screening programme, *i.e.*, effectively advising no increased surveillance over that of the general population. The United Kingdom guidelines allow for deviation from this rule in that the low risk group may be offered no surveillance or a further procedure at 5 years. Of note, the United Kingdom NHS Bowel Cancer Screening Programme (BCSP), while following United Kingdom guidelines (BSG, 2010 and NICE, 2011), offers no surveillance in this group.

Recent data from Norway suggest a significant reduction in CRC mortality at 7.7 years in "low risk" patients after a single screening examination^[68]. However, the definition of "low risk" used in this study

differs from that used in current guidelines as the study authors used cancer registry data and so did not have access to details of polyp size or number. Therefore, all patients with “multiple” polyps or with histology showing either villous architecture or high-grade dysplasia were classified as “high-risk”. This definition makes comparison with other studies difficult.

Intermediate risk

Current guidelines differ most in recommendations for individuals with intermediate risk. It is in this group of patients that the benefit of surveillance is most uncertain.

Patients with 3 or 4 diminutive adenomas at index colonoscopy would be offered a surveillance procedure at 3 years according to United Kingdom, European, and United States guidelines. However, there is little evidence that this group of patients carries any significantly increased CRC risk compared to the general population.

There is evidence for the increased risk of identifying further adenomas at first surveillance in patients classified as intermediate risk at index procedure. However, the relative risk varies within this group of individuals dependent upon factors such as polyp size, patient age, and the presence of advanced adenoma at the index procedure, *i.e.*, with the varying definition of intermediate risk^[69]. Evidence for an effect of surveillance on CRC incidence and mortality is lacking.

Serrated lesions

American and European guidelines include serrated polyps in their recommendations, which are not specifically dealt with in United Kingdom guidelines.

Serrated polyps are known to be more challenging to identify at colonoscopy and their predilection for the proximal colon is thought in part to explain the relatively lower protective effect of colonoscopy on incidence of right-sided CRCs^[10].

Significant variability in detection of these lesions by endoscopists and their classification by pathologists has caused evidence on their natural history and risk profile to be lacking. However, further study and increased awareness of these lesions is likely to lead to further recommendations for surveillance in individuals found to have serrated polyps.

DISADVANTAGES AND LIMITATIONS OF SURVEILLANCE

At present, surveillance procedures account for 20%-30% of capacity in endoscopy departments: approximately the same proportion as primary screening procedures^[70-73]. It is likely that demand for surveillance procedures will increase in line with more widespread implementation of screening programmes,

rising adenoma detection rates associated with modern endoscopes and rising quality standards, and the increased recognition and surveillance of serrated lesions.

While colonoscopy is a generally safe procedure, there is a risk of major complications^[74]. As such, the decision to proceed with surveillance colonoscopy must be informed by both the risk of CRC and the risk of a complication related to the procedure. Additionally, even an uncomplicated colonoscopy may represent considerable burden on the patient, who undergoes bowel preparation, time off work, and potential discomfort during the procedure. Fear of pain during the procedure is known to reduce the uptake of screening colonoscopy^[75,76]. For surveillance programmes to be effective, uptake must be maximised. By definition, individuals invited for surveillance already have personal experience of colonoscopy. This experience is likely to inform the individual's decision on whether to undergo a surveillance procedure, highlighting the importance of patient experience during colonoscopy.

WHEN TO STOP SURVEILLANCE

The decision to discontinue surveillance is guided in current literature only on the criterion of the patient's chronological age^[77]. It is known that rates of complications and post-procedure hospital admission are increased with advancing age and multi-morbidity. Advancing age also reduces the potential survival benefit in surveillance: as progression from adenoma to carcinoma is likely to take around 10 years, patients with a life expectancy of a similar or shorter time have little chance of benefit from a surveillance colonoscopy.

However, the use of chronological age alone is an over-simplification of the decision to discontinue surveillance: a decision which must balance the relative risks for the individual.

Patients found at their initial procedure to have an advanced adenoma, have a CRC risk similar to that of the general population after just one surveillance follow-up colonoscopy^[23,59]. Further study is needed to identify more detailed criteria to guide the decision on continued surveillance.

ADHERENCE

There is strong evidence that adherence to current guidelines by physicians is highly variable^[78]. Some surveillance procedures are performed earlier than advised, some late, and some not performed at all. Clinical guidelines are only a guide to clinicians and many will choose to advise a different approach for an individual patient.

Also, patients may choose not to be subjected to surveillance procedures for multiple reasons including their experience of colonoscopy and the perceived benefits of surveillance. The subject of patient choice

Table 4 Adenoma surveillance

| Findings at index procedure | Suggested initial surveillance interval |
|---|---|
| No adenomas | No surveillance |
| 1-2 adenomas with no advanced neoplasia | No surveillance |
| 3-4 adenomas with no advanced neoplasia | 3 yr |
| ≥ 3 adenomas and advanced neoplasia | 1 yr |
| ≥ 5 adenomas | 1 yr |

in surveillance is an area requiring further study.

FURTHER STUDY

As discussed in the introduction to this paper, progression from adenoma to cancer usually occurs over many years. As such, the benefits of surveillance of colonic adenomas in reducing morbidity and mortality can only be realised over the long term. The introduction of surveillance programmes has become widespread only in recent years, so far limiting the available data on long-term follow-up. The known increased risk of CRC in patients found to have adenomas would make a randomised trial comparing surveillance to no surveillance unethical. Therefore, further study of the data from the era of widespread adenoma surveillance is needed to better inform future practice.

Current guidelines base recommendations on data collected prior to the widespread implementation of population screening programmes and prior to the use of robust quality metrics in colonoscopy. These factors may significantly alter the population classified within each risk group and so have a major impact on the outcomes of each group. More contemporary data from the era of high quality colonoscopy and population screening may allow more accurate risk stratification to better utilise limited colonoscopy resources in the future.

Future of adenoma surveillance

The Table 4 summarises suggested surveillance intervals based on current knowledge on risk stratification by polyp factors.

Polyp factors may be used, as in current guidelines, to determine surveillance interval. However, including other patient factors in this assessment may allow more accurate risk stratification. Possible factors include age, sex, family history of colorectal cancer, smoking status, or obesity.

Additionally, this combination of polyp and patient factors may further inform the decision on whether to continue with any further surveillance after the first surveillance procedure, as it is the first surveillance procedure that has greatest effect in reducing the future risk in the highest risk patients.

CONCLUSION

Internationally, increasing numbers of patients are embarking upon a course of surveillance colonoscopies due to the polyps discovered at the time of a previous examination. Each colonoscopy involves the burden of bowel preparation, potential anxiety and discomfort, and risk of complication for the patient. In many health settings, colonoscopy is a finite resource and so must be recommended only with a strong indication.

It is believed that individuals with non-advanced adenomas have no significantly increased risk of colorectal cancer compared to the general population. In addition, patients found at their initial procedure to have an advanced adenoma, have a CRC risk similar to that of the general population after just one surveillance follow-up colonoscopy^[23,59].

As shown in this review, there is some retrospective evidence to support surveillance procedures in patients at the highest risk of CRC. For those at lower risk, further evidence is needed to better stratify risk and so inform discussions between the individual and their clinician on whether surveillance colonoscopy is appropriate.

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2016 Hepatocellular Carcinoma: Global view

Combined locoregional treatment of patients with hepatocellular carcinoma: State of the art

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Abstract

In recent years, a combination of intervention therapies has been widely applied in the treatment of hepatocellular carcinoma (HCC). One such combined strategy is based on the combination of the percutaneous approach, such as radiofrequency ablation (RFA), and the intra-arterial locoregional approach, such as trans-arterial chemoembolization (TACE). Several types of evidence have supported the feasibility and benefit of combined therapy, despite some studies reporting conflicting results and outcomes. The aim of this review was to explain the technical aspects of different combined treatments and to comprehensively analyze and compare the clinical efficacy and safety of this combined treatment option and monotherapy, either as TACE or RFA alone, in order to provide clinicians with an unbiased opinion and valuable information. Based on a literature review and our experience, combined treatment seems to be a safe and effective option in the treatment of patients with early/intermediate HCC when surgical resection is not feasible; furthermore, this approach provides better results than RFA and TACE alone for the treatment of large HCC, defined as those exceeding 3 cm in size. It can also expand the indication for RFA to previously contraindicated "complex cases", with increased risk of thermal ablation related complications due to tumor location, or to "complex patients" with high bleeding risk.

Key words: Hepatocellular carcinoma; Combined treatment; Chemoembolization; Ablation; Microwave

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Core tip: A combination of imaging-guided local percutaneous and transarterial treatments of hepatocellular

carcinoma is a promising strategy for tumors exceeding 3 cm in diameter and for multifocal tumors that are not amenable to resective surgery and for which the use of a single locoregional treatment option is often inadequate. In this paper, we assessed the indications, the technical aspects, the clinical efficacy and the safety of the different strategies of combining local non-surgical treatments for hepatocellular carcinoma complicating liver cirrhosis. The aim of this review is to explain the technical aspects of different combined treatments and to analyze and comprehensively compare the clinical efficacy and safety of the combined treatment and monotherapy, either chemoembolization or RF ablation alone, in order to provide clinicians with an unbiased opinion and valuable information.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world. It is responsible for an estimated 1 million deaths annually and, in most cases, complicates the clinical course of liver cirrhosis, resulting in a poor prognosis due to its rapidly infiltrating growth^[1,2]. A careful multidisciplinary assessment of tumor characteristics, liver function, and physical status is required for proper therapeutic management. However, consensus about a common treatment strategy for patients with HCC has not been reached worldwide, even if several proposals have been published. The most recent one is the Barcelona-Clinic Liver Cancer (BCLC) staging classification and treatment schedule that is currently used for clinical management of patients with HCC.

According to the BCLC staging system, surgical approaches, including surgical liver resection (SR) and liver transplantation (LT), as well as image-guided tumor ablation, such as radiofrequency ablation (RFA), are regarded as potentially curative treatments for HCC but are only recommended in patients with early stage tumor. Patients diagnosed with intermediate stage HCC are candidates for trans-arterial chemoembolization (TACE), which has proven to control symptoms and prolong survival^[1]. However, it is important to emphasize that this option is considered as a palliative and not a curative treatment, characterized in most instances by an unsatisfactory long-term outcome due to the inability to achieve complete tumor necrosis. Furthermore, repeated TACE is often required to completely eradicate the residual tumors, but its efficiency is limited and the rate of tumor recurrence or

relapse after initial remission or stable disease is very high^[3-6].

However, the early stage also includes patients with a single large HCC exceeding 3 cm in diameter, and the intermediate stage includes many patients with very different presentations of HCC. Indeed, patients with 4 small HCC nodules, with multinodular unilobar or bilobar disease and well-compensated liver function, are all classified as intermediate stage. The principal purpose of research in this field should be to increase the rate of patients who are suitable for non-surgical curative treatment and, consequently, to reduce indications for palliation alone^[7].

In this scenario, the purpose should be to expand the indication for RFA, that is a curative treatment for nodules smaller than 3 cm, by increasing its effectiveness in the treatment of single larger HCC nodules and for use during the intermediate stage. To this end, a combination of intervention therapies has been widely developed and performed in recent years. One such combined strategy is based on the association of the percutaneous approach, such as RFA, and the intra-arterial locoregional approach, such as TACE^[8]. Several types of evidence have indicated the feasibility and benefit of combined therapy, despite some studies reporting conflicting results and outcomes^[9].

Hence, the aim of this review was to explain the technical aspects of different combined treatments and to analyze and comprehensively compare the clinical efficacy and safety of this combined treatment option and monotherapy, either TACE or RFA alone, in order to provide clinicians with an unbiased opinion and valuable information.

RATIONALE

It is well known that RFA is only indicated for early stage HCC patients with fewer than 3 tumors, due to the local range of the treatment action, and with tumors less than 3-cm in size, due to a complete response rate lower than 50% in larger lesions, which is clearly poor for a treatment intended to cure the tumor. Furthermore, a high rate of local recurrence in lesions exceeding the 3 cm threshold has been demonstrated even after an initial complete post-treatment response^[10].

To expand indication for RFA, we should look at the limitations of RFA. The first consideration is the number of lesions; due to its local range of action, patients with multiple monolobar nodules should be considered less ideal for RFA than TACE, which acts by combining ischemia, anoxia and chemotoxicity at the tumor level with a range of action that could be regional or global. On the other hand, when considering the lesion size, the RFA induced volume of coagulation necrosis should be increased, with the potential result of also completely treating lesions exceeding the 3 cm threshold. We know that the extent of coagulation

necrosis is a function of the energy delivered into the tumor, of the local tissue interaction and of the negative factor of heat loss due to perfusion-mediated tissue cooling. Indeed, lesions bordering a large vessel (> 3 mm) may not achieve complete necrosis due to the thermal protection provided by the adjacent blood flow, a phenomenon termed "heat sink". Blood flow promotes heat loss, and reducing or eliminating blood flow during the RFA procedure is known to increase the volume of the ablative zone as confirmed more than 10 years ago by Rossi *et al.*^[11]. More specifically, the authors demonstrated the strict relationship between the shape and size of radiofrequency induced thermal lesions and hepatic vascularization. This *ex vivo* study brought the same group to test this assumption *in vivo*, performing RFA after the occlusion of tumor blood supply, resulting in a significant increase in RFA coagulation necrosis^[12]. This study was essential in demonstrating that, in order to increase the volume of coagulation necrosis (ablation zone size), arterial occlusion of the tumor feeding vessels obtained using balloon occlusion, embolization, and chemoembolization can be combined with RFA.

On the basis of these results, we should alter our final goal of looking for a new bio-kill formula. We can achieve tumor death not only from using the RFA induced thermal damage or the TACE derived ischemic-cytotoxic injury. These treatments can be combined in order to overcome the drawbacks when they are applied alone.

COMBINATION OPTIONS STRATEGY

Several studies have evaluated a multimodal approach to increasing the effectiveness of single treatments for single large or intermediate stage HCC. The available data suggest that combined therapy with RFA and TACE is superior to RFA or TACE alone in preventing the incomplete necrosis of HCC and in improving patient survival, but it is not clear which is the best combination of these two procedures^[13-15].

The first and more common option is represented by TACE followed by RFA. TACE can reduce the cooling effect of hepatic blood flow by decreasing hepatic arterial flow and increasing the necrotizing effect of RFA therapy at the tumor level. Furthermore, the edematous change in the tumor tissue induced by ischemia and inflammation after TACE is expected to enlarge the area of tumor necrosis during RFA treatment, thereby increasing the ablation safety margin and reducing local recurrence.

The second option is to perform RFA followed by TACE. Instead of using only lethal heating, which is obtained with RFA, you can actually try to obtain a sustained anticancer effect from the sublethal heating created in the large area surrounding the heating zone. In this area we have a number of phenomena, including increased blood flow, increased vascular permeability and effects on multiple cell targets. TACE

performed after RFA could increase its therapeutic effect acting on the large zones of sublethal heating obtained during RFA application in tissues surrounding the electrode. In detail, the chemotherapy drug should be concentrated on a relatively small volume of residual viable neoplastic tissue, characterized by reduced cell resistance to the drug due to previous exposure to sublethal heating. Furthermore, the delivery of a chemotherapy drug could be enhanced by the reactive hyperemia induced by RFA application. The rationale of performing TACE after RFA was based on the hypothesis that there could be a loss in efficacy of the drugs used during TACE when they are exposed to high temperatures^[16]. Furthermore, TACE performed before RFA could have a negative impact on the quality of the ultrasonography (US) images; as a matter of fact, after TACE, the lesions become hyperechoic, thereby hampering the identification of viable tissue in the tumor area.

The last option, described in our previously published paper, could be to perform a single-step combination therapy, applying RFA to the lesion during balloon-occlusion of the hepatic artery supplying the tumor, thereby enhancing the thermal damage, followed by selective TACE to enhance the cytotoxic injury^[17]. In detail, balloon occlusion of the tumor arterial supply increases the area of coagulation necrosis (ablation zone size) obtained with RFA, reducing arterial blood flow and minimizing heat loss, as already shown by other authors^[12,18].

A demonstration of the superiority of one approach over the other is not possible due to the lack of randomized comparative studies.

TIME-INTERVAL OPTIONS STRATEGY

To date, there has been no clear consensus about the time interval between TACE and RFA for balancing local therapeutic efficacy and safety. In a study by Choe *et al.*^[19], it was reported that the time-interval between TACE and RFA treatments should be chosen carefully to achieve a balance between successful tumor eradication and adequate preservation of liver function. A longer time interval between the two treatments might preserve liver function because sufficient time is allowed for hepatic functional recovery^[19]. However, this extended time prolongs the hospital stay required or may increase the number of patient admissions to the hospital. Conversely, a short interval can lead to better local efficacy because of the more synergistic effect of the combination of TACE and RFA. However, a short interval might increase the potential risk of liver function injury, mainly in cirrhotic patients with mild to moderate liver dysfunction.

In our opinion, only using a single-step "combined" approach makes it possible to obtain and amplify the synergistic effects of RFA and TACE. This approach entails further relevant advantages, such as the reduction of hospitalization days, decrease in patient

discomforts, and cost saving due to the performance of both procedures in the same session. However, in many published papers, the treatments were performed during different sessions, separated by a time-interval of 1-30 d^[10,20,21]. In fact, the administration of treatments in sequential order is common practice in clinical medicine, particularly when a treatment fails; it could also be performed per protocol independently from the partial effectiveness or failure associated with a specific therapy. In other words, instead of administering different therapies together, there is planned sequential administration based on some specific effects induced by each therapy, which provides additional benefits over time. Based on this definition, it could be more appropriate and correct to define this approach as "sequential treatment" and not "combined treatment". In our opinion, when dealing with the association of different locoregional HCC treatments, it should be mandatory to distinguish between sequential and combined treatments in order to provide more comparable results to the scientific community.

TREATMENT PROCEDURES

All of the combined treatments should be performed in a single-step approach, using antibiotic prophylaxis, patient monitoring and anesthesiological assistance, in an angiographic suite that has the structural characteristics of an operating room.

TACE

Hepatic artery angiography is usually performed through a right common femoral approach to map liver vascular anatomy, check for arteriovenous shunts and identify the arterial tumor supply. A superselective catheterization and chemoembolization is performed using a coaxial technique and by placing a 2.7-Fr microcatheter (Progreat; Terumo, Tokyo, Japan) into the distal segmental hepatic artery feeding the HCC. In the case of balloon-occluded RFA, a 0.014-inch guide wire is advanced into the segmental hepatic artery feeding the lesion, enabling optimal guidance of a low-profile 4-5 mm monorail percutaneous transluminal angioplasty (PTA)-balloon. Conventional chemoembolization is performed by infusing an emulsion of chemotherapeutic agent (Epirubicin-Doxorubicin/Cisplatin/Mytomicin-C) and iodized oil (Lipiodol Ultra Fluid; Mitsui, Tokyo, Japan), followed by embolization performed with gelatin sponge particles (Gelfoam; Pfizer, Tokyo, Japan). Drug-eluting bead TACE is performed with a slow injection of a 100-300 μ m DC-Bead (Terumo, Tokyo, Japan) loaded with Epirubicin (Farmorubicin® 50 mg Powder) until the complete intended dose is administered and slow flow is observed.

RFA

Before or after TACE, RFA is usually performed using

US-guidance with the patient under sedation with Fentanyl citrate (0.1-0.2 mg, Phentanest; Daiichi Sankyo, Tokyo, Japan) and local anesthesia. In detail, US-guidance has been most widely implemented as the standard guiding modality for RFA (Figure 1). The advantages of US are several, including easy availability, lower cost and real-time multiplanar imaging capability. However, ultrasound also has serious drawbacks. For multiple overlapping ablations, the characteristic hyperechoic area of microbubbles generated by previous ablation cycles often obscures the index tumor and may hinder accurate placement of the electrode for subsequent ablation cycles. Furthermore, ultrasound-guided targeting is difficult for tumors in sonographic blind spots, such as the liver dome. In addition, for combined treatment, prior chemoembolization may alter the sonographic conspicuity of the index tumor owing to variable uptake of iodized oil and the chemotherapeutic agent in the tumor and the adjacent hepatic parenchyma. For this reason, computed tomography (CT) fluoroscopy has also been used to guide RFA alone or RFA combined with TACE (sequential approach)^[22-24]. It provides several contiguous axial images through near real-time image reconstruction during the interventional procedure. When the CT plane includes both the electrode path and the index tumor, the needle advancement into the tumor can be monitored in real-time. One major drawback of this guiding modality is the high radiation dose to both patient and operator, which is on the order of centigrays per second of exposure, whereas conventional fluoroscopy is on the order of centigrays per minute of exposure^[25]. This concern is obviously magnified for cases requiring multiple overlapping ablations. Furthermore, targeting the index tumor tends to be more time-consuming than other guidance modalities, such as ultrasound and conventional fluoroscopy. For a tumor in the dome of the liver, oblique advancement of the electrode is preferred to avoid violation of the thorax or the pleura. However, such an oblique approach might have been technically cumbersome with CT fluoroscopy guidance owing to a limited range of CT gantry tilting. Although the transthoracic approach for dome lesions has been generally accepted as safe, pneumothorax can complicate 38%-70% of cases, of which 18%-40% will require chest tube placement^[13,26,27]. Alternatively, the oblique approach for a dome lesion under CT guidance with coronal/sagittal reformatted imaging is also useful to avoid this complication.

Another potential guiding-technique is represented by biplane fluoroscopy^[28]. For combined treatment, this technique has several potential strengths. First, the visible fluoroscopically index tumor can be easily targeted regardless of location and successfully ablated with a single session procedure. Furthermore, registration and fusion of intraprocedural ultrasound with pre-procedural CT, magnetic resonance imaging (MRI) or positron emission tomography images

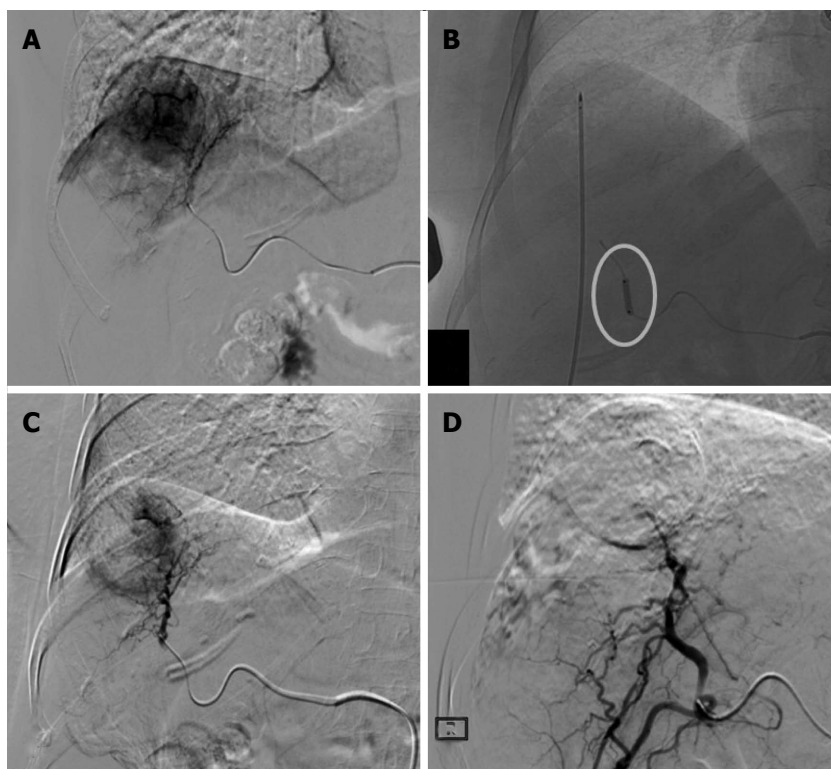


Figure 1 Treatment procedure. A: Hepatocellular carcinoma in SVIII (4.5 cm in size) confirmed on digital subtraction angiography; B: Radiofrequency ablation (RFA) electrode placed into the tumor under ultrasonography-guidance with ablation performed during balloon-occlusion (circle); C: Post-RFA digital subtraction angiography showing the central devascularized area with peripheral reactive hyperemia; D: Complete devascularization obtained with superselective Drug-eluting bead trans-arterial chemoembolization.

have been reported as feasible for thermal ablation of liver tumors^[29,30]. With these fusion techniques, inconspicuous dome HCCs on ultrasounds could be targeted for percutaneous RFA. Second, proper needle placement into the tumor can be more confidently made because biplane fluoroscopy provides real-time orthogonal projectional imaging for the simultaneous delineation of the electrode and the index tumor. Enhanced targeting confidence may shorten the overall procedure time, especially for cases requiring multiple overlapping ablations. In addition, fluoroscopy provides much higher resolution and artifact-free images than CT or CT fluoroscopy, allowing for more precise overlapping ablations. Unlike ultrasounds, microbubbles generated by previous cycles of ablation do not obscure the radio-opaque index tumor in fluoroscopy. Third, this method allows for a greater degree of freedom for electrode insertion than ultrasounds or CT guidance. This is pertinent not only for liver dome tumors as described above but also for subcapsular tumors that may be better accessed through an oblique approach with biplane fluoroscopy than with ultrasound or CT to avoid direct puncture and to minimize the risk of bleeding or seeding^[31]. However, a major drawback of this guiding method is obviously that fluoroscopy cannot provide cross-sectional imaging with the soft-tissue contrast available when using ultrasound or CT. Therefore, ultrasound as an accessory guidance modality is required to

avoid a traversal of critical intrahepatic or extrahepatic structures during targeting and to estimate the ablation zone. Future perspectives could be based on the use of MRI to guide RFA alone or in combination with TACE in a sequential approach^[21].

CLINICAL INDICATIONS

Combined vs curative treatments

A recent meta-analysis showed that RFA plus TACE significantly improved the overall survival (OS) rates at 1 and 3 years compared with RFA alone in patients with a single HCC, without significant differences in major complications. Subgroup analyses by tumor size showed that RFA plus TACE significantly improved the OS at 1, 3 and 5 years compared with RFA alone for patients with HCC larger than 3 cm. However, there was no advantage for patients with HCC smaller than 3 cm; the reason for this may be that RFA alone can already achieve complete necrosis in treating small (< 3 cm) HCC nodules, suggesting that adding TACE to RFA could be redundant^[32].

There are conflicting results in the literature when analyzing the comparison between combined treatment and surgical resection (SR). Some retrospective studies have suggested that TACE-RFA may yield OS rates comparable to those from SR^[8,12,33]. Yamakado *et al.*^[34] reported that patients with early-stage HCC who underwent TACE-RFA had OS and disease-free survival

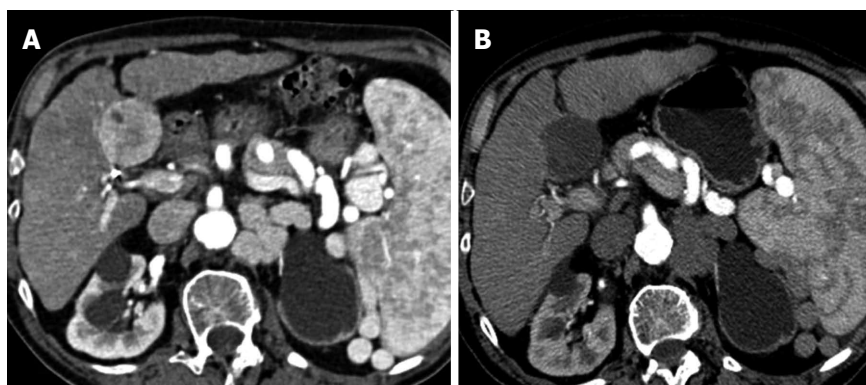


Figure 2 Complex lesion. A: Hepatocellular carcinoma in SV (5 cm in size), located on the intra-abdominal free-surface, adjacent to a gastrointestinal structure; B: Combined treatment allows obtaining a central safe necrosis with radiofrequency ablation (RFA); subsequent post-RFA trans-arterial chemoembolization was used to treat a peripheral portion of the tumor, obtaining a safe complete response.

(DFS) rates similar to those found in patients who underwent SR. In contrast, Kagawa *et al.*^[41] reported that compared with SR, TACE-RFA in patients with early-stage HCC yielded a similar rate of OS but a lower rate of DFS. In a study by Takuma *et al.*^[35], the OS and DFS rates after TACE-RFA in patients with early stage HCCs within the Milan criteria were significantly lower than those observed after SR. These findings may be explained by differences in baseline patient characteristics; in fact, after adjusting for propensity score matching, patients who underwent TACE-RFA had a similar OS rate but had poorer DFS compared to patients who underwent SR. The difference in DFS rates may be mainly due to local tumor progressions, which are higher after TACE-RFA. Based on published papers, it seems that TACE-RFA is safe and may confer an OS rate comparable with that of SR after adjusting for potential confounders. However, SR improves the DFS rate compared with the rate attained with TACE-RFA. We can conclude that combined treatments may be considered an effective treatment modality in patients with early/intermediate HCC when SR is not feasible.

Combined vs palliative treatments (TACE)

The combined use of TACE and RFA may have an advantage over TACE alone in the treatment of HCC because they are mutually complementary, thereby significantly improving the efficacy, quality of life and long-term survival of HCC patients^[33,36,37]. In detail, TACE plus RFA increases the chance of peritumoral satellitosis clearance, reduces the possibility of tumor recurrence, thereby enhancing the possibility of complete tumor necrosis and conceivably improves the OS rate. In a recent study, we demonstrated that balloon-occluded RFA immediately followed by DEB-TACE was effective at achieving prolonged local disease control in single large HCCs (> 3 cm), with a sustained complete response in 62.5% of the treated lesions, a 2-year cumulative HCC recurrence rate of 48.1%, and an overall survival rate of 91.1%,

which is significantly better than that achieved in a comparable control group of patients treated with DEB-TACE alone^[38]. It is worth noting that these promising results were obtained in a single-step procedure without significantly increasing the procedural time, and the benefit of combined therapy was not offset by any important side effects or worsening of liver function, as none of our patients experienced an increased Child-Pugh score at 1-mo post treatment. Furthermore, in patients with lesions > 5 cm, a large necrotic area was obtained in most cases, and a sustained CR was achieved in about half of the cases with a mean number of only 1.3 procedures per patient. This implies the potential reduction of the therapeutic procedures number, and consequently of the liver function failure risk. Finally, in some cases, the combined therapy appeared to be promising as an effective bridge treatment to liver transplantation.

Complex lesions/complex patients

Combined treatments could expand the indication for RFA to previously contraindicated cases^[39]. In detail, it could be possible to effectively also treat complex lesions with RFA, *i.e.*, hepatic tumors adjacent to the diaphragm with a consequent high risk of thermal injury, or tumors located next to the intra-abdominal free surface, or proximal to the hepatic portal region. As a matter of fact, the aim to ensure a safety margin is tempered by the high risk of damaging big arterial or portal vein vessels, bile ducts, or intestinal loops with subsequently serious complications, such as hepatic infarction, biloma, abscesses or intestinal perforation. On the other hand, less aggressive treatments, using lower RFA power or shortened exposure time to the RFA needle, may result in local recurrence. Taking this into consideration, in these cases the RFA should be limited to the tumor portions located far from the diaphragm or intra-abdominal free surface as well as adjacent to vascular/biliary structures; these tumor portions can then be treated with post-RFA chemoembolization in order to obtain an effective and

secure safety margin (Figure 2). Finally, it's noteworthy that using this single-step approach could make it possible to also safely treat "complex patients" with high risk for bleeding complications without requiring blood transfusion or other prophylactic treatment. As a matter of fact, transarterial chemoembolization performed after RFA could effectively and immediately treat any eventual RFA-induced hepatic bleeding.

CONCLUSION

The combined use of TACE and RFA (combined treatment) is a safe and effective option in the treatment of patients with HCC. In detail, combined treatments may be considered an alternative treatment modality in patients with single large or multinodular HCC when surgical resection is not feasible. In particular, this approach seems to provide better results than RFA and DEB-TACE alone for the treatment of large HCC exceeding 3 cm in size, significantly improving the efficacy, quality of life and long-term survival of patients. Finally, it could also expand the indication for RFA to previously contraindicated "complex cases", in which the application of RFA alone entails an increased risk of complications, or to "complex patients" with high risk of RFA-related bleeding complications.

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2016 Hepatocellular Carcinoma: Global view

Hepatocellular carcinoma and hepatitis B surface protein

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Abstract

The tumorigenesis of hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC) has been widely studied. HBV envelope proteins are important for the structure and life cycle of HBV, and these proteins are useful for judging the natural disease course and guiding treatment. Truncated and mutated preS/S are produced by integrated viral sequences that are defective for replication. The preS/S mutants are considered "precursor lesions" of HCC. Different preS/S mutants induce various mechanisms of tumorigenesis, such as transactivation of transcription factors and an immune inflammatory response, thereby contributing to HCC. The preS2 mutants and type II "Ground Glass" hepatocytes represent novel biomarkers of HBV-associated HCC. The preS mutants may induce the unfolded protein response and endoplasmic reticulum stress-dependent and stress-independent pathways. Treatments to inhibit hepatitis B surface antigen (HBsAg) and damage secondary to HBsAg or the preS/S mutants include antivirals and antioxidants, such as silymarin, resveratrol, and glycyrrhizin acid. Methods for the prevention and treatment of HCC should be comprehensive.

Key words: Hepatitis B surface protein; Hepatocellular carcinoma; PreS/S mutants; Endoplasmic reticulum stress; "Ground Glass" hepatocytes

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Core tip: The tumorigenesis of hepatitis B virus-associated hepatocellular carcinoma (HCC) has been widely studied. The preS/S mutants are considered "precursor lesions" of HCC. Different preS/S mutants induce various mechanisms of tumorigenesis, such as transactivation and an inflammatory response. The preS2 mutants and type II "Ground Glass" hepatocytes represent novel biomarkers of HCC. The preS mutants may induce the unfolded protein response and endoplasmic reticulum stress-dependent and stress-

independent pathways. Treatments to inhibit hepatitis B surface antigen (HBsAg) and damage secondary to HBsAg or the preS/S mutants include antivirals and antioxidants. Methods for the prevention and treatment of HCC should be comprehensive.

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INTRODUCTION

There are approximately 387 million carriers of hepatitis B virus (HBV) worldwide, and one-quarter of them will develop hepatocellular carcinoma (HCC)^[1]. In HBV-endemic regions, chronic hepatitis B (CHB) is a primary risk for HCC^[2]. Epidemiological studies have provided overwhelming evidence for a causal role of CHB infection in HCC development^[3]. The risk for developing HCC increases from 6 to 37 times in patients with different statuses of HBV infection compared with control subjects^[4-8]. In one study, the relative risk of HCC was increased by approximately 100-fold in HBV carriers compared with noncarriers^[9]. Most of the HCC cases occur at the advanced stage or the anti-HBe-positive phase, with the peak incidence occurring in the sixth decade^[10]. However, studies of HBV-induced tumorigenesis are widely debated^[3].

STRUCTURE AND ROLE OF SURFACE PROTEINS IN HBV

The HBV genome of hepadnaviruses is a relaxed circular, partially double-stranded DNA (RC-DNA) structure^[11]. The RC-DNA is converted into a template for the transcription of viral RNAs - covalently closed circular molecules - in the nucleus^[12-14]. Three major unspliced transcripts, with sizes of 3.5 kb, 2.4 kb, and 2.1 kb, and a less abundant 0.8 kb transcript with a common polyadenylation site, all code for the viral proteins; the 3.5 kb pregenomic RNA also serves as a template for viral replication through a reverse transcription mechanism^[14]. The 3.2 kb HBV genome has four overlapping open reading frames (ORFs)^[3]: the preC/C ORF encodes the e antigen (HBeAg) and core antigen; the P ORF encodes the terminal protein (TP) and viral polymerase that possesses DNA polymerase, reverse transcriptase, and RNase H activities; and the X gene encodes hepatitis B x protein (HBx) for virus replication. The spliced viral transcript encodes a viral protein termed the hepatitis B spliced protein (HBSP)^[15]. The preS/S ORF overlapping the HBV polymerase ORF encodes the three viral surface proteins. Three co-terminal envelope proteins termed

large (LHBs, including the preS1 + preS2 + S domain), middle (MHBs, including the preS2 + S domain), and small (SHBs, the S domain alone) surface proteins, respectively^[16]. Additionally, truncated and mutated preS2/S (the LHBs and truncated MHBs) or HBx proteins are produced by integrated viral sequences defective for replication^[17,18]. The viral surface proteins are important for the HBV structure and life cycle because HBsAg reflects the transcriptional activity of the cccDNA^[19]. The small surface proteins (SHBs) are major components of the virion envelope and the nucleocapsid-free subviral particles^[20]. The HBV envelop proteins and capsids (with the HBV genome) are assembled on the viral particles in the endoplasmic reticulum (ER) and are discharged from the cell^[21,22]. The viral surface proteins may interact with a host cell receptor to initiate the infection^[3]. However, more than a dozen host-binding proteins to the preS1, preS2, or S domain have been identified^[22]. PreS1, but not preS2, which is myristoylated at the glycine residue in position 2, is essential for virus infection^[23,24]. The preS1 domain has a receptor binding site that contains the essential aa residues 9-18 and recognizes the asialoglycoprotein receptor on the surface of human hepatocytes or HCC cells^[25-27]. HBV initially combines with heparan sulfate proteoglycans (HSPGs) that are trapped within the liver in the space of Disse^[20]. Sodium taurocholate cotransporting polypeptide (NTCP) is a binding partner for the myristoylated peptide 2-48 of the preS1 domain. Additionally, hepatitis delta virus uses HBV envelope proteins for its transmission^[16]. However, the initial phases of HBV infecting hepatocytes, namely virion attachment, uncoating, and entry, are not completely identified^[3]. Another important role of HBV surface proteins is in tumorigenesis, particularly in HCC, which has complex and heterogeneous features^[3,28].

HBV PROTEINS AND GENOME IN HEPATOCARCINOGENESIS

HBV tumorigenesis involves inflammation and liver regeneration, HBV gene mutations, and viral (mutant) oncoproteins^[4,13,29,30]. HCC-associated signaling pathways include the Wnt/b-catenin signaling^[31], the p14ARF/p53 pathway^[32], transforming growth factor alpha (TGF- α) signaling, Ras/mitogen-activated protein kinase (MAPK) signaling, and phosphatase and tensin homolog/Akt and mammalian target of rapamycin (mTOR) pathways^[33].

Both HBeAg and HBV genotype C infections are considered risk factors of HCC^[3,34]. Because reverse transcriptase lacks proofreading activity, HBV gene mutations occur more frequently than in other DNA viruses^[35]. Various mutations can predict HBV-associated HCC during long-term infection^[35]. HCC-associated mutations include the preS region in HBV genotype C, C1653T in enhancer II, as well as T1753V and A1762T/G1764A in the basal core promoter in HBV genotype C. These mutations alone or in combination can predict

HCC development in 80% of all cases^[36-38]. HBSP can promote carcinogenesis^[39] as well as hepatoma cell motility and invasion^[40]. Occult HBV infection (OBI, positive for HBV-DNA, negative for serum HBsAg) may produce proteins with transforming properties that contribute to hepatocellular transformation^[41,42]. HBV DNA integration may frequently regulate key cellular pathways of carcinogenesis^[42], contribute to cis- or trans-activation^[3,43-47], and influence gene families that are involved in cell survival, proliferation, and immortalization^[42].

Both HBx and HBs (mutant) proteins are designated "viral oncoproteins". The HBx protein has been studied extensively^[3]. Tumorigenesis of the HBx protein may influence the regulation of the cell cycle, signaling pathways, DNA repair^[1,48-50], chromosomal instability^[51], cell transcription^[52,53], proliferation, and inflammation and immune responses^[54-56]. The HBs (mutant) proteins, primarily preS mutants, are recognized as "precursor lesions of HCC"^[57] and a risk factor for the post-operative recurrence of HCC^[58]. The tumorigenesis of HBs (mutant) proteins has also been widely studied.

CLINICAL ASSOCIATION BETWEEN HEPATITIS B SURFACE PROTEINS AND HCC

HBsAg has primarily been considered a marker of HBV infection. Since the two methods for HBsAg quantification, the Architect HBsAg assay (Abbott Diagnostics, Abbott Park, IL, United States)^[59] and Elecsys HBsAg II quant assay^[60], first became available, serum HBsAg levels have been found to reflect the activity of intrahepatic cccDNA^[61]. These methods may evaluate HBV replication more accurately.

HBsAg levels vary significantly according to the different courses of HBV infection^[62]. They have proven useful in judging the natural disease course and guiding treatment in addition to HBV DNA and HBV envelope antigen and antibody^[61]. CHB is classified into five phases: the "immune tolerant phase", "immune active phase", "inactive HBV carrier state", "HBeAg-negative CHB phase", and "HBsAg-negative phase"^[63,64]. In HBeAg-positive patients, the HBsAg levels are associated with fibrosis and immune tolerance; lower HBsAg levels are associated with moderate to severe fibrosis^[65,66]. In HBeAg-negative patients, an HBsAg level less than 1000 IU/mL was associated with a lower risk of HCC^[67]. The absolute lowest risk with a cumulative risk for HCC decreased less than 1% over 9 years after the patients had anti-HBs and anti-HBe seroconversion^[68]. However, the prediction^[68] may not apply to preS mutants because these mutants may account for the different levels of HBsAg^[61] and have a close association with HCC.

EPIDEMIOLOGY OF PRES MUTANTS

PreS mutants evolve in the long course of CHB, possibly for immune pressure, or antivirals^[3,7]. The frequency of preS mutants increases successively in the different stages of CHB infection. A meta-analysis showed that it was approximately 10%, 20%, 35%, and 50% in asymptomatic HBsAg carriers, CHB patients, patients with liver cirrhosis, and HCC patients, respectively^[36]. Su *et al.*^[69] reported that these mutants are present in up to 63% of HCC patients. The prevalence of the preS mutants varied in different countries and HBV genotypes; there was a higher prevalence in genotype B and C than in the other genotypes^[70]. The mutants are located in both the preS1 and preS2 regions^[69,71]. The preS2 mutants occur more frequently than the preS1 mutants^[36], possibly because preS1 is essential for virus infection. The preS2 mutants often coincide with changes in human immune cell epitopes^[71].

DIFFERENT PRES MUTANTS INDUCE VARIOUS MECHANISMS THAT CONTRIBUTE TO HCC

The preS1/preS2/S sequence encodes a transcriptional activator with potentially transforming properties^[17,72,73]. These transcriptional effects can activate the protein kinase C-dependent c-Raf-1/MAP1-kinase signal pathway, thus promoting transcription factors to increase the proliferation rate of hepatocytes^[17]. Only the carboxy-terminal truncation of LHBs or MHBs has transactivating properties^[1]. The truncated LHB protein expressed in transgenic mice resulted in the development of HCC^[74]. The transactivating effects of MHBs are mediated *via* sequence-specific binding to DNA^[75,76], thereby stimulating promoter sequences of the c-myc, c-fos, and c-Ha-ras oncogenes^[77,78]. Both LHBs and MHBs proteins have the same transcriptional effects^[73,79].

Subsequent studies have reported tumorigenesis in several mutants. An HBV polymerase rtA181T/surface truncation mutant in a patient with advanced HCC transactivated the simian virus 40 and human c-Myc promoters; the tumorigenic effects of the mutant were identified in nude mice^[80]. Three mutations, sL95*, sW182*, and sL216*, activated cell proliferation and transformational abilities; the sW182* mutant demonstrated potent tumorigenic activity. However, the three mutants could not promote ER stress^[81]. A W4P/R mutation in the LHB region of HBV genotype C may contribute to HCC development in an interleukin (IL)-6-dependent manner only in male patients^[82]. *In vitro*, the MHBst167 mutants interacted with the proteins related to tumor development/progression^[83]. Because the preS mutants varied in different research

groups, it is difficult to investigate the corresponding management.

PRES MUTANTS INDUCE ER STRESS THAT CONTRIBUTES TO HCC

HBV proteins utilize the ER protein folding machinery and cellular secretory pathway^[84]. Therefore, the underlying mechanisms of preS mutants that contribute to HCC are involved in ER stress. Both preS1 and preS2 mutants activate ER stress in hepatocytes^[85]. Different preS mutants activate differential activities of ER stress in hepatoma cells with accumulated LHB proteins^[86].

PreS1 mutants in HBV tumorigenesis

A previous study showed that HBV preS1 mutants demonstrate hepatocarcinogenesis effects through the transactivation of the TGF- α gene^[87]. PreS1 mutant activated higher levels of ER chaperones (Grp78 and 94), calcium release, cyclooxygenase-2 (COX-2), inflammatory cytokines, and oxidative stress intermediates, which tend to result in apoptosis^[85]. The HBV preS2 mutant proteins play a more important role in ER stress.

“Ground glass” hepatocytes accumulated with preS mutants

“Ground glass” hepatocytes (GGHs) comprise abundant particles of surface antigens that accumulate in the ER during CHB infection^[88-90]. Su *et al.*^[85] found that type II GGHs distributed in large clusters express marginal HBs proteins that harbored preS2 mutants and usually emerge at the late nonreplicative stage or in cirrhotic liver; type I GGHs, which accumulate with inclusion-like HBs (small surface protein) proteins and harbor preS1 mutants, are usually scattered sporadically during the replicative phases^[86]. Type II GGHs with preS2 mutants are suggested biomarkers of HCC and can predict recurrence and survival in HBV-infected HCC patients^[91].

The mutated HBV surface proteins cannot be properly folded in the ER, possibly leading to the induction of the unfolded protein response (UPR)^[90]. Both HBV surface proteins and HBx protein can trigger the UPR^[92,93]. HBV SHBs activate the UPR and host autophagy to enhance HBV envelopment and replication^[94]. However, HBV also activates the ER degradation-enhancing mannosidase-like proteins (EDEMs) to enhance the degradation of HBV surface proteins (terminally misfolded glycoproteins), thus relieving ER stress during the UPR^[95]. The mechanisms might maintain the balance between viral loads and host cells to facilitate the persistence of HBV infection. This mechanism may direct us to a new treatment strategy. However, the pathogenic role of the UPR in HBV infection and HCC appears to be complex. A drug (similar to EDEM) that relieves ER stress to prevent

HCC might facilitate persistent HBV infection and *vice versa*. These mechanisms require further study.

Pres2 mutants induce ER stress-dependent and stress-independent pathways

The preS2 mutant protein accumulated in the ER can trigger the ER stress-dependent vascular epithelial growth factor/Akt/mTOR and nuclear factor kappa B/COX-2 signal pathway^[96]. Through this signal pathway, HBx and envelope proteins that accumulate simultaneously in GGHs can enhance the oncogenic effects in transgenic and human HCCs^[97]. mTOR can suppress autophagy and regulate cellular metabolism^[98]. The suppression of autophagy by mTOR^[98] is in contrast to the induction of autophagy during UPR^[94]. This might result in contradictory treatment for HCC and HBV infection^[57].

The LHBs protein with preS2 mutants may also activate an ER stress-independent pathway, ultimately leading to a growth advantage in type II GGHs. The pathway includes c-Jun activation domain binding protein 1 nuclear translocation and activation of p27/retinoblastoma/Cdk2/cyclin A, D pathways, which results in cell cycle progression, cell proliferation, and centrosome over-duplication^[99-101].

Together, the data show that the preS2 mutant protein is a promising gene transactivator and an ER stress inducer, resulting in host genomic instability^[85] and HCC development.

HBV SURFACE PROTEINS INDUCE AN IMMUNE INFLAMMATORY RESPONSE CONTRIBUTING TO HCC

HBV-induced chronic necroinflammation plays an indirect role in hepatocarcinogenicity in the preS/S transgenic mice^[74]. The LHBs proteins in the ER triggered inflammation, abnormal regeneration and transcription, resulting in cancer development^[102]. This mechanism is similar to the contribution of the W4P/R mutation to HCC development in an IL-6-dependent manner^[84]. Another immune-associated carcinogenesis mechanism is viral immune escape induced by HBV mutants^[103]. HBV escape from the host's immune surveillance may favor the clonal proliferation of hepatocytes with the preS mutants^[104].

HBV surface proteins may be detected in immune cells. In dendritic cells, both HBV and HBsAg abrogated the CpG-A/TLR9-induced signal pathway and decreased the levels of co-stimulatory molecules and cytokines; this mechanism may contribute to HBV persistence^[105].

In addition, microRNAs may contribute to carcinogenesis in HBV-induced HCC. Both HBx and HBs proteins can increase microR499a expression, which might play an oncogenic role by targeting MAPK6^[106].

The duration of time between when HBV first enters a host and HCC development is long. A large

Table 1 PreS1/S2/S and hepatocellular carcinoma carcinogenesis

| Function | PreS1/S2/S mutation | Ref. |
|---|--|---|
| <i>A trans-activator function</i> | Integration of HBV preS/S sequences | Casemann <i>et al</i> ^[72] 1990, Kekulé <i>et al</i> ^[73] 1990 |
| Transactivation of NF-κB, or AP and other transcription factors for transactivation, stimulating promoter sequences of the c-myc, c-fos, and c-Ha-ras oncogenes | MHBst | Meyer <i>et al</i> ^[77] 1992, Lauer <i>et al</i> ^[78] 1994 |
| A transcriptional activator, activated tumor promoter pathways via the PKC-dependent c-Raf-1/ Erk2, MAP1- kinase signal pathway, increase the proliferation rate of hepatocytes | LHBs, the preS2 | Hildt <i>et al</i> ^[17,73,79] 1996, 1998, 2002 |
| Transactivator with DNA-binding properties | HBV surface (S) | Alka <i>et al</i> ^[76] 2000 |
| Transactivated the simian virus 40 and human c-Myc promoters truncation mutant | Polymerase rtA181T/surface | Lai <i>et al</i> ^[80] 2008 |
| Activated cell proliferation and transformational abilities | sL95*, sW182*, and sL216* | Huang <i>et al</i> ^[81] 2014 |
| Interacted with the proteins related to tumor development/ progression <i>in vitro</i> | MHBst167 | Li <i>et al</i> ^[83] 2014 |
| Biomarkers of HCC and predictor of recurrence survival | GGHs harbored preS2 mutants in the ER | Malhi <i>et al</i> ^[90] 2011, Su <i>et al</i> ^[85] 2014 |
| <i>Trigger the ER stress-dependent pathway</i> | | |
| VEGF/ Akt/mTOR and NF-κB/ COX-2 signal pathway in GGHs | | Yang <i>et al</i> ^[96] 2009 |
| Enhance the oncogenic effects in transgenic and human HCCs | Co-expressing hepatitis B virus X protein and surface antigens | Wu <i>et al</i> ^[97] 2014 |
| <i>An ER stress-independent pathway</i> | | |
| Activate JAB1 nuclear translocation and activation of p27/ retinoblastoma/ Cdk2/ cyclin A, D pathways, results in cell cycle progression, cell proliferation, and centrosome over-duplication, a growth advantage in type II GGHs | LHBs with preS2 mutants | Wang <i>et al</i> ^[99] 2005, Hsieh <i>et al</i> ^[101] 2011, Wang <i>et al</i> ^[100] 2012 |
| <i>Immune-associated carcinogenesis</i> | | |
| HBV-induced chronic necroinflammation | Pre S/S transgenic mice | Chisari <i>et al</i> ^[102] 1989 |
| The clonal proliferation of hepatocytes with the preS mutants | The HBV mutants escape from the host's immune surveillance | Zhong <i>et al</i> ^[104] 1999 |
| Contributing to HCC in an IL-6-dependent manner only in male patients | A W4P/R mutation in the LHB region of HBV genotype C | Lee <i>et al</i> ^[82] 2015 |
| <i>Other</i> | | |
| Increase microR499a expression, which might play an oncogenic role by targeting MAPK6 | HBs proteins | Xiang <i>et al</i> ^[106] 2014 |

HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; LHBs: Hepatitis B virus large surface protein; PKC: Protein kinase C; MHBst167: C terminally truncated surface antigen middle protein of hepatitis B virus; MAPK: Mitogen-activated protein kinase; ERK: Extracellular Signal-regulated Kinase signaling; GGHs: "Ground glass" hepatocytes; VEGF: Vascular epithelial growth factor; JAB1: c-Jun activation domain binding protein 1; LHBs: MHBs, large (including the preS1 + preS2 + S domain), middle (including the preS2 + S domain) surface proteins.

number of factors participate in carcinogenesis (Table 1). What are the key and special factors? For example, not all inflammation results in cancer. Additionally, are there any other special factors that promote HCC development in an IL-6-dependent manner?

TREATMENTS TO INHIBIT HBSAG AND DAMAGE SECONDARY TO HBSAG ANTIVIRALS

HBV-induced HCC was the first tumor to be prevented by universal immunization against the responsible virus^[1]. The host's immune system can control HBsAg levels^[61]. However, spontaneous HBsAg seroclearance is rarely achieved (an annual incidence of 1%-2% in CHB^[107,108]). At an advanced stage of HCC, the levels of HBsAg are still high, while that of HBV DNA may be negative. This tumor type with a poor prognosis is refractory to chemotherapeutic regimens^[109,110]. In chronic HBV infection, the prophylactic measures or

therapy for HCC include suppression of HBV replication and recovery from molecular abnormalities due to HBV infection. Achieving HBsAg loss and anti-HBs positivity is the final aim of antivirals^[61]. Nucleos(t)ide analog (NA) therapy has antiviral effects that may reduce HCC development and the post-operative recurrence of HCC^[111]. NA treatment affects the reverse transcription of pregenomic RNA but does not affect cccDNA and subgenomic RNA that have translational activity associated with HBsAg levels^[112]. Thus, the current NA therapy hardly clears HBsAg. With NA treatment, it may take 36 to 52 years to achieve HBsAg loss^[113,114]. Lamivudine was the first licensed NA introduced to treat HBV, and it reduced the incidence of HCC when compared to no treatment. However, the development of HCC may increase in cases with lamivudine resistance. The current first-line NAs, entecavir and tenofovir, may also reduce HCC development, but the risk of HCC is not eliminated, even in the patients who remain in virological remission with this therapy^[115]. NA resistance suggests antiviral failure.

Additionally, the drug-resistant HBV strains may have mutant surface proteins with oncogenic effects^[116]. In our study, substitutions in the S region, but not the drug-resistant mutations, possibly resulted in the poor effects of adefovir^[117]. Subsequent studies also showed that preS2 mutants induced resistance to NAs and predicted HCC development^[118]. Treatment with interferon inhibits HBsAg or preS mutant proteins more than NAs^[119-121].

Thus, due to cccDNA, HBV and HBsAg cannot be eliminated^[61]. There are also no measures to manage the integrative HBV DNA. It is impossible to develop sequence-specific antivirals for so many envelope mutants. The rtA181T/surface mutant may occur spontaneously in the absence of antiviral therapy. Its oncogenic potential warrants careful re-evaluation of the current strategy of prolonged antiviral therapy^[80].

However, there are many compounds in development for CHB up to June 22, 2015, such as Myrcludex B (entry inhibitor targeting NTCP), Rep 2139 (REP 9AC, HBsAg release inhibitor), TKM-HBV (HBsAg inhibitor), and BSBI-25 (cccDNA inhibitor)^[122]. These compounds should improve the effects of antivirals and reduce the burden of drug resistance and HCC development^[123].

TREATMENTS BASED ON ER STRESS

Drugs based on the theory of ER stress vary in the prophylaxis and treatment of HCC. The UPR is targeted as primary or adjuvant chemotherapy for HCC, *e.g.*, bortezomib for treating cancer *via* ER stress-induced apoptosis^[124] and mTOR inhibition as a promising strategy for the clinical management of HCC^[125]. However, mTOR inhibition may activate HBV replication in HBV-induced HCC^[57]. mTOR activation may recruit the YY1-HDAC1 complex to feedback suppress transcription from the preS1 promoter (nucleotides 2812-2816)^[57], thus partially explaining the low or negative HBV replication in the HCC stage. Therefore, mTOR inhibitors should be used in combination with antivirals.

To prevent HCC, targets to HBV-induced ER stress provide a strategy in high-risk CHB. Antioxidants may be such ideal agents because they reduce ER stress, thereby improving protein folding^[126]. Natural products, such as silymarin and resveratrol, have been used in HCC. The two drugs can target the ER stress-associated signal pathways^[85]. However, these findings require further verification.

Glycyrrhizin acid (GA) has multiple functions, such as effective hepato-protection and the reduction of elevated transaminases. Glycyrrhizin can suppress ER stress in acute liver injury^[127]. Long-term treatment with glycyrrhizin prevents HCC development in chronic hepatitis C infection^[128]. Glycyrrhizin treatment suppressed the sialylation of HBsAg and secretion of HBsAg in PLC/PRF/5 cells^[129,130]. Therefore, it is also widely administered in CHB infection. A study to determine whether drugs such as GA and extracts from other herbs would influence the preS mutants is

required.

Comprehensive prevention and treatment also include avoiding other risk factors such as aflatoxin B1 and alcohol intake. A prolonged battle against the damage induced by this virus is necessary.

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2016 Hepatitis C virus: Global view

Hepatitis C virus relies on lipoproteins for its life cycle

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Abstract

Hepatitis C virus (HCV) infects over 150 million people worldwide. In most cases, HCV infection becomes chronic causing liver disease ranging from fibrosis to cirrhosis and hepatocellular carcinoma. Viral persistence and pathogenesis are due to the ability of HCV to deregulate specific host processes, mainly lipid metabolism and innate immunity. In particular, HCV exploits the lipoprotein machineries for almost all steps of its life cycle. The aim of this review is to summarize current knowledge concerning the interplay between HCV and lipoprotein metabolism. We discuss the role played by members of lipoproteins in HCV entry, replication and virion production.

Key words: Apolipoproteins; Hepatitis C virus; Lipid metabolism; Lipoproteins; Review

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Core tip: The aim of the review is to summarize current knowledge concerning the interplay between hepatitis C virus (HCV) and lipoprotein metabolism. In particular, the manuscript discusses the role played by members of lipoproteins family in all steps of HCV life cycle.

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INTRODUCTION

Hepatitis C virus (HCV) infection is one of the main causes of chronic liver disease worldwide. It has been estimated that 130-170 million people are chronically infected with HCV^[1-3] with a prevalence, in selected countries, ranging from 0.4% to 12.3%^[4].

Acute infection is spontaneously cleared only in 15%-30% of individuals and the majority of patients develop chronic infection. HCV infection is generally a slowly progressive disease characterized by different liver damages that can progress to life-threatening diseases, such as cirrhosis and hepatocellular carcinoma^[5,6].

The recent advent of highly potent direct-acting antiviral drugs (DAAs), employed in interferon-containing and interferon-free combinations, has led to virus elimination in more than 90% of treated patients^[7]. However, it is yet unclear whether, and how, the virus-induced liver damages are reversible; therefore, it is important to fully elucidate the mechanism of HCV-induced pathogenesis.

HCV does not cause a direct cytopathic effect on host cells and most of the related liver dysfunctions are likely due to the virus-mediated alteration of host processes such as immune responses and several metabolic pathways^[8-10]. In particular, HCV interferes with the host lipid metabolism and cholesterol homeostasis. Several lipids abnormalities have been associated to HCV chronic infection, such as liver steatosis, particularly evident in patients infected with the genotype 3 of the virus, hypobetalipoproteinemia and hypocholesterolemia^[11].

The relationship existing between the virus and the lipid metabolism is very intimate, every step of the viral life cycle relies at least on one member involved in lipid pathways^[12,13].

HCV is an enveloped positive-strand RNA virus, a member of the genus *Hepacivirus* within the family of *Flaviviridae*. HCV enters the cell by receptor-mediated endocytosis involving multiple cell surface molecules (as recently reviewed by Ding and coauthors^[14]). After pH-dependent fusion and uncoating, the 9.6 kb single-stranded RNA genome is translated at the rough endoplasmic reticulum (ER). The resulting polyprotein precursor is processed by cellular and viral proteases into ten mature proteins; core and envelope glycoproteins E1 and E2 are the main constituents of the virus particle, p7 and nonstructural protein (NS) 2 participate in virus assembly, while NS3, NS4A, NS4B, NS5A, and NS5B are sufficient for viral RNA replication and are involved in virus assembly^[15]. Replication takes place in ER-derived membrane spherules called membranous web, which formation and architecture remain to be fully elucidated. Progeny RNA is then packaged into virus particles that exit the cell *via* the

secretory pathway^[16].

Lipoproteins are responsible for lipids packaging and transport through the bloodstream and for their delivery to target tissues. The transported lipids, which are the core of the lipoproteins, are cholesteryl esters (CE) and triglycerides (TG), derived either from the diet or from liver neo-synthesis. They are enveloped by a layer of phospholipids, free cholesterol and proteins (mainly apolipoproteins), which control lipoproteins assembly, transport and metabolism by mediating interactions with receptors, enzymes and lipid transport proteins^[17,18]. Lipoproteins vary in the content of lipids and proteins. Their classification and isolation procedures are commonly based on their density, which reflect their different content of CE, TG, free cholesterol and apolipoproteins. The main lipoproteins particles generated by the liver are the very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), in which apolipoprotein (apo)B-100 (apoB) is the main structural component, and high-density lipoproteins (HDL), where apoA-I is the main structural component^[17,18].

In the endogenous transport pathway, the liver releases TG and CE in the circulation mainly through the generation of VLDL particles^[19]. Mobilized lipid storage pool in the liver, as well as *de novo* synthesis of fatty acids and phospholipids, contribute to hepatic VLDL synthesis. Lipoprotein lipase (LPL) hydrolyses the TG present in the core of circulating VLDL, releasing free-fatty acids (FFA) to the target tissues. A large proportion of the resulting particles (IDL) is efficiently removed from plasma by the hepatocytes through the LDL receptor (LDLR). The remaining part is converted to LDL by a reaction catalyzed by hepatic lipase, which further reduces the amount of TG. Once formed, CE-rich LDL delivers cholesterol to peripheral tissues where are taken up by the LDLR and internalized *via* a clathrin-dependent pathway. Following endocytosis, LDL are degraded into lysosomes and free cholesterol is released and either accumulated within the cells or incorporated into new lipoproteins.

In the reverse transport system, HDL carry the excess of cholesterol from extrahepatic cells to the liver. HDL biosynthesis and maturation are complex multistep processes that involve the secretion of proteins-rich and lipid-poor particles (nascent HDL) and a massive extracellular lipid acquisition of mainly phospholipids and cholesterol. The major lipid components of HDL are CE and phospholipids, while apoA-I and apoA-II are the main apolipoproteins required for normal HDL biosynthesis^[17,18].

Several steps of HCV life cycle are strictly linked to host lipoprotein metabolism, the aim of this review is to describe this close relationship (summarized in Table 1).

HCV ENTRY AND LIPOPROTEINS

Viral particles purified from infected patients sera

Table 1 Components of lipoprotein metabolism involved in hepatitis C virus life cycle

| Name | Lipoprotein association | Main function | HCV life cycle step | Function | Ref. |
|----------|-------------------------|---|---------------------|-------------------------------------|---------------|
| GAGs | | Lipoproteins adsorption | Entry | LVP adsorption | [33-36,40] |
| LDLR | | apoE- and apoB-containing lipoproteins receptor | Entry | LVP binding | [37-44] |
| ApoB | VLDL and LDL | Structural protein of VLDL/LDL | RNA replication | Cellular distribution of lipids | [42] |
| ApoE | VLDL, LDL and HDL | Ligand of LDL receptor | Virion production | ? | [102-105,110] |
| ApoA-I | HDL >> VLDL | Structural protein of HDL | Entry | GAGs/LDLR binding | [35,36,51] |
| ApoH | VLDL >> HDL | Cholesterol efflux | Virion production | ? | [106-111] |
| ApoA-II | HDL >> VLDL | Structural protein of HDL | RNA replication | ? | [82,83] |
| ApoC-I | VLDL, LDL, HDL | Inhibitor of CETP activity | Virion production | ? | [82,110,111] |
| ApoC-II | VLDL, LDL, HDL | Activator of LPL | Entry | ? | [85] |
| ApoC-III | VLDL, LDL, HDL | Inhibitor of LPL and HL | Virion production | ? | [110,111] |
| ACSL3 | | Phosphatidylcholine synthesis for apoB | Entry | Viral and cellular membranes fusion | [58,59] |
| | | | Virion production | ? | [110,111] |
| | | | Virion production | ? | [110,111] |
| | | | Virion production | ? | [110,111] |
| | | | Virion production | ? | [105] |

GAGs: Glycosaminoglycans; LDL: Low-density lipoproteins; LDLR: LDL receptor; ApoB: Apolipoprotein B-100; VLDL: Very low-density lipoproteins; HDL: High-density lipoproteins; LVP: Lipoviral particles; LPL: Lipoprotein lipase; HL: Hepatic lipase; CETP: Cholesteryl ester transfer protein; ACSL3: Cyl-coenzyme A synthetase 3.

revealed that HCV has a spherical morphology of different sizes (range 40-70 nm diameter), with an enveloped membrane displaying the two surface viral glycoproteins E1 and E2^[20,21]. HCV exists as a mixture of infectious and noninfectious particles, both *in vivo* and *in vitro*, and, very interestingly, virions found with a very low buoyant density (range 1.10-1.14 g/mL) displayed the highest infectivity^[22-26].

The high buoyant density is mainly due to the association with apoB-containing lipoproteins (VLDL/LDL) to form the so-called lipoviral particles (LVP). Different lipoproteins components, such as cholesterol, TG, apoB, apoE, apoA-I and apoCs, have been found in the LVP of HCV infected patients^[27,28]. *In vitro* produced HCV particles have confirmed these associations^[26,29,30].

HCV LVP enter into the cells *via* a multi-step endocytosis that requires a growing number of receptors, co-receptors and host factors, which probably are responsible for the hepatotropism of the virus. The long list of these host proteins includes two binding factors glycosaminoglycans (GAGs) and low-density lipoprotein receptor (LDLR), four receptors CD81, scavenger receptor class B type 1 (SR-BI), claudin-1 (CLDN1), occludin (OCLN), and several co-receptors epidermal growth factor receptor (EGFR), ephrin receptor A2 (EphA2), the cholesterol transporter Niemann-Pick C1-like 1 (NPC1L1), transferrin receptor 1 (TfR1) and the cell-death-inducing DFFA-like effector b (CIDEB). For an exhaustive description of all known HCV receptors and their involvement in HCV cell entry, we refer the reader to recent reviews and references therein^[14,31]. Here we will focus on molecules involved in lipoprotein metabolism.

HCV entry into the cells is a process that requires spatial and temporal control of these cellular cofactors. The putative mechanism consists of three main steps

(1) viral attachment to the hepatocyte; (2) receptor-mediated endocytosis; and (3) endosomal fusion. Members of the lipoprotein metabolism seem to be involved in all these steps.

Attachment of the virus to the host cell is firstly obtained by the interactions with GAGs and LDLR present on the surface of the cells, which are known to mediate lipoprotein metabolism^[32]. It has been demonstrated that HCV binds to the GAGs present on heparan sulfate (HS) proteoglycans (HSPGs) and syndecan 1 and syndecan 4 have been reported to be involved in this process^[33,34]. The minimum HS oligosaccharide length required for HCV infection is a decasaccharide with the N- and 6-O-sulfation. Very interestingly, it has been reported that apoE is responsible for this process, while viral glycoproteins, although capable of binding to GAGs, are not involved^[35,36].

The LDLR, which transports the cholesterol-rich LDL intracellularly *via* clathrin-mediated endocytosis^[37], is involved in this first step of HCV entry. However, its exact role in HCV infection still remains controversial; it is not clear whether it acts as a HCV receptor or as a facilitator of initial attachment to hepatocyte surface or for other steps of the virus life cycle, such as viral replication^[38-44]. It is worth to note that the internalization of infectious particles and lipoproteins mediated by LDLR displays different kinetics, thus suggesting distinct uptake steps/pathways for HCV and lipoproteins^[42].

The capture of viral particles is mediated by other members of the lipoprotein metabolism. In fact, after the initial interaction with GAGs and LDLR, HCV utilizes SR-BI, a major receptor of high-density lipoproteins (HDL) that can bind also apoB-containing lipoproteins (VLDL/LDL) and oxidized forms of LDL^[45,46]. SR-BI is a

glycoprotein with two N- and C-terminal cytoplasmic domains separated by a large extracellular domain, which is involved in lipoprotein metabolism, mediating the uptake and the intracellular delivery of the cholesterol esters (CE). Interestingly, the SR-BI-mediated intracellular lipid transportation is different from that of the LDLR. In fact, it binds lipoproteins on the cell surface and delivers CE intracellularly without internalization of the intact lipoprotein particle (as reviewed by Shen and coauthors^[47]). This could partially explained the aforementioned distinct internalization pathways described for HCV and lipoproteins^[42].

The interaction between HCV and SR-BI is thought to mediate the dissociation of lipoproteins from LVP, likely through the SR-BI-mediated cholesterol transfer activity, and to induce conformational changes in the E2 glycoprotein, exposing its CD81 interaction domains^[48,49]. Interestingly, it has been reported that HDL increases HCV entry into the cells by accelerating their endocytosis through SR-BI activation, with the consequence of decreasing the neutralizing effect of the anti-HCV antibodies^[50]. On the other hand, apoB-containing lipoproteins competed and effectively inhibited the interaction between HCV and hepatocytes and, as reported for LDLR, the binding to SR-BI is not mediated by E2 but mainly, although not exclusively, by apoE^[51].

Together, these studies suggest that the first step of HCV entry is regulated by the complex interactions occurring between lipoproteins components, lipoproteins receptors (*i.e.*, GAGs, LDLR and SR-BI) and HCV envelope glycoproteins.

After the attachment to the cells, HCV binds its receptors, the tetraspanin CD81 and the tight junction proteins CLDN1 and OCLN, leading to cellular internalization of the virus through a clathrin-dependent endocytosis process. After the binding to HCV, CD81 moves toward the tight junctions and interacts with CLDN1. This movement depends on the activation of several transduction pathways such as EGFR, Ras and Rho GTPases, which trigger the actin-mediated lateral membrane diffusion of HCV-CD81 complexes^[52-54]. It is reported that also Tfr1 is engaged at a post-CD81 binding step in HCV entry and it is involved in the viral entry. Although the exact mechanism of action is unknown, the Tfr1 inhibition decreases significantly the infection of HCV derived from cell culture (HCVcc) and HCV pseudoparticles (HCVpp), thus suggesting that it is involved in viral internalization. Interestingly, the cell-to-cell spread is less dependent on this molecule^[55]. Similarly, OCLN is required for HCV entry and it acts after the GAGs and SR-BI post binding step and prior to endosomal acidification, thus suggesting that the tight junction region is the last to be encountered by the virion before cellular internalization^[56].

The interaction of HCV-CD81 complexed with CLDN1 induces clathrin-mediated endocytosis. Following

uptake, HCV co-receptor complexes are trafficked to RAB5A containing endosomes for HCV fusion^[57]. Interestingly, receptor-specific antibodies and HCV particles increased CD81 and CLDN1 endocytosis, thus supporting a model wherein HCV stimulates receptor trafficking to promote viral particle internalization.

After viral internalization, the interaction between E2 and CD81 induces a yet unknown fusion mechanism between the viral glycoproteins and the endosomal membrane in a low-pH environment. Interestingly, this process is favoured by apoC-I, an exchangeable apolipoprotein that predominantly resides in HDL^[58,59]. ApoC-I specifically enhances the infectivity of HCVcc and HCVpp as well as of HCV isolated from viremic chimpanzees. ApoC-I increases the infectivity *via* a direct interaction with the HCV glycoproteins. Interestingly, the hypervariable region 1 (HVR1), located at the N-terminus of the HCV E2 glycoprotein, is the essential viral component for the apoC-I-mediated activity^[58]. ApoC-I activity does not rely on SR-BI or CD81, thus not influencing the binding or the internalization steps of HCV entry. ApoC-I instead enhances the pH-dependent fusion rates between viral and target membranes, as measured by a HCVpp/liposome fusion assay^[58].

Following fusion, HCV genomic RNA is released into the cytosol, where it is directly translated to produce viral proteins and initiate viral replication.

HCV REPLICATION AND LIPOPROTEINS

HCV RNA replication is a multi-step process regulated by several viral and cellular proteins^[60]. It is well established that the minimal viral proteins necessary and sufficient for viral replication are NS3-4A, NS4B, NS5A and NS5B, together with 5' and 3' untranslated regions^[15]. HCV replication occurs within a dense cluster of rearranged intracellular membranes referred to as membranous web. It is composed by double-membrane vesicle structures, most likely derived from the ER^[61]. This membranous matrix is the center of HCV replication. In fact, it contains all the non-structural viral proteins necessary for replication as well as the newly synthesized viral RNA^[62]. It is worth to note that, although the membranous web is induced by all HCV replicase factors (NS3-5B)^[61], the sole expression of NS4B and NS5A induces very similar membrane alterations^[63,64]. HCV, through NS5A, hijacked the isomerase activity of cyclophilin A (CypA)^[65,66] and the membrane-deforming ability of proline-serine-threonine phosphatase interacting protein 2 (PSTPIP2)^[67] for remodeling intracellular membranes and, thus, enhancing HCV replication.

The replication step of HCV life cycle is highly linked to the host lipid metabolism processes. RNA replication occurs in membranes rich in cholesterol and sphingolipids, two lipids not abundant in the ER membrane^[68]. Since HCV hijacks host lipid metabolism at different levels, it is likely that these lipids are

transported to the membranous web, rather than that the replication complexes take place in specific area of the ER enriched in cholesterol and sphingolipids. In fact, HCV is able to alter the lipid composition of membranes affecting the subcellular localization of the lipid kinase phosphatidylinositol-4-kinase III α (PI4KIII α), which leads to a different distribution of its product phosphatidylinositol-4-phosphate (PI4P) from the Golgi compartment and plasma membrane to the cytoplasm^[69,70]. HCV utilizes the redistribution of PI4P to alter the lipid composition of the membranous web, attracting sphingolipids and cholesterol through the recruitment of the PI4P-interacting lipid transfer proteins four-phosphate adaptor protein 2 (FAPP2), which is also a glycosphingolipid-binding protein, and oxysterol-binding protein (OSBP), respectively^[71,72].

In addition to these structural and molecular alteration of membranes, HCV also induces *de novo* lipid and membrane biosynthesis modulating the expression of a number of genes involved in lipid metabolism. This is likely mediated by the transcriptional activity of the sterol regulatory element-binding protein (SREBP) pathway^[73,74]. In this regards, it has been well described how HCV infection is able to alter the lipidomic profile of hepatocytes^[75].

While the lipid metabolism involvement in HCV replication has been well demonstrated, the role of members of the lipoprotein machineries is far to be fully proved. It has been reported that LDLR is necessary for viral RNA replication. The use of a neutralizing antibody against LDLR after HCV RNA electroporation into Huh7 cells induced a decreased RNA production. The treatment with this antibody induced a changed in the cellular lipid profile, with an increase of CE level and a change in phospholipids [*i.e.*, increased phosphatidylethanolamine (PE) and a lower phosphatidylcholine (PC) content]. Therefore, it is likely that LDLR is necessary to the virus to have the adequate amount and variety of lipids at the membranous web^[42].

Although how LDLR contributes to HCV life cycle, in general, and to the viral entry step, in particular, is still controversial, its importance is undisputed. The role of this receptor in viral life cycle is further emphasized by the direct or indirect ability of HCV to modulate LDLR expression by both increasing its gene transcription and inhibiting its PCSK9-mediated protein degradation^[44]. Interestingly, the HCV-mediated PCSK9 regulation has been reported to be different in patients infected with the genotype 3 compare to those with genotype 1, thus suggesting that HCV can affect lipoprotein metabolism in a genotype specific-manner^[76].

This hypothesis is indirectly supported by the observation that hepatoma cells transfected with core proteins of the different HCV genotypes displayed a genotype-specific intracellular TG accumulation^[77]. This accumulation is probably caused by the virus-mediated inhibition of the microsomal triglyceride transfer

protein (MTTP), a key enzyme in the apoB-containing lipoproteins assembly pathway^[78]. It has been also found that the HCV genotype could also affect the circulating levels of apolipoproteins. In chronic patients the infection with genotype 1b was found to be an independent factor significantly associated with higher levels of apoA-II and apoE, and lower levels of apoC-II and apoC-III, while genotype 2 infection was associated only with lower levels of apoC-II and apoC-III^[79]. Moreover, the HCV genotypes influence the levels of LDL-cholesterol differently in patients with different IL28B polymorphisms, as well as the lipid-related genes expression in cultured cells^[80]. These different effects on lipoproteins machinery, together with other pathogenic effects^[81] could explain, at least partially, the HCV genotype-specific steatogenic effects.

Another connection between the lipoprotein metabolism and the RNA replication step of the HCV life cycle was discovered in our laboratory. We found that apoA-I is involved in the replication step of HCV. In fact, the downregulation of apoA-I induces significant decrease of viral RNA levels in either replicon carrying cells and in the HCVcc infected cells^[82].

Although apoA-I is the major structural protein of the HDL, we focused our attention on this exchangeable apolipoprotein because a decreased association of apoA-I to the circulating LDL of HCV infected patients was found by a proteomic analysis. This result suggests that the function of apoA-I necessary for HCV replication could be linked to a its putative role in the biogenesis of apoB-containing lipoproteins rather than that of HDL. This is indirectly supported by the finding that the siRNA-mediated downregulation of apoA-I induces a significant reduction of HCV RNA only at later time point (day 4-6 post-silencing), when a decreased levels of apoB secretion was observed (unpublished results).

The apoA-I involvement in HCV replication were confirmed by others using different replicon system and siRNAs^[83], thus lowering a possible off-targets effect, as recently underlined by a work on MOBK1B by Rice's group^[84]. Interestingly, Saito's laboratory found that one of the transcriptional effect of a histone deacetylase inhibitor treatment (SAHA) was the down-regulation of apoA-I and also the up-regulation of osteopontin (OPN), which are *per se* sufficient to induced the inhibition of HCV RNA replication^[83].

Another apolipoprotein that has been described to influence HCV replication is apoH (also known as b2-glycoprotein I), which was able to limit RNA replication using human liver slices as a HCV infection model^[85]. Although, the authors have not proved a direct effect on HCV replication, treatment with apoH reduced HCV production while not affecting HCV entry^[85]. A negative effect of apoH on the virus is also supported by the positive correlation between high plasma levels of apoH and viral clearance, both in spontaneous remission and in response to pegylated-interferon/ribavirin therapy in HCV patients^[86]. Interestingly, patients carrying the

favorable IL28B rs12979860 CC SNP correlated with high plasma concentration of apoH, thus unveiling this apolipoprotein as a quantitative trait associated with IL28B^[86].

Remarkably, apoH is part of the LDL^[87] and it is known to influence the size and the lipid composition of LDL^[88-90]. This further reinforces the idea that proteins affecting the generation of apoB-containing lipoproteins could have also an effect on HCV replication. However, since the inhibition of either apoB expression or MTTP function does not affect viral replication, this suggests that the distribution or the quality of lipids related to the lipoprotein machinery could influence HCV RNA production.

HCV VIRION PRODUCTION AND LIPOPROTEINS

The last step of the intracellular HCV life cycle is the formation of viral particle. The dynamic of virus assembly is challenging to track, suggesting that this process is either rare or rapid. However, it is now well recognized that the HCV virion biogenesis strictly relies mainly on lipid droplets (LD), the storage sites for neutral lipids in cells, and on the apoB-containing lipoprotein machinery^[91-93].

HCV particles production is a coordinated and complex process regulated spatially and temporally by all viral proteins and host factors. Virion assembly is coordinated between the synthesis of new positive RNA strands, its encapsidation and the acquisition of envelop, likely *via* budding into the ER^[94].

The RNA replication site and the formation of nucleocapsid has to be separated to avoid competition for the binding of viral RNA. This is obtained by the localization of the core protein on the surface of cytosolic LD, probably through the presence of two amphipathic helices and a palmitoylated conserved cysteine residue. The MAPK-regulated cytosolic phospholipase A2, group IVA (PLA2G4A) is important for the core recruitment at the LD and for the specific cleavages of lipids with arachidonic acid, which is essential for the production of highly infectious viral particles^[95]. LD localization of core may be also enhanced by the diacylglycerol acyltransferase-1 (DGAT-1)^[96], an enzyme involved in LD morphogenesis that is also known to influence VLDL biogenesis^[97]. The LD localization of core is an essential step. In fact, it regulates the recruitments of the other viral components and cellular factors, which regulate the transfer of both the newly replicated viral genomes from the membranous web and the HCV glycoproteins E1 and E2 from the ER, to the assembly site^[94,98,99]. Although this step is not fully elucidated, it is likely regulated through protein-protein interactions between multiple viral proteins and a yet not fully unveiled list of cellular proteins, as recently reviewed elsewhere^[94,100].

Nascent virions, following maturation and acquisition

of the typical low density, exit the cell *via* the secretory pathway. Although, the exact mechanisms are still poorly understood, it is now evident that assembly and secretion of HCV particles are associated with the VLDL/LDL pathway. There are different experimental evidences supporting this model.

Notably, in humanized livers of transplanted SCID/Alb-uPA mice, HCV infection occurs only when the engraftment of human hepatocytes is sufficient to obtain a human-like lipoproteins profile while it is not correlated to the number of human hepatocytes^[101].

In Huh7 cells, the isolation of membrane vesicles in which HCV replicates lead to the enrichment in different members of the apoB-containing lipoproteins such as apoB, apoE and MTTP^[102]. Interestingly, the impairment of VLDL/LDL production through the downregulation of apoB or the inhibition of either MTTP, which stabilizes apoB by transferring lipids during its translation, or long chain acyl-coenzyme A synthetase 3 (ACSL3), which mediates the phosphatidylcholine synthesis required for apoB secretion, lead to a decreased HCV particles production^[102-105].

However, the direct requirement of apoB is controversial. Other researchers did not find a dependency of HCV production on apoB but rather on the activity of apoE^[106-108]. Moreover, it has been reported that MTTP inhibitors at low doses, which are effective for apoB secretion inhibition, are ineffective for HCV production, while at higher concentrations those inhibitors blocked apoE expression and secretion and, consequently, suppressed the generation of viral particles^[106]. The ectopic expression of apoE, but not apoB or MTTP, was found necessary also for the production of infectious HCV trans-complemented particles in human non-liver cells^[108]. Moreover, apoE but not apoB was found necessary also for the cell-to-cell transmission of the virus. In fact, either the silencing of apoE in donor cells, but not in acceptor cells, inhibited the cell-to-cell viral spread^[109].

A recent work of Matsuura's lab smooth out the controversy. They showed that apoB and apoE redundantly participate in the formation of infectious HCV particles^[110]. They generated Huh7 cells knock out for either or both apoB and apoE by zinc finger nucleases and found that the single knock out cells have a slightly reduction of HCV virions, while the apoB/apoE double knock out severely impaired the formation of infectious viral particles. More interestingly, they showed that the overexpression of different exchangeable apolipoproteins (*i.e.*, apoA-I, apoA-II, apoC-I, apoC-II, apoC-III, apoE but not apoH) in the double knock out Huh7 cells rescued the capability of producing viral particles^[110].

These results were independently confirmed in complemented HCV virus production experiments using the non-permissive 293T/miR-122 cells transfected with HCV, in which the expression of cDNAs encoding for all members of the apoA and apoC family,

but not apoD, complemented HCV virus production, although at lower levels compared with apoE^[111].

These results support the data obtained by different labs in which a requirement of other apolipoproteins for HCV production has been reported.

For instance, apoJ, a small heat shock protein that prevents unfolded secretory protein aggregation identified as a VLDL-associated protein^[112], was found involved in efficient infectious HCV virion production^[113]. ApoJ silencing decreased the HCV virion production, without affecting the HCV RNA replication, which could be restored upon reconstitution with a siRNA-resistant apoJ. Most likely, apoJ is involved at the step of virion assembly, since it was found to interact with core and NS5A, stabilizing the dual protein complex. Interestingly, immunofluorescence analysis showed that HCV infection induces a cellular redistribution of apoJ from Golgi to LD at the ER-Golgi membrane contact site^[113].

Our lab found that the silencing of apoA-I induced a significant decrease in HCVcc production^[82], although at lower levels respect of that of apoE (unpublished data). As described for other cellular components also the distribution of apoA-I was affected by HCV. We found that the apoA-I specific association to LDL was reduced in the circulating lipoproteins of HCV infected patients. Although it is known that the exchange of apolipoproteins among lipoprotein particles and interconversion of particles occurs in the plasma compartment, we found that the HCV-induced decreased association of apoA-I with LDL has a cellular origin. The HCV-associated LDL-specific reduction in apoA-I was also observed in the different HCV cellular models (*i.e.*, HCVcc, genomic and subgenomic replicons). The results obtained with subgenomic replicon-carrying Huh7 cells, which recapitulate only the viral RNA replication step, indicate that the sole NS viral proteins are sufficient to impair the apoA-I/LDL association, thus reinforcing the hypothesis that VLDL/LDL biogenesis and viral replication could share common sites and bridging molecules. However, although apoA-I is known to be associated to the circulating VLDL and LDL^[114,115], its role in the physiology of these lipoproteins is yet unknown. A possible role of apoA-I in the viral life cycle could be to drive HCV components in the right cellular compartments, and its binding activity to NS5A seems to support this hypothesis^[116,117].

Moreover, as above mentioned, Fukuhara and colleagues found that different apolipoproteins are sufficient for HCV viral production in culture. More specifically, they found that the amphipathic α -helices present in the different apolipoproteins possess a redundant role in the assembly of HCV, through a direct interaction with the viral particles at the post-envelopment step^[110].

Interestingly, similar results were found for the replication step of HCV life cycle. The N-terminal amphipathic helix of NS5A, which motif is very

similar to that of apolipoproteins, bound specifically to PI 4,5-bisphosphate [PI(4,5)P₂], inducing a conformational change that stabilizes the interaction between NS5A and TBC1D20, which is required for HCV replication^[118].

Since phosphoinositides bind and regulate localization of proteins *via* a variety of structural motifs, these results support the hypothesis that the requirement of the different apolipoproteins is related to the proper cellular localisation of viral components, which is necessary for the exploitation of lipid, in general, and of the lipoprotein metabolism, in particular. In other words, HCV may modulate the production of lipoproteins in the host cells to make this pathway more appropriate for viral maturation.

This point of view is supported by the observation that there is no correlation between the ability to generate VLDL and the production of LVP. In fact, the VLDL-producing HepG2 cells generated LVP similar, for both density and apolipoproteins content, to those generated by the VLDL-deficient Huh7.5 cells^[119].

However, it is important to note that dissimilarities exist in the molecular composition of LVP found in HCV patients or generated in culture; for instance serum LVP could be immunoprecipitated by anti-apoB antibodies while the interaction with HCVcc is less efficient^[29].

These discrepancies should be kept in mind because may have major implications for our understanding of HCV assembly and secretion. In fact, the exact model for the LVP composition is not yet defined. It has been proposed either a single-particle model, in which HCV exists as a hybrid particle fused to a VLDL/LDL, or as a two-particles structure, in which the virion is surrounded by lipoproteins (Figure 1).

CONCLUSION

HCV interacts with and hijacks host cell machineries and pathways to generate a chronic and productive infection. It is now well established that HCV has an intimate relationship with the host lipid metabolism, which has a role in all the steps of the viral infectious cycle. In particular, HCV modulates the production of lipoproteins in the host cells to make it more effective for viral production, propagation and persistence.

The virus circulates in the bloodstream as a highly lipidated LVP, although it is not yet known whether fused with or surrounded by lipoproteins (Figure 1). HCV utilizes the lipoproteins pathways for cell entry, virus assembly and, possibly also for RNA replication. Moreover, it is becoming recognized that its strict resemblance with VLDL/LDL may contribute to the viral immune evasion strategies, such as masking viral epitopes or escaping from anti-HCV neutralizing antibodies directed against either viral proteins or the entry factors involved in lipoproteins pathways (*i.e.*, CD81 and SR-BI as reviewed by Vercauteren and coauthors^[120]).

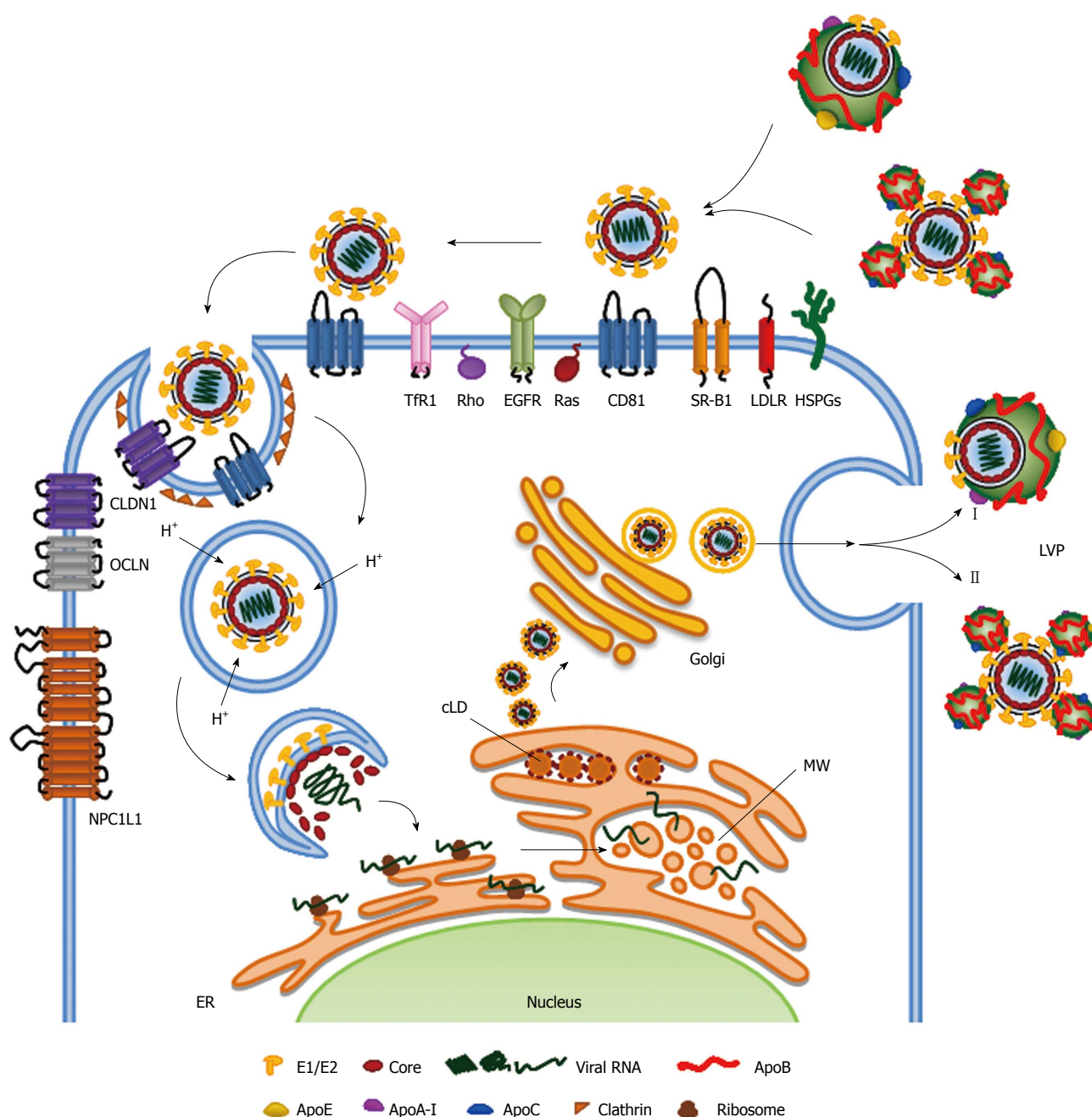


Figure 1 Hepatitis C virus life cycle. Following the initial binding of hepatitis C virus (HCV) to glycosaminoglycans present on heparan sulfate proteoglycans (HSPGs), to low-density lipoprotein receptor (LDLR), to scavenger receptor class B type 1 (SR-B1) and to CD81, the viral particles utilize different proteins, such as epidermal growth factor receptor (EGFR), Ras, Rho ephrin receptor A2 (EphA2), transferrin receptor 1 (TfR1), cholesterol transporter Niemann-Pick C1-like 1 (NPC1L1), claudin-1 (CLDN1) and occludin (OCLN), for entering into the cell by clathrin-mediated endocytosis. After the pH-dependent fusion between viral and target membranes, HCV RNA is released into the cytosol and translated at the rough ER, giving rise to a polyprotein that is then cleaved into mature viral proteins. Viral proteins together with host cell factors, induce the formation of the membranous web (MW), composed by vesicles as well as cytosolic lipid droplets (cLD) where the RNA replication occurs. Assembly of HCV particles probably starts in close proximity to the ER and lipid droplets. The viral envelope is acquired by budding into the ER at sites of lipoprotein synthesis. HCV particles are thought to be released via the constitutive secretory pathway in association with components of lipoproteins in order to produce a mature form of lipoviroparticles (LVP). This lipidation might occur either during budding (hybrid particle model; I) or during egress via interaction between the virion and lipoproteins (dual-particle model; II).

What has not been investigated yet is the possible involvement of lipid/lipoprotein metabolism in the dysregulation of the immune response mediated by HCV, as recently reported for the hepatitis B virus^[121]. Recent evidences showing that LVP affect dendritic cells maturation^[122], ApoE3 blocks the antiviral effect of ficolin-2^[123] and the VLDL and LDL of chronic

infected patients induce an altered intracellular lipid production^[124] suggest that the HCV-induced modification of lipoprotein metabolism could be involved in the regulation of the immune response.

Overall, decoding the multiple interactions that HCV establishes with the lipoproteins pathways is mandatory to obtain a deeper knowledge of HCV

biology and pathogenesis, a step necessary to understand and to manage the reversibility of liver damages upon DAAs-mediated viral clearance.

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2016 Hepatitis C virus: Global view

Anti-rods/rings autoantibody generation in hepatitis C patients during interferon- α /ribavirin therapy

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Abstract

Chronic inflammation associated with hepatitis C virus (HCV) infection can lead to disabling liver diseases with progression to liver cirrhosis and hepatocellular carcinoma. Despite the recent availability of more effective and less toxic therapeutic options, in most parts of the world the standard treatment consists of a weekly injection of pegylated interferon α (IFN- α) together with a daily dose of ribavirin. HCV patients frequently present circulating non-organ-specific autoantibodies demonstrating a variety of staining patterns in the indirect immunofluorescence assay for antinuclear antibodies (ANA). Between 20% to 40% of HCV patients treated with IFN- α and ribavirin develop autoantibodies showing a peculiar ANA pattern characterized as rods and rings (RR) structures. The aim of this article is to review the recent reports regarding RR structures and anti-rods/rings (anti-RR) autoantibody production by HCV patients after IFN- α /ribavirin treatment. Anti-RR autoantibodies first appear around the sixth month of treatment and reach a plateau around the twelfth month. After treatment completion, anti-RR titers decrease/disappear in half the patients and remain steady in the other half. Some studies have observed a higher frequency of anti-RR antibodies in relapsers, *i.e.*, patients in which circulating virus reappears after initially successful therapy. The main target of anti-RR autoantibodies in HCV patients is inosine-5'-monophosphate dehydrogenase 2 (IMPDH2), the rate-limiting enzyme involved in the guanosine triphosphate biosynthesis pathway. Ribavirin

is a direct IMPDH2 inhibitor and is able to induce the formation of RR structures *in vitro* and *in vivo*. In conclusion, these observations led to the hypothesis that anti-RR autoantibody production is a human model of immunologic tolerance breakdown that allows us to explore the humoral autoimmune response from the beginning of the putative triggering event: exposure to ribavirin and interferon.

Key words: Rods and rings; Autoantibodies; Hepatitis C; Ribavirin; Interferon- α

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Core tip: Between 20% and 40% of hepatitis C virus patients treated with interferon- α and ribavirin develop autoantibodies showing a peculiar antinuclear antibodies pattern characterized as rods and rings (RR) structures. In those patients, the first appearance of anti-RR autoantibodies occurs around the sixth month of treatment and reaches a plateau around the twelfth month. The main target of anti-RR autoantibodies is the inosine-5'-monophosphate dehydrogenase 2 (IMPDH2) enzyme, critical in *de novo* GTP biosynthesis. In cell culture, IMPDH2 inhibition by ribavirin promotes its aggregation into RR structures. These observations led to the hypothesis that anti-RR autoantibody production represents a human model of immunologic tolerance breakdown that allows us to explore interesting aspects of the humoral autoimmune response from the beginning of the putative triggering event.

Keppeke GD, Calise SJ, Chan EKL, Andrade LEC. Anti-rods/rings autoantibody generation in hepatitis C patients during interferon- α /ribavirin therapy. *World J Gastroenterol* 2016; 22(6): 1966-1974 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i6/1966.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i6.1966>

INTRODUCTION

Liver inflammation caused by infection with the hepatitis C virus (HCV) remains a major health challenge. HCV is transmitted by parenteral contact with contaminated blood, frequently through medical procedures. HCV is a small RNA virus 40 to 100 nm in diameter^[1]. It has a single-stranded RNA genome that is used directly as messenger RNA in protein synthesis. This positive single-stranded RNA is copied to the negative strand form, which is used as a template for the production of new virus copies. It replicates in the cytosol and endoplasmic reticulum of the infected cells, usually hepatocytes, producing ten viral proteins. Some of these viral proteins inhibit apoptosis and others inhibit interferon effects. The pathological effects of HCV on the liver are mainly caused by the action of the host immune system on infected hepatocytes^[2].

Until recently, in most countries, the standard treatment for hepatitis C consisted of weekly injections of 180 mcg of interferon alpha (IFN- α) 2a or 1.5 mcg/kg of IFN- α -2b, typically together with daily 15 mg/kg ribavirin for 48 to 72 wk^[3,4]. IFN has potent antiviral activity but does not act directly on the virus or replication complex. Instead, it acts by inducing IFN-regulated genes (ISGs) that provide a non-specific antiviral response^[5,6]. Ribavirin is a synthetic guanosine analogue that acts directly against RNA and DNA viruses, probably by inhibiting the virus-dependent RNA polymerase. As a guanosine analogue, ribavirin is intracellularly phosphorylated to generate the monophosphate (RMP), diphosphate (RDP), and triphosphate (RTP) forms. RMP is a competitive inhibitor of inosine-5'-monophosphate dehydrogenase 2 (IMPDH2), which leads to depletion of GTP required for the intracellular synthesis of viral RNA^[7]. The incorporation of RTP instead of GTP by the virus-dependent RNA polymerase leads to inhibition of viral replication or to the production of defective virions. However, RTP has been shown to be a weak inhibitor of many viral polymerases^[8]. RTP can also be incorporated into viral RNA, forming a template for pairing to CTP and UTP with equal efficiency. The frequency of transitions G→A and A→G in the viral genome will then increase, leading to lethal mutagenesis^[9,10]. Therefore, ribavirin alone has no significant effect on HCV, but has a valuable adjuvant effect when used in combination with IFN- α therapy^[11].

Autoantibodies are immunoglobulins directed against self-antigens. They can disturb cellular physiology and cause tissue damage by several mechanisms, such as (1) blocking membrane receptors; (2) causing cytolysis by means of antibody-dependent cytotoxic activity; (3) immune complex formation; and (4) complement activation, among others^[12]. The presence of non-organ-specific autoantibodies in the sera of HCV patients is common. The proportion of ANA-positive HCV patients can vary from 7% to 50%, with an average of 20% to 30%, depending on the population studied and the methodology used. Some HCV patients also present autoantibodies normally associated with autoimmune liver diseases such as autoimmune hepatitis (AIH) and primary biliary cirrhosis^[13,14]. Altogether, these observations suggest that chronic hepatitis C infection is a strong autoimmunogenic condition^[15].

Molecular mimicry, imbalance of effector T cells and regulatory T cells, and direct action over B lymphocytes are possible mechanisms leading to autoimmune manifestations of HCV^[16]. CD81 on the surface of B lymphocytes is a natural ligand for HCV envelope 2 (E2) protein. B lymphocyte-specific protein CD21, a receptor for the complement C3d fragment, is closely related to CD81. The B cell threshold for polyclonal activation is lowered considerably when HCV E2 coated by C3d engages CD81 and CD21, favoring misleading B cell activation against autoantigens. In addition, the B lymphocyte activating factor (BAFF) is upregulated

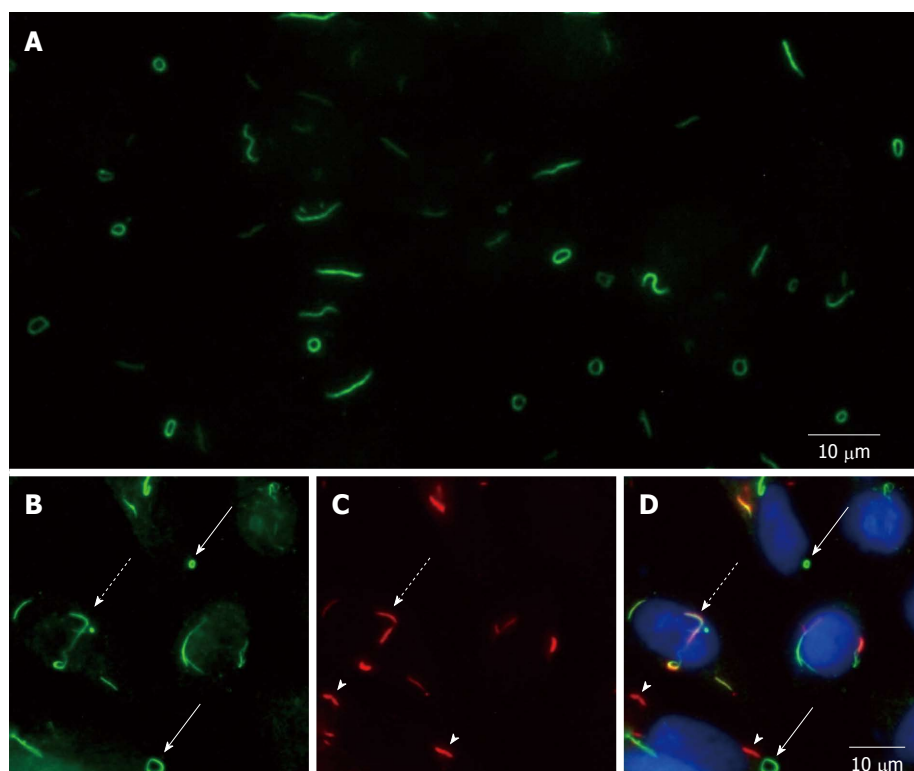


Figure 1 Inosine-5'-monophosphate dehydrogenase 2 and cytidine triphosphate synthetase enzymes can aggregate into rods and rings structures. A: Representative image of the RR pattern observed in a Euroimmun HEP-2 slide; B-D: HEP-2 cells were cultivated with DON treatment and labeled by indirect immunofluorescence with anti-RR-positive HCV serum (B) and rabbit anti-CTPS1 antibody (C). Merged image of panel B and C plus DAPI (D). IMPDH2-based (solid arrows), CTPS-based (arrowheads), and mixed RR structures (dotted arrows) can be observed. (A-D) All data and images were obtained in our own laboratory from assays performed by Keppeke GD. RR: Rods and rings; DON: 6-diazo-5-oxo-L-norleucine; HCV: Hepatitis C virus; IMPDH2: Inosine-5'-monophosphate dehydrogenase 2; CTPS: Cytidine triphosphate synthetase.

during HCV infection. BAFF binds CD19, a transducer of activation signal into the cell, adding to the production of autoantibodies and cryoglobulins^[15,17,18].

Since autoantibodies against rods and rings (RR) structures have been observed by several laboratories, the aim of this article is to review the recent reports revealing the main characteristics of anti-RR autoantibody production by HCV patients, including its clinical relevance and close relationship with IFN- α plus ribavirin treatment. The major characteristics of RR structures and their molecular constituents are also discussed.

HCV TREATMENT INDUCES AUTOANTIBODIES AGAINST RR STRUCTURES

About 30% of HCV patients treated with IFN- α plus ribavirin (IFN- α /ribavirin) develop autoantibodies that recognize cytoplasmic and nuclear structures resembling rods and rings (RR) (Figure 1A) in the indirect immunofluorescence assay for antinuclear antibodies (ANA)^[19-21]. Despite occurring in high titers, anti-RR autoantibodies have not yet been clearly linked with demographic, clinical, or virological features^[20,22-24]. Instead, by analyzing sequential

samples from several patients, we showed that anti-RR autoantibody production is closely related with IFN- α /ribavirin therapy^[20,25]. Anti-RR autoantibodies initially appeared around the sixth month of treatment in nearly half the patients (47%); the anti-RR titers also increased during treatment, reaching their highest levels towards the end of the standard therapy at twelve months. After treatment completion, there was a decrease in anti-RR titer in half the patients while titers remained steady in the other half^[20]. A recent publication by Novembrino *et al*^[22] also reported anti-RR titer decline after treatment cessation. They reported that the frequency of anti-RR increased in parallel with therapy duration, with rates of 9%, 38%, and 53% at weeks 12, 24, and 48, respectively^[22].

Since the first reports on autoantibodies against RR structures in HCV patients came out, important questions have been raised regarding the clinical relevance of such autoantibodies. A summary of the available data from the literature is presented in Table 1. One of the earliest studies by Covini *et al*^[21] found that these autoantibodies were more prevalent in patients who did not respond to therapy or relapsed (HCV viral load increased six months after end of treatment) when compared with patients that eliminated the virus completely (33% vs 11%, $P = 0.037$)^[21]. The publication from Novembrino *et al*^[22] mentioned above

Table 1 Summary of findings relating the presence of anti-rods and rings autoantibodies to hepatitis C virus treatment outcome

| Publication | Patient cohort | Results | Conclusions |
|--|---|--|---|
| Covini <i>et al</i> ^[21] (2012) | Italian cohort: REL/NR <i>n</i> = 30; SVR <i>n</i> = 45; (total = 75) | The prevalence of anti-RR antibody was significantly higher in REL/NR (33%) than in SVR (11%, <i>P</i> = 0.037) | Higher prevalence of anti-RR in REL |
| Keppeke <i>et al</i> ^[20] (2012) | Brazilian cohort: Anti-RR reactivity <i>n</i> = 39; No anti-RR reactivity <i>n</i> = 86; (total = 125) | The proportion of NR was equivalent in the 39 patients with anti-RR reactivity (77%) when compared with the 86 anti-RR negative (64%, <i>P</i> = 0.150) | No association between anti-RR reactivity and treatment outcome |
| Carcamo <i>et al</i> ^[19] (2013) | United States cohort: <i>n</i> = 47; Italian cohort: <i>n</i> = 46; (total = 93) | In the United States cohort, NR/REL had significantly higher anti-RR titers compared to SVR (about 1:3200 <i>vs</i> 1:100, <i>P</i> = 0.0016) In the Italian cohort, REL had significantly higher titers when compared to NR and SVR (<i>P</i> = 0.004 and <i>P</i> = 0.015, respectively) | Higher titer of anti-RR in REL |
| Novembrino <i>et al</i> ^[22] (2014) | Italian cohort: SVR <i>n</i> = 53; REL <i>n</i> = 27; NR <i>n</i> = 8; (total = 88) | Anti-RR reactivity was significantly more frequent in REL (56%) than in SVR (30%) or NR (12%) (<i>P</i> = 0.0282) | Higher prevalence of anti-RR in REL |

NR: Non-responders, patients who did not respond to therapy; REL: Relapsers, hepatitis C virus viral load increased six months after end of treatment; SVR: Sustained virological response, patients that eliminated the virus completely.

reported a higher frequency of anti-RR autoantibodies in relapsers when compared with patients that achieved sustained virological response (SVR) (56% *vs* 30%, *P* = 0.0282). Since these two studies found a higher prevalence of anti-RR reactivity in relapsers, it should be mentioned that relapsing patients are usually submitted to a second or third round of IFN- α /ribavirin treatment. We discuss above that longer exposure to the treatment increases the chance that the patient will produce anti-RR autoantibodies. In a previous study, we found no association between the presence of anti-RR autoantibodies and the response to anti-HCV treatment with IFN- α /ribavirin in a cohort of 125 patients^[20]. This difference between the studies may be related to the origin of the cohorts studied and SVR rates, since Covini *et al*^[21] and Novembrino *et al*^[22] studied Italian patients achieving SVR of approximately 60%, while we studied Brazilian patients with SVR at approximately 30% (Table 1).

The main target of anti-RR autoantibodies has been demonstrated in several studies, using different methods, to be the IMPDH2 enzyme^[19,21,23-26]. In a 2013 report from Carcamo *et al*^[19], 96% of samples from a cohort of 46 Italian patients with anti-RR reactivity recognized a 55 kDa band in immunoprecipitation (IP) corresponding with IMPDH2 mobility. In the same study, they also analyzed an American cohort of 47 patients; however, only 53% of American patients recognized a similar 55 kDa band in IP^[19]. When we tested a group of Brazilian samples using the same methodology, 12 of 15 patients (80%) recognized the 55 kDa IMPDH2 band^[25]. Probst *et al*^[26] developed a cell-based indirect immunofluorescent assay with HEK293 cells expressing recombinant IMPDH2. Using this assay, they found that all 33 anti-RR-positive

samples they examined recognized recombinant IMPDH2. Additionally, we performed a sandwich ELISA assay where the native antigen was captured by affinity-purified polyclonal anti-IMPDH2 antibody and found that 37 of the 53 (70%) anti-RR-positive samples presented reactivity above the cut-off^[25]. Finally, double-labeling immunofluorescent studies showed that anti-RR autoantibodies label the same RR structures as a commercial anti-IMPDH2 antibody, but not filamentary structures labeled by an anti-cytidine triphosphate synthetase (CTPS) antibody, a critical enzyme in pyrimidine biosynthesis that aggregates into filamentary RR-like structures^[23,27-29]. Altogether, these data indicate that IMPDH2 is a major target of anti-RR autoantibodies.

RR STRUCTURES AND THEIR FUNCTIONS

Over the last few years, a number of reports have described the ability of CTPS and IMPDH2, rate-limiting enzymes in the cytidine and guanine nucleotide biosynthesis pathways, respectively, to form large polymers^[23,30-34]. Under certain conditions, these enzymes aggregate into structures in the shape of rods 3-10 μ m in length and rings 2-5 μ m in diameter (Figure 1). These structures have been designated rods and rings (or RR) when the structures are composed mainly of IMPDH2, or *cytoophidia* (Greek for "cellular snakes") and CTPS filaments when the structures are composed mainly of CTPS, by different laboratories^[23,28,29,35]. The first mention of RR-like structures dates back to 1987, when Willingham *et al*^[36] published that they immunized Balb/c mice with Schmidt-Ruppin Rous sarcoma virus-transformed

Balb 3T3 cells and obtained a monoclonal antibody that labeled cytoplasmic structures very similar to RR structures in indirect immunofluorescence. The putative antigen/structure was named “nematin” due to the worm-like appearance of the observed structures.

Enzyme aggregation into non-membrane-bound large bodies is a common feature in eukaryotic cells^[37]. Although it is not known whether all aggregates represent functional entities or enzymatically inactive storage depots, examples of assembled polymers are discussed as a result of: (1) pathologic damage to enzymes (*e.g.*, sickle-cell hemoglobin); (2) enhanced enzymatic activity (*e.g.*, acetyl-CoA carboxylase); (3) formation of structural and functional elements (*e.g.*, actin fibers and microtubules); and (4) as a means to store catalytic potential (*e.g.*, CTPS filaments)^[37].

The function of RR structures is still unknown. To our knowledge, no study has specifically addressed the enzymatic activity state of the IMPDH2 enzyme while aggregated into RR. However, four very recent reports draw apparently contradicting conclusions regarding the enzymatic state of the CTPS enzyme when presented in the filamentary *cytoophidia* form. Three of the reports, from Barry *et al.*^[38], Aughey *et al.*^[39], and Noree *et al.*^[40], agreed that the aggregation of CTPS into *cytoophidia* downregulates enzymatic activity^[38-40]. Strohlic *et al.*^[41], on the other hand, demonstrated that CTPS within the *cytoophidia* structures is catalytically active during *Drosophila* oogenesis^[41]. Thus, the current hypothesis is that the assembly and disassembly of RR/*cytoophidia* structures allows for a highly sensitive control of enzymatic activity by keeping enzymes in active/inactive forms. This could be an important mechanism of regulation of the indispensable GTP/CTP biosynthesis pathways.

The observation that some RR structures disassemble after injection of anti-IMPDH2 antibody into live cells indicates that IMPDH2 molecules are the major building blocks of IMPDH2-based RR structures^[27]. However, it also indicates that the binding among IMPDH2 molecules to form RR structures is not very strong, allowing its disassembly by putative chemical tension, allosteric interactions, or other unknown mechanisms generated by the binding of several antibodies. These observations reinforce the hypothesis that assembly and disassembly of RR structures represent highly sensitive maneuvers to control enzymatic activity as described in the previous paragraph^[27].

Aggregation of IMPDH2 vs CTPS

Several publications demonstrated the ability of IMPDH2 and CTPS to aggregate into large filamentary structures; however, those studies were focused on only one of these enzymes at a time^[23,30-32]. While studying both enzymes simultaneously, we demonstrated the independent formation of IMPDH2-

based (structures composed mainly of IMPDH2) and CTPS-based (structures composed mainly of CTPS) filamentary structures within the same cell. We also reported that after treatment with glutamine antagonist 6-diazo-5-oxo-L-norleucine (DON), both enzymes can interact in the formation of “mixed” RR structures that display a mosaic of IMPDH2 and CTPS aggregation (Figure 1B-D)^[29].

IMPDH is involved in purine biosynthesis, catalyzing the nicotinamide adenine dinucleotide (NAD⁺)-dependent oxidation of inosine-5'-monophosphate (IMP) to xanthosine-5'-monophosphate (XMP), which is then converted into guanosine triphosphate (GTP), a precursor of the guanine nucleotide^[42,43]. Humans express two distinct versions of IMPDH with 84% sequence resemblance and similar kinetic properties, encoded by different genes: *IMPDH1* and *IMPDH2*^[43]. Both IMPDH1 and IMPDH2 are expressed constitutively in most tissues, however IMPDH2 is highly expressed in cancer cells and proliferating tissues^[44-46]. Therefore, IMPDH has been targeted by immunosuppressive drugs such as mycophenolate (mycophenolic acid or MPA). CTPS is involved in pyrimidine biosynthesis, catalyzing the final step in the biosynthesis of the nucleotide cytosine by converting uridine triphosphate (UTP) into cytidine triphosphate (CTP)^[47,48]. In humans, two versions of CTPS are encoded by different genes: the *CTPS* gene for the enzyme CTPS1 and the *CTPS2* gene for the enzyme CTPS2. Both are expressed constitutively in all tissues, as they are related to cellular growth and development, but have been shown to be overexpressed in cancer tissues, making them candidate targets for anti-cancer chemotherapy^[48,49].

IMPDH2 and CTPS seem to respond differently to conditions that induce their aggregation into RR/*cytoophidia*, such as the increase in intracellular concentrations of nucleotides^[32,33]. In the presence of excess guanosine, IMPDH2-based RR formed by DON disassembled, but not CTPS-based *cytoophidia*^[29]. This indicates that there are likely two distinct aggregation models for IMPDH and CTPS. RR and *cytoophidia* show very similar characteristics of formation and behavior, such as the morphological characteristics of rods and rings predominantly localizing to the cytoplasm and occasionally being observed within the nucleus as shorter, thinner structures. However, it has not yet been determined if the mechanisms that regulate the aggregation of each enzyme into RR/*cytoophidia* are related or not. While some progress has been made in the study of the enzymatic activity of CTPS filaments, the enzymatic state of IMPDH2 in its aggregated form is still totally unknown.

TOLERANCE BREAKDOWN: THE ANTI-RR CASE

Self-immune tolerance breakdown with autoantibody production is a multifactorial process that involves

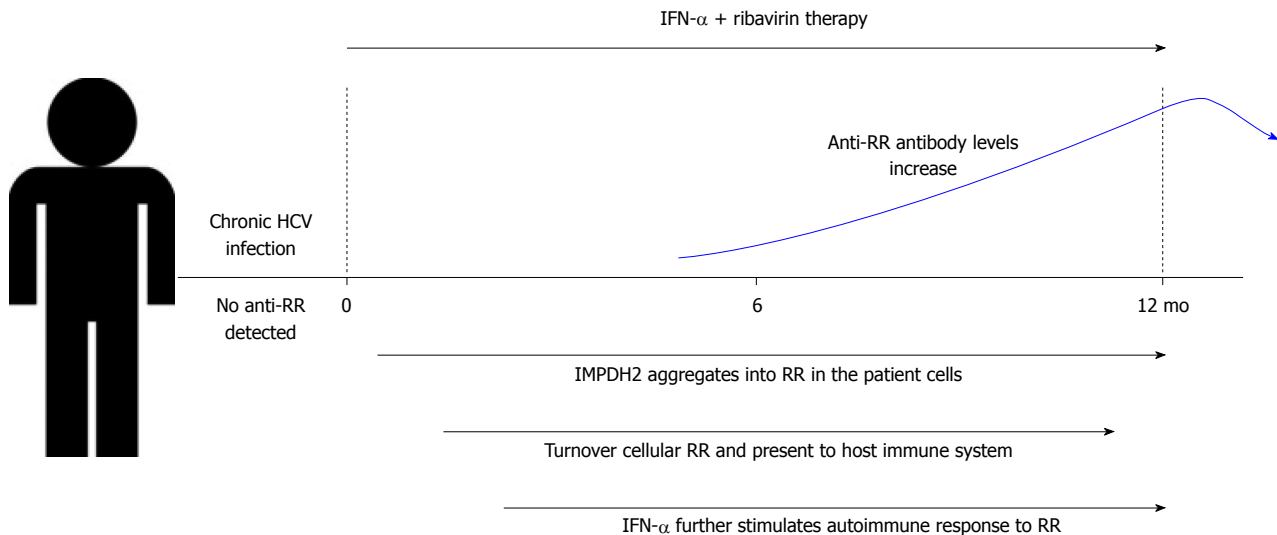


Figure 2 Anti-rods and rings autoantibody production in patients with chronic hepatitis C virus infection. Ribavirin therapy leads cells to present RR structures, while interferon- α stimulates the host immune system. These factors, plus others yet to be confirmed, could contribute to the tolerance breakdown with autoantibody production against RR structures, whose levels increase during treatment. RR: Rods and rings; HCV: Hepatitis C virus; IFN: Interferon.

intrinsic and extrinsic aspects. Intrinsic aspects depend on individual characteristics and certain abnormalities which may involve genes related to the major histocompatibility complex and several molecules involved in the control of the innate and adaptive response, as well as the hormonal environment. Extrinsic aspects could be various xenobiotics such as bacterial and viral infections or physical and chemical agents such as UV light exposure, pesticides, and drugs (including medications^[50]). Improper nutrition and lack of exercise are also possible contributors^[51,52].

The generation of anti-RR/IMPDH2 autoantibody appears to depend on inhibition of the target enzyme by treatment with ribavirin. In a previous study from our laboratory, none of 166 treatment-naïve HCV patients showed anti-RR reactivity. In fact, anti-RR/IMPDH2 antibodies were exclusively observed in patients who had undergone IFN- α /ribavirin therapy^[20]. The absence of anti-RR in HCV patients prior to IFN- α /ribavirin therapy was also described in other studies^[21,22]. However, it is possible that the immunological abnormalities associated with HCV infection and administration of IFN- α that stimulate the host immune system establish the conditions for ribavirin to act as an activator for the breakdown of tolerance with generation of anti-IMPDH2 autoantibodies (Figure 2). Indeed, we noticed that systemic lupus erythematosus patients treated with mycophenolate mofetil, an inhibitor of IMPDH2, do not develop anti-RR antibodies, except in extremely rare cases^[20,53,54]. In other words, the production of autoantibodies to IMPDH2 is unlikely to result from the inhibition of IMPDH2 and formation of RR alone (Figure 2)^[54].

In view of the facts that anti-RR autoantibodies primarily target IMPDH2, that inhibition of IMPDH2 by ribavirin leads to its aggregation into RR structures, and that HCV patients undergoing ribavirin treatment

produce anti-RR/IMPDH2 antibodies, we hypothesize that this represents a human model of immunologic tolerance breakdown followed by autoantibody production. We explored such a model, aiming to determine the temporal kinetics of the humoral autoimmune response to IMPDH2 in patients from the onset of treatment with IFN- α /ribavirin. We demonstrated that regarding titer, avidity maturation, and isotype levels, the humoral autoimmune response to IMPDH2 resembled that of a conventional humoral response to infectious agents, although at a considerably slower pace in titer increase and avidity maturation, as well as in isotype class switch, since these changes occurred over months in contrast to a time frame of weeks in the case of an infectious challenge^[25,55]. The temporal kinetics of the humoral autoimmune response is not readily accessible in human diseases, because we do not know when the triggering event occurs. The model of anti-RR/IMPDH2 autoantibody induced by ribavirin treatment provides a unique opportunity to study this aspect of the autoimmune response in humans. This difference may be related to the peculiarities in the adjuvant milieu in autoimmune and infectious diseases. The conventional infectious process is fueled by the strong adjuvant effect of the innate immune response associated with the inflammation caused by exposure to pathogen-associated molecular patterns (PAMPs) related to infectious agents. In the scenario of an autoimmune response, on the other hand, these elements are lacking or are present in minor proportions, thus possibly conveying different kinetics of the specific autoimmune response against self-antigens. Another element that might contribute to a slower pace in the maturation of the autoimmune humoral response is the existence of an array of counter-regulatory mechanisms that contribute to the maintenance of tolerance to self, including regulatory T and B cells.

CLINICAL RELEVANCE OF ANTI-RR AUTOANTIBODY

The possible clinical impact of anti-RR antibodies has been investigated by several laboratories, but no association has been found with disease severity, clinical evidence of autoimmunity, viral load or strain, or intensity of liver inflammation and injury^[20,22-24]. On the other hand, as outlined above, some studies indicate that the presence of anti-RR autoantibodies, especially at high titer, are more frequently observed in HCV-treated patients classified as relapsers. This association was observed in the cohorts of Italian and American patients^[19,21,22], but no such trend was observed in the Brazilian cohort^[20]. These observations could suggest that the presence of anti-RR autoantibodies indicates a higher chance for poor response to IFN- α /ribavirin therapy, and might support interruption of the treatment and a switch to the new protease inhibitors available for HCV therapy.

However, we emphasize that there is no established evidence for this reasoning. The association observed in the Italian and American cohorts is marginally significant from a statistical point of view, and there is considerable overlap between responders and relapsers with respect to the presence of anti-RR reactivity. In addition, no such association was found in the larger Brazilian cohort. In fact, we propose that the marginal association observed in some cohorts may operate from a different perspective. The production of autoantibodies against RR/IMPDH2 is stimulated by IFN- α /ribavirin treatment, save rare exceptions^[53]. Ribavirin has been shown to induce IMPDH2 to aggregate into RR structures *in vitro*^[23,29] and *in vivo* (Keppeke and Andrade, unpublished data). The strict association between anti-RR reactivity and IFN- α /ribavirin treatment in HCV patients strongly suggests that the ribavirin-induced IMPDH2 aggregate is the triggering immunogen in this drug-induced autoimmune reaction. It is therefore conceivable that longer exposure to the treatment would result in a higher chance of anti-RR autoantibody development. Unpublished observations from our laboratory show that up to approximately 70% of the patients treated for a second or third time present positive anti-RR reactivity as opposed to an approximately 40% frequency in patients treated for the first time. This finding adds strength to the hypothesis that longer treatment means a higher chance to produce anti-RR autoantibodies. Relapsers are patients that often need to receive successive rounds of treatment with IFN- α /ribavirin. In view of this reasoning, we propose that the higher proportion of anti-RR reactivity in relapsers observed in the Italian cohort might be attributed to the longer period of exposure to ribavirin in these patients. This hypothesis must be appropriately challenged in prospective follow-up studies with a large and heterogeneous cohort of patients. In the

meantime, it might be appropriate to closely follow anti-RR-positive patients with more frequent viral load measurements.

In conclusion, the autoantibody response against IMPDH2 elicited by ribavirin treatment in hepatitis C patients has allowed us to explore interesting aspects of immunological tolerance breakdown in humans from the beginning of the triggering event. In addition, anti-RR autoantibodies turned out to be invaluable tools in the investigation of the intriguingly large cytoplasmic and nuclear structures known as rods and rings. The molecular constitution of these RR/*cytoophidia* structures thus far appears to be largely based on the IMPDH2 and/or CTPS enzymes. Our laboratory and others have had the opportunity to verify that the RR structures may occur in many physiological and pathological instances. Currently, our efforts are dedicated to understanding the biological significance and the biochemical mechanisms involved in the process of aggregation of enzymes, especially the IMPDH2 enzyme, into RR structures. Future studies should also investigate why IMPDH2 is preferentially targeted by the immune system of HCV patients under IFN and ribavirin therapy, the role of IMPDH2 aggregation into RR filaments in this phenomenon, and to establish animal models for anti-RR tolerance breakdown as observed in HCV patients.

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2016 Laparoscopic Surgery: Global view

Laparoscopic and robot-assisted laparoscopic digestive surgery: Present and future directions

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Abstract

Laparoscopic surgery is applied today worldwide to most digestive procedures. In some of them, such as cholecystectomy, Nissen's fundoplication or obesity surgery, laparoscopy has become the standard in practice. In others, such as colon or gastric resection, the laparoscopic approach is frequently used and its usefulness is unquestionable. More complex procedures, such as esophageal, liver or pancreatic resections are, however, more infrequently performed, due to the high grade of skill necessary. As a result, there is less clinical evidence to support its implementation. In the recent years, robot-assisted laparoscopic surgery has been increasingly applied, again with little evidence for comparison with the conventional laparoscopic approach. This review will focus on the complex digestive procedures as well as those whose use in standard practice could be more controversial. Also novel robot-assisted procedures will be updated.

Key words: Laparoscopy; Robotic surgery; Colectomy; Esophagectomy; Gastrectomy; Obesity surgery; Liver resection; Pancreatectomy; Laparoscopic training

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Core tip: Laparoscopic surgery is increasingly used in the treatment of digestive diseases. New procedures are performed and novel technologies are applied. In addition, robot-assisted laparoscopic surgery has appeared as a useful tool for the digestive surgeon. The aim of this paper is to up-date the recent advances and scientific evidence supporting clinical practice.

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Manuel-Palazuelos C, López-Useros A, Gómez-Fleitas M. Laparoscopic and robot-assisted laparoscopic digestive surgery: Present and future directions. *World J Gastroenterol* 2016; 22(6): 1975-2004 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i6/1975.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i6.1975>

INTRODUCTION

Laparoscopic surgery has spread worldwide these days and many digestive procedures have become the standard practice. The rapid development in technology and improvement in surgeon skills have allowed that virtually every complex surgical technique, usually performed through open approaches, has been accomplished laparoscopically. Numerous reports favoring this approach have been published recently.

Caution interpreting literature is, however, necessary. There is a risk for selection and publication bias. Selection bias is possible because most favorable (in terms of location, number and tumor size) cases could have been operated on through a laparoscopic approach. Publication bias could also happen, since it is generally recognized a trend to report studies only with good outcomes and those exhibiting significant differences, which can lead to an overestimation of the laparoscopic approach. This publication bias can also be induced by some journal editors, who could prefer to publish papers with positive and spectacular outcomes more than negative and poor results. In addition, most published studies have been performed at large academic institutions by experienced surgeons, which could not reflect the current general practice.

Although feasibility of most laparoscopic procedures in general surgery has been shown, some have concerns about reproducibility, at least for the most complex techniques.

There are also concerns about costs of minimally invasive techniques in the era of cost containment. The advantage could come from shortening hospital stays and possibly diminishing some complications, but the disadvantage is more expensive surgical material and longer operating room times.

All of these considerations can be applied with robotic-assisted surgery, a novel approach applied today in most surgical procedures, and this approach is becoming used more and more.

The scope of this review tries to update most digestive surgical procedures, which precludes a systematic review. Outstanding papers were reviewed, but we especially focused on most of the recent work.

MINIMALLY INVASIVE COLORECTAL SURGERY

Laparoscopic procedures started in 1902 when George

Kelling, a German surgeon, used a laparoscope to assess the peritoneal cavity in a dog^[1]. This procedure was followed by different studies until 1987 when Mouret in France performed the first video assisted laparoscopic cholecystectomy. In 1993 this technique was established by consensus as the gold standard for the treatment of cholelithiasis^[2]. Since then, laparoscopy has been successfully used to perform funduplications, appendectomies, splenectomies, nephrectomies and a long etcetera.

Colorectal cancer is the second cause of death in western countries^[3]. Despite the progress in the different medical treatments and radiotherapy, surgery remains as the only potentially curative approach for this disease. The implementation of laparoscopic surgery in the field of colorectal surgery faced bigger difficulties because of the need of working in a broader surgical field that involved several quadrants, moving apart the small bowel, dissecting the retroperitoneal planes and removing a big sized specimen^[4]. Despite all these difficulties, Jacobs *et al*^[5] in 1991 reported the first laparoscopic sigmoidectomy for colon cancer starting a new era for colorectal surgery.

The enthusiasm for laparoscopic colorectal resections for cancer suffered a setback when in 1993 Alexander *et al*^[6] reported the first case of wound recurrence three months after a right colectomy for colon cancer. Tomita *et al*^[4] published their experience in laparoscopic colorectal resections in 1999 reporting a 1% wound recurrence similar to the one reported in open surgery. We have needed more than 20 years in order to have clinical evidence to demonstrate the benefits of laparoscopic surgery over open surgery in the treatment of rectal tumors.

A prospective comparative study performed at the Barcelona Clinic Hospital comparing laparoscopic and open colonic resections with 219 patients with a 98 mo mean follow-up, demonstrated that the laparoscopic surgery group had a faster recovery after surgery and less morbidity with a longer survival and a lower local recurrence rate^[7,8].

Several multicentric prospective randomized studies have shown the advantages of laparoscopic colonic surgery in terms of complications, postoperative recovery and oncological safety (COST, COLOR and CLASSIC)^[9-11].

There is not yet enough evidence in the case of rectal surgery. The conclusions of many retrospective studies make us think that this approach will have several advantages but solid data is still lacking.

Some studies such as the prospectively performed by Ströhlein *et al*^[12] comparing laparoscopic and open surgery, conclude that the laparoscopic surgery group has the same oncological results, with faster recovery and a shorter hospital stay, finding statistically significant differences in mid and lower rectal tumors. This study presents a high conversion rate of 22%.

Braga *et al*^[13] published a prospective and randomized study with 168 patients comparing open

and laparoscopic surgery for rectal cancer with a 5-year follow-up. This study concludes that postoperative morbidity and postoperative stay were significantly lower in the laparoscopic group. Survival and local recurrence were similar in both groups with better scores in the quality of live tests in the laparoscopic group after the first year. Long term costs were similar in both groups. Conversion rate in the laparoscopic group was 7.5%.

In a review of the literature presented by Indar and Efron^[14], the results of several prospective randomized trials were analyzed. From this review we can say that laparoscopic rectal surgery is safe, with good results in terms of morbidity, leaks and hospital stay. The conversion rate ranged from 3% to 29%. A higher conversion rate could be due to a lack of experience of the surgeons even though in some studies this rate was not reduced with a progression in the learning curve^[15]. The factors associated with conversion in the CLASSIC trial^[16] were a high body mass index (BMI), inaccessibility of the tumor, fixation of the tumor, and loss of definition of the surgical planes. Pugliese *et al.*^[17] have shown a significant increase in the anastomotic leak rate in the converted cases. Other alarming data is the increase of local recurrence rate up to 16% in those cases converted during laparoscopic surgery for rectal cancer, as shown by Ströhlein *et al.*^[12]. Scheidbach *et al.*^[18] presented a prospective study with 1409 patients with a 10-year follow-up about the impact of conversion in colorectal laparoscopic surgery. They observed statistically significant differences in terms of a higher blood loss, more postoperative complications, more anastomotic leaks and a decrease of the survival rate in the cases converted to open surgery.

The COREAN Trial was a randomized and prospective study performed by three Korean centers with a wide experience, recruiting 170 patients in the laparoscopic arm and 170 patients in the open group, with a 3-year follow-up. There was a very low conversion rate of 2% maybe because of the huge laparoscopic experience of these centers. There were no statistical difference in the oncological results in both groups and in the surgical specimen quality. The laparoscopic group showed a faster recovery with less need of postoperative pain-killers, a faster recovery of oral intake and a better quality of life 3 years after surgery^[19].

In a meta-analysis published by Anderson *et al.*^[20] including 1403 cases of laparoscopic rectal surgery and 1577 cases of open rectal surgery, there was no difference in terms of survival or oncological results.

The Finnish trial published by Kellokumpu *et al.*^[21] comparing open surgery vs laparoscopic surgery for rectal cancer concluded that the laparoscopic approach offered a faster recovery after surgery, lower blood loss and a shorter in hospital stay with less complications in the long term. Survival and local recurrence

were similar in both groups. Conversion rate in the laparoscopic group was up to 22%.

In the cohort retrospective study from Brazil, Melani *et al.*^[22] analyzed 84 rectal cancer patients that had undertaken surgery between 2000 and 2003, 50% with a laparoscopic approach and 50% with an open procedure. Follow-up was longer than 5 years in both groups and they found no difference in terms of complications, survival and oncological results. No conversion rate data was provided and the authors admitted a selection bias since the study was not randomized.

Park *et al.*^[23] published a comparative study of two groups with mid-low rectal tumors in which they performed ultralow intersphincteric dissection with an open approach vs a laparoscopic approach. Oncological and survival results were similar in both groups after a 3-year follow-up, but the authors concluded that the laparoscopic surgery group had less postoperative morbidity, a shorter hospital stay and a faster functional recovery.

In 2013, the COLOR II trial results after a 3-year follow-up were published. 1103 patients from 30 centers were recruited and randomized either to laparoscopic or open surgery^[24]. The authors conclude that the laparoscopic group patients had less blood loss, a shorter time to start bowel movements and a shorter hospital stay, with statistically significant differences. No difference in specimen quality was observed. Postoperative morbidity and mortality were also similar. Conversion rate was 17% even though the surgical teams were selected among centers with experience in laparoscopic colorectal surgery.

In a recently published study^[25], the authors analyze data from 3 randomized and controlled trials that compare laparoscopic vs open surgery for rectal cancer. The mean follow up was of 124.5 mo in the laparoscopic group and 136.6 mo in the open group. Disease free survival after a 10-year follow-up were similar in both groups. There was a tendency towards a lower local recurrence in the stage III tumors treated with laparoscopic surgery.

In summary, we can say that laparoscopic colonic surgery is feasible and completely comparable to open surgery, with advantages in postoperative complications and hospital stay. Rectal laparoscopic resection is also feasible and the studies performed to date suggest that short and long-term outcomes are comparable to open surgery.

Robotic colorectal surgery

Despite the advantages of the laparoscopic approach for colorectal surgery, this approach has some limitations such as loss of the 3D vision, limitations in the freedom degrees of the surgical instruments, the amplification of the physiological tremor and the "fulcrum" effect.

The implementation of robotic technology avoids

this disadvantages and improves the ergonomics of the surgeon^[26]. Uhrich *et al*^[27] proved that the uncomfortable positions during laparoscopic surgery increase surgeons fatigue and iatrogenic injuries.

The development of robotic surgery started in the mid-80s of the 20th century mainly focused in the development of tele-surgery. The FDA approved the use of Da Vinci system (Intuitive Surgical, Sunnyvale, CA, United States) in 2000, and nowadays is the only robotic system available for minimally invasive surgery.

The first article published about robotic colonic surgery using Da Vinci Surgical System was from Weber *et al*^[28] reporting a right colectomy and a sigmoidectomy for benign diseases. At the same time, other authors published their first robotic colonic surgery case reports^[29-31].

In 2004, D'Annibale *et al*^[32] reported 53 patients that had undertaken robotic colonic resections. In this group, 22 were resections for oncological reasons. They concluded that the operative and postoperative results were similar to those obtained with conventional laparoscopic instruments. Braumann *et al*^[33] published the first 5 robotic colonic resections performed in Germany in 2005.

Pigazzi *et al*^[34] and Hellan *et al*^[35] published the first article reporting robotic rectal resections in 2006. These authors reported the first series of 39 consecutive resections for rectal cancer, concluding that this technique is safe and feasible.

In Asia, the first robotic rectal surgery with total mesorectal excision was performed by Baik in June 2006^[36]. The same author published the first extended resection with hysterectomy^[37]. Ng *et al*^[38] reported the first robotic abdominoperineal resection in Hong Kong.

Few data are available regarding the value of robotic colonic resections but the results seem to be similar to those reported by conventional laparoscopic approaches^[39]. No final conclusions can be made at this moment.

Robotic rectal resections for rectal cancer

There are not many articles regarding robotic rectal resections in the scientific literature. Hopefully, the ROLARR (Robotic vs Laparoscopic Resection for Rectal cancer) trial results will be available in a few months and will give some valuable information.

Baek *et al*^[40] analyzed the results of 64 rectal resections for cancer with no operative mortality, a mean blood loss of 200 mL, a conversion rate of 9.4% and a leak rate of 7.7%. The mean of harvested lymph nodes was 14.5, the distal margin was 3.4 cm and the circumferential margin was negative in all cases. They found a local recurrence in 6 patients after an interval of 23 mo. The disease free survival rate was 73.7%.

Luca *et al*^[41], performed a study analyzing sexual and urinary function in 74 patients after robotic rectal resections concluding that robotic instrumentation

helped to preserve sexual and urinary function after total mesorectal excision.

Biffi *et al*^[42], studied the blood loss during robotic rectal resections and reported that only one case of their series of 49 patients required blood transfusion, by contrast with patients with open surgery who required blood transfusion in 12 cases of 105.

Shiomi *et al*^[43], in a study with 113 consecutive robotic rectal resections, 12 of them having T4 tumors, observed no conversion and no hospital mortality. The frequency of Clavien III/IV complications was 2.7%. They concluded that robotic instrumentation was helpful in performing advanced dissections with a very low morbidity and conversion rates.

Robotic vs laparoscopic surgery for rectal cancer comparative studies

deSouza *et al*^[44] compared the results obtained with robotic surgery in 36 consecutive cases against those obtained in 46 cases with laparoscopic assisted surgery using a hand port for the splenic flexure. The authors conclude that robotic total mesorectal excision is feasible and safe, and comparable with open surgery in terms of perioperative and anatomopathological results. There was a statistically significant difference in the tumor location, with more mid and low rectal tumor in the robotic group.

Kwak *et al*^[45] in another retrospective study concluded that the results of robotic surgery were comparable to those obtained with a laparoscopic group. This study was performed by a single surgeon with a huge experience in minimally invasive surgery for the treatment of rectal cancer. He recognizes a selection bias in this study and concludes that prospective, multicentric and randomized studies are necessary.

A very interesting study from Kang *et al*^[46], compared three groups of patients with mid and low rectal tumors treated with either open, laparoscopic or robotic approach. They observed that the robotic group had a faster postoperative recovery with a lower hospital stay, less pain and better specimen quality. The disease free survival rate was similar in all groups three years after surgery.

Fernandez *et al*^[47], retrospectively compared a group of patients treated with a robotic approach vs a group treated with a laparoscopic approach. They performed a low anterior resection in all cases with low anastomosis. They observed no difference in blood loss, postoperative morbidity or surgical specimen quality but, nevertheless, they recognized that the robotic group had lower tumors with a more advanced disease and more chemo radiation. The conversion rate was 17% in the laparoscopic group and 8% in the robotic group.

Patriti *et al*^[48] performed a study comparing 29 patients with robotic rectal resections against 37 treated with a laparoscopic approach with a one-year

follow-up. They obtained similar results in both groups with a higher conversion rate in the laparoscopic group: 7% vs 0%.

Lin *et al*^[49], performed a meta-analysis concluding that robotic surgery is clearly superior in terms of conversion rate. Another meta-analysis of studies comparing robotic vs laparoscopic surgery for rectal cancer performed by Trastulli *et al*^[50] suggested that the robotic surgery group had a statistically significant difference in conversion rate without significant differences in operation time, hospital stay, postoperative morbimortality or surgical specimen quality. The meta-analysis performed by Ortiz-Oshiro *et al*^[51] had similar conclusions.

In the systematic review of the literature performed by Scarpinata and Aly^[52] excluding the studies that referred to colonic resections, they suggested that there is evidence that robotic surgery may offer a better short term results, mainly in obese or male patients. It also may be better in those cases with previous radiotherapy and lower tumors. There was no evidence of any difference in terms of leakages, circumferential margins or preservation of the autonomous function.

A study performed by a Taiwanese group^[53], compared the postoperative results of 64 patients with ultralow anterior resection and intersphincteric dissection. Twenty-eight patients had undertaken a conventional laparoscopic approach and 36 a robotic approach. They found statistically significant differences in terms of surgical time - longer in the case of robotic surgery - and in the number of definitive stomas, which was 46.4% in the laparoscopic group vs 19.4% in the robotic surgery group. The authors conclude that this kind of procedure is feasible and safe with robotic instrumentation, with better functional outcomes and that surgical time will diminish as the experience of the surgeons increases.

Casillas *et al*^[54], analyzed the results of robotic colorectal surgery performed in a single institution by a single surgeon. They compared 200 laparoscopic cases vs 144 robotic cases. They observed a shorter hospital stay and a lower complication rate in the case of robotic surgery.

Park *et al*^[55], performed a prospective study with 217 patients that undertook minimally invasive surgery for rectal cancer. 133 patients had robotic surgery while 84 had a conventional laparoscopic approach. There were statistically significant differences in favor of the robotic approach in terms of hospital stay and conversion rates (0% vs 7.1%). Overall survival rate and disease free survival rate were similar in both groups with a 5-year follow-up. Saklani *et al*^[56] have reported similar results in a 3-year follow up study.

In a recently published meta-analysis, Xiong *et al*^[57] made a comparative analysis between laparoscopic and robotic rectal surgery in terms of safety and efficacy. They identified 8 studies with an overall number of

1229 patients, 554 robotic cases vs 675 laparoscopic cases. The authors concluded that the robotic approach is safe and feasible, but they did not find statistically significant differences in circumferential margins or in sexual function after surgery.

In summary, rectal robotic excision is feasible and safe, is comparable to laparoscopic surgery in terms of short and long-term outcomes, with some advantages such as shorter hospital stay, lower conversion rates and better functional results. Some particular conditions such as lower rectal tumors, male and/or obese patients or locally advanced rectal tumors may be indications that could benefit the most from the robotic approach.

Robotic colorectal surgery learning curve

The use of robotic instrumentation in rectal surgery requires not only training in surgical technique but also training in the use of the robotic system. This training has the specific handicap of the loss of the tactile feedback. The procedure is performed from a console far from the patient. This requires an excellent coordination between the surgeon and the surgical assistant.

The advantages that robotic technology provides make, for an expert surgeon in open surgery, less necessary the previous training in conventional laparoscopic surgery. The surgeon expert in conventional laparoscopic procedures has to make a specific training in robotic surgery. No differences have been observed in the learning curve for robotic surgery in surgeons with previous training in laparoscopic surgery vs those without that training.

Giulianotti *et al*^[58] observed that the robotic surgery learning curve was short for easy procedures as suture knotting or instrument use. They also observed that the training for advanced surgical procedures required previous experience in open and laparoscopic surgery.

Very few studies analyze the learning curve in robotic surgery. Bokhari *et al*^[59] estimated following the Cumulative Summation (CUSUM) technique that 50 robotic rectal procedures are necessary for a surgeon to be proficient in this procedure. Sng *et al*^[60] have recently published an article reporting a multiphasic learning curve. In the first phase (around 35 initial cases) the surgeon performs selected easy cases. In the second phase, with a worse CUSUM, the surgeon performs more complex cases (to 100 cases). Finally the surgeon enters in a consolidation phase.

Buchs *et al*^[61] have reported that the learning curve can be reduced using simulation in an animal/cadaver model or through the visualization of video clips or attending to courses.

In a recent study, Byrn *et al*^[62] compared their initial 43 robotic rectal surgery cases with the following 42 cases observing a significant reduction of surgical time and costs.

Other considerations concerning training are made

at the end of the paper.

Cost-effectiveness studies

One of the most criticized aspects of robotic colorectal surgery is the increase of cost per procedure. Cost analysis may vary depending on the criteria we use and the items we analyze. Rectal cancer process is very long and the analysis of costs of this process may vary a lot if we analyze just the perioperative period or a 5-year period including stoma cost, quality of life or local recurrence. The cost analysis also varies a lot depending on the health care system characteristics. There are not yet high quality articles that are conclusive about the cost issue.

Delaney *et al*^[63] reported a significant increase of in-hospital costs with robotic colorectal surgery: 2946 dollars per laparoscopic procedure vs 3721.5 dollars per robotic resection. This increase was mainly attributed to the increase in intraoperative costs.

However Rawlings *et al*^[64] did not find any statistically significant difference when they analyzed intraoperative, staff and surgical time costs.

Park *et al*^[55] compared robotic with laparoscopic rectal cases with 150 laparoscopic rectal cases and concluded that the robotic approach increased the perioperative costs. These authors recommended cost effectiveness studies including long-term results, oncological results and functional outcomes. Bodner *et al*^[65] have reported similar results.

MINIMALLY INVASIVE ESOPHAGECTOMY

The esophageal cancer frequency - mainly adenocarcinoma - is increasing worldwide, presently being the 8th in the incidence ranking and 6th in mortality^[66].

For patients with advanced loco-regional disease (T2-T4a and N +, stages II and III), controlled randomized trials and a large meta-analysis have shown a clear benefit in disease-free survival in patients treated with chemo-radiotherapy and surgery^[67]. However, esophagectomy is one of the most complex procedures of the gastro-intestinal surgery, with a high postoperative morbidity and mortality, mainly of respiratory origin^[68]. The potential advantages of minimally invasive procedures, especially regarding a decrease in pulmonary complications, have been studied for the last 4 decades^[69]. Minimally invasive esophagectomy (MIE) includes conventional pure laparoscopy/thoracoscopy, hybrid procedures (celiotomy/thoracotomy; celiotomy/thoracoscopy), hand-assisted surgery and, more recently, robot-assisted esophagectomy.

The first thoracoscopic esophagectomy was performed in 1992 by Cuschieri^[70]. Later, other experiences have been published: some reporting less than 5 cases of MIE Ivor Lewis each^[71], larger series of three-field esophagectomy -McKeown technique such as that of Luketich *et al*^[72] in 2003 with 222 patients, that of

Palanivelu *et al*^[73] in 2006 with 130 cases or others including hybrid procedures^[74]. Three meta-analysis have compared MIE with open esophagectomy and find a benefit of MIE in a hospital stay, respiratory complications and overall morbidity^[75-77]. Current evidences suggest that MIE is a feasible and safe technique with benefits in the short term. As a result, indications have been expanded from the Barrett's esophagus with high grade dysplasia to locally advanced tumors after neoadjuvant therapy, which are also the indications of open esophagectomy^[74]. Some aspects such as oncological outcomes, anastomosis location or patient positioning are, however, controversial. There are to date only a few works reporting long-term oncological outcomes and they have a short follow-up and a small number of patients, but they have failed, up to now, to show benefits by comparison with open esophagectomy^[78,79]. Probably, because of this, and despite the MIE procedures increasing, today only 30% of esophagectomies are performed worldwide through minimally invasive approaches, with 20% in 2009 in Japan or 19% in the United Kingdom in the last 8 years^[80-82].

Patient positioning influences the MIE technique. In the MIE as well as in the open technique, the left lateral decubitus has been more frequently used, although the prone decubitus is now being increasingly used. In 2012, the first controlled randomized trial analyzing this position was published^[83]. Fifty-nine MIE patients who were operated on in prone decubitus were compared with 56 patients treated by esophagectomy through a thoracotomy in semi lateral left decubitus. A significant decrease in respiratory complications in the MIE group was found by comparison with the open group (9% vs 29%). It is not clear whether the benefits are due to the position or the minimally invasive approach or, more probably, a combination of both. However, recent studies comparing both positions^[84,85] show that the prone decubitus provides some advantages such as better oxygenation and exposition of the surgical field, which lead to improved postoperative outcomes. As a result, the prone decubitus is being increasingly used.

Regarding the anastomosis location, the McKeown technique with cervical anastomosis, using thoracoscopy only for the esophageal mobilization, has been the most used procedure to date since a lower skill is needed by comparison with the intrathoracic anastomosis. However, the McKeown technique is associated with high complication rates such as recurrent laryngeal nerve injury, and anastomosis leak. As a result, there is a tendency to perform more intrathoracic anastomoses. In 2012 a large series of 1000 MIE patients compared 481 cases treated by the McKeown technique with 530 with the Ivor Lewis procedure^[86]. The outcomes in both groups were similar with low mortality rate and a similar number of retrieved lymph nodes, but the authors

concluded that Ivor Lewis MIE is preferable because a lower frequency of recurrent laryngeal nerve injury and a mortality rate of 0.9%, not higher than that of McKeown. Similar outcomes were found in another study from 2014 comparing 103 Ivor Lewis MIE with 185 McKeown MIE^[87], with significantly better results found in the first group: overall morbidity (16.5% vs 31.4%), respiratory complications (8.7% vs 25.9%), anastomosis leak (1.9% vs 13%), anastomosis stenosis (0% vs 4.9%), and recurrent laryngeal nerve injury (1% vs 7.0%).

Nevertheless, intrathoracic anastomosis is difficult to do through a minimally invasive approach, so there is a limited number of papers reporting more than 50 cases each in the last 2 years^[88-92].

Robot-assisted esophagectomy

Some limitations of the MIE can be overcome by the aid of the robotic systems, which provide some advantages such as a tridimensional view of a field selected for the surgeon instead of the assistant-, the 7 degrees of freedom allowing movements similar to open surgery, the tremor suppression resulting in a better precision, and a better ergonomics which leads to less surgeon fatigue. All of these advantages are even greater in small surgical fields, with few instrument exchanges as the thoracic phase of an esophagectomy is.

The first robot-assisted MIE using the Da Vinci system was published by Kernstine *et al.*^[93] in 2004. A few short series, between 6 and 47 cases, have been reported since then, always with cervical anastomosis^[94-99]. The first robot-assisted Ivor Lewis series were published from 2013 to now, reporting 22^[100], 17^[101], and 50^[102] cases, respectively, all with the patient in lateral decubitus. In 2014 our group published the first series of robot-assisted Ivor Lewis in prone decubitus with intrathoracic manual anastomosis^[103]. We feel that the prone position makes the dissection and lymphadenectomy easier, in an optimal field. We had no respiratory complication in 39 cases, although a stapled anastomosis, either transthoracic or transoral, is more difficult. The robotic assistance makes a manual intrathoracic anastomosis easier and allowed us to use the prone position and its potential advantages without flawing short and long-term oncological outcomes, as we recently reported in a series of 21 cases^[104].

Despite these potential advantages, the evidence to show any possible superiority of the robot-assisted MIE over either open esophagectomy or conventional thoracoscopic esophagectomy is still very limited. Since 2012 a single center controlled randomized trial has been ongoing in the Netherlands to compare the robot-assisted and open esophagectomy^[105], with a recruitment prevision of 112 patients - 56 per arm and a follow-up of 5 years. However, for stronger evidence, multicentric trials with a large number of patients is

needed.

MINIMALLY INVASIVE GASTRECTOMY

Gastric cancer incidence is decreasing but is still fourth in the world ranking and accounts for 10% of the overall cancer deaths. There are wide geographic variations, not only in incidence but in their clinical features also. Fifty percent of world cases are diagnosed in Asia and it is the most common cancer in South Korea^[106]. In the western world there is a trend for tumors to be advanced at diagnosis, to locate more proximally, to be histologically diffuse, and the patients tend to be older - 10 years more on average, to have an increased body mass index and more comorbidities^[107]. Because of these differences, two classification systems have been developed - the Japanese Gastric Cancer Association (JGCA) and the Union for International Cancer Control (UICC/TNM)^[108,109]. Also, different concepts concerning the optimal surgery, the reconstruction type and the minimally invasive surgery implementation have arisen between the West and the East. Fortunately, unification of the classification systems and approximation of the lymphadenectomy extent have been achieved in the last decade. As a result, the D2 lymphadenectomy has been implemented in the West^[110,111] and in the East now is accepted that no more than a D2 lymphadenectomy is mandatory^[112].

The same as in other procedures, minimally invasive gastrectomy (MIG) in case of cancer could show some advantages. These techniques were pioneered by Kitano *et al.*^[113] in 1992 with a laparoscopic-assisted gastrectomy. Since then, the development has been determined, not only by the tumor features - location and stage, but also by the above mentioned differences between the West and the East. In general, the high incidence or early tumors in the East led to 7341 distal and 1103 total laparoscopic gastrectomies in 2009 in Japan, and 3783 laparoscopic procedures in South Korea, in contrast with 245 in Spain between 2005 and 2008 and 133 in the United Kingdom between 2011 and 2012^[114].

Laparoscopic distal gastrectomy

For tumors in early stage and distal location, literature is profuse in retrospective studies, case series and comparative studies but there are seldom randomized controlled trials (RCTs) comparing Laparoscopic distal gastrectomy (LDG) with open gastrectomy and they have a limited case number^[115-122]. Despite the limitations, LDG appears to have advantages over open gastrectomy in terms of postoperative pain, recovery of gastrointestinal and pulmonary function, hospital stay and return to normal activity. The complexity of a proper lymphadenectomy - especially if a D2 is mandatory, and the concerns about oncological safety has slowed down its generalization^[123].

Additional evidence has been provided by several

meta-analysis^[124-127], some of them with more than 3000 patients including advanced stages^[128-131] and the most recent with 2144 cases^[132]. All coincide in the LDG perioperative advantages - blood losses, overall morbidity, hospital stay - and that oncological results are not inferior to those of open surgery, although more operative time is spent.

Currently two multicentric phase III RCTs are ongoing, one from the Japan Clinical Oncology Group^[133] and another from the Korean Laparoendoscopic Gastrointestinal Surgery^[134]. The joined recruitment prevision is more than 2300 patients and the results are expected for 2015.

At the moment, and after the international expert group meeting, the LDG is accepted for distal tumors cT1-2 cN0 and the laparoscopic total gastrectomy (LTG) for proximal tumors cT1 cN0, although no consensus was achieved for other stages^[135].

Literature data concerning LDG in locally advanced tumors (stage II and III) are even scantier. Only two RCTs have compared MIG and open gastrectomy^[136,137]. Two meta-analysis, have been published: the first included 7 studies with 174 laparoscopic and 278 open gastrectomies^[138], and the second analyzed 10 studies with 495 laparoscopic and 544 open gastrectomies, both with D2 lymphadenectomy^[139]. Both coincide in that the minimally invasive approach in advanced gastric cancer is associated with a longer operative time, but less blood losses, pain, postoperative complications and hospital stay. Similar lymph node number and overall survival were found in the two approaches.

A recently published phase II prospective clinical trial with 157 patients concluded that laparoscopic gastrectomy with D2 lymphadenectomy for advanced gastric cancer is technically feasible and safe, with acceptable morbidity and mortality rates^[140].

More high quality RCTs comparing open and laparoscopic gastrectomy are needed, as well as multicentric studies. Even so, some aspects will probably not be able to be applied to the West due to the above mentioned clinical and histological differences.

Laparoscopic total gastrectomy

The Laparoscopic total gastrectomy (LTG) spreading has been slower worldwide, even in Korea and Japan, due to the need of an esophagogastric anastomosis, which is technically demanding, and because of the low incidence of the proximal gastric cancer in the East. The first relatively large series appeared in 2009, such as that of Shinohara *et al.*^[141] with 57 patients or the multicentric study from South Korea by Jeong *et al.*^[142] of 131 laparoscopic-assisted total gastrectomies. Both conclude that LTG is feasible and safe - morbidity of 31% and 19%, respectively with no mortality, it is possible to retrieve sufficient lymph nodes - 46 and 35, respectively - although with long operative times - 4.5 h on average.

In the West, the European pioneer groups in this field, such as Dulucq *et al.*^[143] or Huscher *et al.*^[144] published a series of only 8 and 11 LTG, respectively. In 2013, a specialized center, the MSKCC published a series of 17 cases^[145].

Three recent meta-analysis^[146-148] also suggest that, in skilled hands, LTG has better perioperative outcomes than the open procedure in terms of blood losses, pain, resumption of oral intake, hospital stay, with no inferiority concerning lymph node retrieved and survival. However, the operative times are longer.

A phase II multicentric prospective trial (KLASS-03) in patients with stage I gastric cancer is currently ongoing, with the aim of assessing perioperative morbidity and mortality of LTG by comparison with the open procedure^[149].

Robot-assisted gastrectomy

The main advantages of the robotic systems have already been discussed. In the case of gastric cancer, these potential advantages lie in a better precision for the lymphadenectomy and an improved skill for intracorporeal anastomosis^[150]. Hashizume *et al.*^[151] published the first Robot-assisted gastrectomy (RAG) in 2002, but because the procedure is technically complex and the equipment is expensive, the spreading has been slow. Several groups have compared the RAG with the laparoscopic and open techniques. The Yonsei University group published in 2009 the initial experience with 100 patients, extended to 236 cases more (73% subtotal and 27% total) in 2011 of RAG compared with 591 laparoscopic (81% subtotal and 19% total). They concluded that RAG seems to have better short-term results with comparable oncological outcomes^[152,153]. Other published series until 2012 support these conclusions, although the largest one reports less than 40 cases^[154,155]. The first meta-analysis, also from 2012, compared 268 RAG with 650 laparoscopic gastrectomies^[156]. Significant differences were not found in overall morbidity, hospital stay, or number of lymph nodes retrieved.

Interesting, to make the most of the benefits of the robotic assistance, the morbidity due to anastomotic leak must be minimal. A study analyzing postoperative complications in 5839 patients (4542 open, 861 laparoscopic and 436 robotic gastrectomies) concluded that, even in expert hands, minimally invasive techniques are associated with an increased risk of anastomotic leak by comparison with open gastrectomy, although the overall morbidity and mortality rates were similar^[157].

Since 2012, seven new meta-analysis have been published, analyzing between 404 and 762 RAG patients^[158-164]. All concluded that RAG was associated with lower blood loss and shorter hospital stay, with an adequate lymphadenectomy, by comparison with the laparoscopic and open gastrectomy, although with longer operative times.

More high quality studies are needed to clearly define the role of RAG.

MINIMALLY INVASIVE BARIATRIC SURGERY

Obesity is a world health problem of epidemic dimension in western countries^[165,166]. A WHO report estimates that more than 1600 million people are overweight and 400 million people have frank obesity^[167]. The increase in obesity prevalence is associated with a rise in the associated disease prevalence such as diabetes mellitus, hypertension, hyperlipidemia, obstructive sleep apnea, cardiovascular and pulmonary disorders, some tumors, osteoarticular disorders, and depression^[167,168]. Life expectancy of obese patient is approximately reduced by 12 years^[167]. Bariatric surgery has been shown to be the most effective treatment to achieve significant and sustained weight loss^[169-173] and also improves every cardiovascular risk factor, with the exception of the hypercholesterolemia^[174]. These comorbidities lead to an increased consumption of health resources, and as a result, to increased costs of obesity treatment.

The therapy of this disorder includes both medical and surgical treatment. The former is based on a multidisciplinary approach with the participation of endocrinologists, dieticians and psychologists. The aim is to achieve a change in the life style, promoting physical exercise and healthy nutritional habits with the support of multiple drugs of limited efficacy^[175,176]. The current surgical techniques can be divided into restrictive (adjustable gastric banding and sleeve gastrectomy), malabsorptive (biliopancreatic diversion) and mixed (gastric bypass)^[177,178]. Since 1993, these techniques are being increasingly performed laparoscopically with preference over the open approach^[179]. Laparoscopy in obesity surgery offers several advantages: less pain, lower frequency of wound infection and incisional hernia, less postoperative complications, shorter hospital stay, faster recovery and better cosmetic results^[180]. Several early studies on vertical banding gastroplasty^[181], adjusted gastric banding^[182], and Roux-en-Y gastric bypass^[183-186], support some of these advantages, although the mortality rate did not decrease, probably because of the limited number of cases in each series. Later, several reviews^[187,188], including the 2009 Cochrane study, compared laparoscopic and open surgery, but no statistically significant difference was found regarding mortality, morbidity, reoperations or weight loss.

Two reviews of observational studies conclude that the frequency of incisional hernia and wound infection are lower in laparoscopic surgery, although lacking a direct comparison with open procedures. The systematic review by Reoch *et al.*^[180] analyzed 6 randomized studies including 510 patients with a minimum follow-up of 12 mo. The risks of reoperation,

wound infection, incisional hernia, anastomotic leak and cause of death were studied. They found that in the laparoscopically treated patients a 75% and 89% decrease of wound infection and incisional hernia risk, respectively. The risk of reoperation, anastomotic leak, and death cause were, nevertheless, similar in the laparoscopic and open surgery groups. Another review of 361 studies including 85048 patients^[189] analyzed the 30 d mortality and found a 0.28% rate for biliopancreatic diversion and duodenal switch and 1% for revisional surgery.

The restrictive procedures are associated with lower mortality rates than the mixed techniques, and the malabsorptive procedures have the highest mortality. In the meta-analysis by Buchwald *et al.*^[189] a higher mortality is found in open surgery by comparison with the laparoscopic procedures, with the exception of BPD/DS. Higher complication rate has, however, been reported in laparoscopic cases^[190]. Flum *et al.*^[191] published a population-based study of Medicare beneficiaries and found a laparoscopic surgery mortality at 30 d, 90 d and 1 year of 2%, 2.8% and 4.6%, respectively as well as higher mortality for individuals older than 65 years.

Morino *et al.*^[192] studied the mortality risk factors of several bariatric procedures, such as gastric bypass, banding gastroplasty, adjusted gastric banding, biliopancreatic diversion, biliointestinal diversion and other procedures in a 13871 patient retrospective analysis. They concluded that the laparoscopic approach significantly reduces the mortality risk, and the surgical technique, the open approach, a prolonged operative time, associated comorbidities and the surgeon experience increased the risk.

Mortality associated to laparoscopic bariatric procedures has been shown to be lower in centres with more than 100 cases per year (0.3%) than in those with less than 100 (1.2%)^[193].

In summary, the laparoscopic approach significantly reduces the overall mortality risk, since the hazard of thromboembolism as well as other complications decreases by comparison with open surgery. However the evidence level of long term outcomes of most review studies are low since many patients are lost to follow-up in many series.

Robot-assisted bariatric surgery

Robot-assisted bariatric surgery (RABS) has been used since 1998, when a gastric band was put from the distance^[194], and shows some advantages over the laparoscopic techniques, which have some limitations related with a poor ergonomics due to a limited instrument mobility due to the abdominal wall width, and hepatomegaly. RABS suppresses the position port limitation as well as the physiological tremor and confers a better ergonomics^[195]. The three dimensional view allows a more precise dissection^[196] and a decrease in blood loss. A shorter learning curve

by comparison with conventional laparoscopy has been claimed^[197].

Several studies report that RABS is safe with lower complications than the laparoscopic techniques. Edelson *et al.*^[198] compared 287 robotic and 120 laparoscopic gastric banding cases, and they did not find any significant difference in intraoperative or postoperative complications, hospital stay or operative time; the time was, however, significantly lower in patients with a BMI > 50 kg/m² operated on through a robotic approach (91 min vs 103 min). Fourman *et al.*^[199] reported similar findings in a literature review of RABS which included gastric banding.

The gastric bypass has been used since 2000, with satisfactory outcomes^[200]. Lower complication rates than those of the laparoscopic approach, without mortality or anastomotic leaks have been published^[199,201-205]. Others also report significantly less anastomotic failures with RABS^[206]. Skilled teams have achieved similar operative times and even shorter^[199,206,207], with comparable long-term results concerning weight loss and comorbidity improvement.

In a review of 1686 patients comparing laparoscopic and robotic bypass, similar results were found regarding anastomotic leaks, postoperative complications, operative time and hospital stay^[208]. However, an advantage was found in a decrease of the anastomosis stenosis rate at 6 mo. Most groups coincide in that anastomosis leak is lower with RABS, although without reaching statistical significance.

The vertical gastrectomy or sleeve gastrectomy (SG) has become in one of the most popular bariatric procedures due to its effectiveness in reducing weight. Overweight losses as high as 61% after 24 mo have been reported^[209], as well as comorbidity improvement such as diabetes resolution in 47%-66%^[210,211]. Other advantage are the lower operative time needed, the shorter hospital stay and the lower frequency of complications, by comparison with the laparoscopic GB. Since the use of robotic surgery has been limited to the most complex procedures - revisional surgery and gastric bypass, there are only a few studies on SG^[199,211-217] which do not show any significant difference concerning complication frequency - stenosis, bleeding-, mortality or hospital stay. One study reports a lower fistula frequency and a shorter stay in the robotic cases, but without statistical significance^[218]. This technique, when performed with robot assistance, is associated with longer operative times and is more expensive, and thus is controversial its generalized use. However, it is proposed as a way to learn robotic skills before performing the gastric bypass.

The Scopinaro biliopancreatic diversion (BPD) and the biliopancreatic bypass with duodenal switch (DS) are more effective than the gastric bypass in achieving weight loss and improvement of obesity associated comorbidities^[219,220] but due to their complexity, higher complication rates and the need of nutritional control,

are the least used. The first results of these procedures performed through a laparoscopic approach were published by Ren *et al.*^[221] in 2000, with a 2.5% mortality. In a systematic review, a 30-d mortality of 0.1% for restrictive procedures, 0.5% for gastric bypass and 1.1% for BPB/DS were reported. Sudan *et al.*^[222] reported a series of 59 robotic BPB/DS without mortality, anastomotic leaks, bleeding, sepsis or pulmonary thromboembolism.

The main criticism to RABS is its high cost, as well as the longer operative time, especially because of the preoperative docking time needed. This however, can be minimized with increasing experience of the surgical team.

LIVER SURGERY

Nowadays, laparoscopic resection is increasingly being performed for both benign and malignant liver lesions. This review will focus on the latter.

There are several options to perform a laparoscopic liver resection (LLR): totally laparoscopic, hand-assisted and hybrid resection. In the latter, the liver is mobilized laparoscopically, with the hilar dissection and parenchymal division performed through an abdominal incision, usually epigastric as described by Koffron *et al.*^[223]. Hand-assisted laparoscopy and the hybrid technique have been recommended as a bridge to the totally laparoscopic technique as the first steps of surgeons not familiar with complex laparoscopic procedures^[224]. There is not sufficient data supporting the superiority of any technique over the other in terms of operative time, blood losses or complication rates^[225].

Every type of liver resection has been performed: from non-anatomic resection to segmentectomy or right lobectomy, removing from one tumor node to multiple nodes^[226]. Pedicle control can be done, the same as in open surgery, in order to minimize bleeding.

Laparoscopic liver surgery is associated with some potential benefits by comparison with the open technique. There is a better view due to magnification and favorable vision angles. This is the case of the adrenal glands and the area around the inferior vena cava, since these structures are located on the dorsal side of the liver and are best seen by a laparoscope inserted through the umbilicus^[227]. There is also less bleeding with less transfusion needs, as most papers show^[226], explained in part by the laparoscopic magnification, and decreased venous oozing from the cut surface under pneumoperitoneum^[227]. Another explanation is a longer portal clamping time by comparison with open surgery, as reported in some works^[228], although this is not seen in others^[226]. The lower analgesia requirements are due to less postoperative pain^[228]. A lower frequency of postoperative complications has been claimed; several papers have reported a trend to decreased complications

rates, although without significant differences^[229-232]. Particularly, a lower overall incidence of pulmonary complications has been reported^[233]. A recent published metaanalysis reports a significant decrease in the complication rate of laparoscopic cases^[226], but other studies find similar complications in laparoscopic and open groups^[228]. Other advantages are shorter in-hospital stays - due to less pain and less complications-, better cosmetic results. Lower frequency of incisional hernia. Further resections, if necessary, or even salvage transplantation in the case of hepatocellular carcinoma (HCC) are probably easier and this could increase the re-resection rates. All of this without compromising the oncologic aims as free borders (R0 resection)^[228]. Increased liver regeneration has been reported in living donor patients operated by laparoscopy by comparison with open procedures^[234]. Although the reason is unclear and these findings have to be confirmed, the diminished acute-phase stress response and improved immune system function reported after laparoscopic surgery could explain this in part.

There are however some concerns regarding the laparoscopic technique. The first is the problem of venous gas embolism^[230]. Since the pneumoperitoneum rises intrabdominal pressure, an increased risk of CO₂ embolism is possible, although because of its greater solubility than nitrogen, it is much safer. Also, the use of argon beam coagulation could increase the risk of argon gas embolism. As a result, some authors recommend its avoidance or extremely cautious use^[224], only over minor bleeding points and opening one port to allow venting excessive gas pressure. Concerns also remain regarding the oncologic adequacy of LLR compared with open liver resection as well as failure to detect occult lesions, which is especially important in the case of metastases. This will be discussed later. There is a potential risk of tumor dissemination (port metastasis, peritoneal carcinomatosis). In case of major bleeding, the restriction on movements make the suture difficult^[227], leading to important risks. On the other hand, laparoscopic procedures might result in additional hospital costs due to the need for laparoscopic instrumentation, and possibly longer operative times. However, these costs can be offset by shorter lengths of stay. Estimates of costs in some centers find that the laparoscopic approach is not associated with higher costs^[230].

The advent of minimally invasive liver surgery could result in overuse of these procedures. Some authors have stated with caution that laparoscopic procedures could lead to their use in cases where surgery is not indicated and therefore that laparoscopic procedures should only be used when an open procedure is clearly indicated. However, some authors argue that in some cases, especially when faced with diagnostic and therapeutic dilemmas, laparoscopic procedures might be considered instead of conservative nonsurgical

management^[231].

Most published papers report case series of laparoscopic resections, usually comprising a small number of patients. To date there are no published randomized clinical trials. In addition, few works have reported comparisons with open resection. This is probably due to the difficulty of putting together a team skilled in both advanced laparoscopic and open hepatic surgery.

Conversion rates are variable. The reasons are oncological, bleeding, strong adhesions due to previous surgery and no progression for anatomical reasons.

Transection methods are variable but any energy device or staplers can be used. For deeper transection, an ultrasonic aspirator (CUSA or equivalent) or the clamp-crushing technique can be used^[224].

Indications for LLR are the same as those of open surgery: large and symptomatic benign lesions, diagnostic concerns and, especially malignant tumors. The latter comprise any type of malignancy, but most frequently, colo-rectal metastases, HCC and cholangiocarcinoma. Our discussion will be focused on the malignant lesions.

Recently, the Second International Consensus Conference published their recommendations for LLR. They found these were not inferior to open resection in mortality, postoperative complications, margin negativity, overall survival, and costs. Laparoscopy was superior in length of stay. Also, technical recommendations were provided. They state that minor LLR (less than 3 segments) is "confirmed to be a standard practice in surgery but is still in an assessment phase". They also state that "major LLR is an innovative procedure and is still in an exploration or learning phase and has incompletely defined risks"^[224].

LIVER METASTASES

When several years ago laparoscopic LLR began, some concerns regarding liver metastasis resections arose: will free margins can be achieved? Will small metastases be found^[235]? No trials comparing open and laparoscopic metastasis resection were available in 2009 when the few available studies reported an 80%-87% 3-year overall survival^[235]. Nowadays we have some studies which allow comparing several aspects of open and laparoscopic surgery.

As mentioned above for liver resections in general, most published papers concerning metastasis reports a negligible or nihil mortality with a trend to a lower complication rate in laparoscopic series^[226,232], although this is not seen in other works^[229,236]. Also, the bleeding and transfusion needs are significantly lower than in open surgery^[226,229,237-239].

The mean operation time ranges from 180 to 377 min - depending on the resection extent - which is similar to the time spent in open resections^[226,229,237-239].

The length of stay ranges from 3.7 to 7.3 d^[226,229,237-239]

and 18.3 in a study from Japan^[230] but is significantly shorter in laparoscopic resections in all of them.

Concerning oncologic outcomes, the papers report R0 resection rates ranging 82.7%-100%, not different from those obtained in open resections^[226,229,232]. The long-term outcomes - overall and disease-free survival - are also similar. The 5-year overall survival rate found in the metaanalysis by Schiffman *et al*^[226] is 51.4%, although rates as high as 76% have been reported^[226]. In addition, non-significant differences have been found when compared with survival after open procedures^[226,229,232,237]. Importantly, no significant difference between laparoscopic and open resections disease-free survival has been found^[226,237], reflecting that no missed metastasis was left behind after laparoscopic procedures.

HCC

Resection is the usual therapy for HCC in non-cirrhotic patients. However, most cases in the western world arise in cirrhotic patients. This implies to deal with a liver with some functional impairment as well as more fibrotic tissues. Current guidelines recommend resection only in solitary tumors when portal hypertension has been excluded and serum bilirubin is normal^[240]. Although the best candidates are those patients with tumors up to 5 cm of diameter, resection in bigger tumors is also acceptable^[240]. Anatomic resections are recommended because of its better survival rate than wedge resections^[230,240].

In 2007 some benefits - reduced blood losses and morbidity in cirrhotic patients as compared with open resections, especially with lower frequency of postoperative ascites - were already recognized for the laparoscopic approach^[241]. The possible benefits of laparoscopy in cirrhotic patients can be due to preservation of the abdominal wall and round ligament, which avoids interruption of collateral circulation and, therefore, preventing a rise in portal pressure; less mobilization and manipulation of the liver which reduces liver trauma; avoidance of exposure of the abdominal viscera, which allows to restrict fluid requirements and decreases electrolytic and protein losses; and by reduction of intraoperative blood losses.

A recent metaanalysis of studies dealing with HCC in both cirrhotic and non-cirrhotic patients found lower rates of bleeding and transfusion requirements in laparoscopic resections, by comparison with open procedures^[242]. There were no significant differences in operation time. Concerning complications, lower rates of postoperative ascites and liver insufficiency were found. However, the frequency of other complications such as bile leakage, postoperative bleeding, intra-abdominal abscess, and mortality was similar^[242,243]. These results ought to be interpreted cautiously since not all the analyzed patients were cirrhotic. The length of hospital stay appeared to be similar after both approaches^[242].

Concerning oncologic results, significant differences in free margin rates have not been found^[242], although in a non-randomized study it was significantly higher in LLR than in open surgery^[244]. Both overall and disease-free survival have been shown to be similar in cirrhotic patients^[242-244]. Also, tumor recurrence seemed similar^[242-244].

As a result, LLR for hepatocellular carcinoma appears feasible and safe both in cirrhotic and non-cirrhotic patients, provided the functional status of the latter is acceptable. Also, oncologic long-term results are not inferior to those of open surgery.

CHOLANGIOCARCINOMA

Laparoscopy can be used in cholangiocarcinoma both for staging work-up and for therapy.

Cholangiocarcinoma (CC) has considerable rates of unresectability due to the common invasion of vessels, secondary and tertiary biliary duct divisions, presence of distant lymph node metastases and peritoneal metastases. Staging laparoscopy (SL), often combined with ultrasound, detects many of this settings, therefore avoiding unnecessary laparotomies and has been used with staging purposes for many years. The usefulness appears to have decreased considering reports from 2002 and from 2011, showing a drop both in efficacy (41.8% to 14%) and accuracy (72% to 32%)^[245]. This decrease can be explained by the continuous improvement in imaging techniques which detect today minimal disease. As a result, today the most extended opinion is that SL only is indicated in case of concerns of unresectable disease on imaging techniques or in patients with high risk of holding it, as T2/T3 cases of the Jarnagin-Blumgart staging system are^[246].

On the other hand, experience with laparoscopic treatment of CC is short, with most papers reporting only a few cases of laparoscopic^[247,248] or robot-assisted laparoscopic treatment^[249]. Two case series report 14 cases each. Yu *et al*^[250] treated 8 Bismuth I tumors by local excision and 6 Bismuth II cases by partial hepatectomy. The R0 resection rate was 100% in the former but only 60% in the latter. Importantly, they do not perform caudate lobe excision. Overall, there was no mortality and there was 35.7% of biliary fistula. They report two tumor recurrences. Gumbs *et al*^[251] report a multicenter experience of 9 intrahepatic and 5 perihilar CC. The former were treated by partial hepatectomy as well as 3 of the perihilar tumors, with a conversion rates of 11% and 20%, respectively. No caudate lobe resection was carried out. The R0 resection rate was 77.7% in the intrahepatic and 80% in the perihilar CC. In the intrahepatic CC the mortality and morbidity rates were 11% and 33%, respectively, whereas in the perihilar CC there was no mortality or complications. The survival rates were 66.6% at 22 mo in the intrahepatic and 100% at 10 mo in the perihilar CC.

Concerning robot-assisted surgery, there is an anecdotal published case of hilar CC treated by extended right hepatectomy and bile duct excision^[249]. They had free margins with no complications.

This scanty experience allow us to conclude that laparoscopic treatment of CC is feasible and that the rates of mortality, morbidity and survival are comparable to the open surgical procedures.

In the absence or randomized control trials is difficult to reach any conclusion concerning superiority of laparoscopic over open resection. For the moment, only clinical data mainly coming from case series performed in highly specialized centers show comparable oncological results and some advantages in hospital stay.

LIVING-DONOR LIVER TRANSPLANT

Right lobe living donor liver transplantation is the usual way of adult-to-adult live liver transplantation. Laparoscopic approach for liver procurement has been used in a few reports. The procedure has been performed as totally laparoscopic right or left hepatectomy^[252-257] and laparoscopically assisted using a hand port system^[256,257]. Also, the hybrid technique has been performed^[256] because some authors claim a shortening in the prolonged operative times of the procedure with this technique^[256]. The reported donor outcomes were satisfactory with low complication rates -most of them minor- and without mortality^[252-257].

Some studies have compared the laparoscopic and open techniques. Baker *et al*^[234] studied donor right hepatectomies and found in the donor similar rates of complications, estimated blood losses, and hospital stays, as well as shorter operative times and higher liver regeneration volumes in the laparoscopic group. Kim *et al*^[258] studied left lateral sectionectomies and found a significantly shorter hospital stay and time to oral diet in the laparoscopic group. Duration of operation, blood loss, warm ischemia time and complications were comparable, with no deaths in any group.

In summary, every liver resection procedure seems safely feasible through a laparoscopic approach provided the surgeon has proper training in both complex laparoscopic and liver procedures. The learning curve for this training has been estimated in 60 cases^[259]. Short and long-term results for metastases and hepatocellular carcinoma are as good as in open procedures, with shorter in-hospital stay and a trend for less complications and intraoperative bleeding. The Second International Consensus Conference considers that there are insufficient evidence from few centers to give any recommendation^[224].

ROBOTIC HEPATECTOMY

Theoretical advantages of robotic over laparoscopic hepatectomy are increased freedom degrees of the

instrument movements, abolition of the physiologic hand tremor and 3-dimensional view. Also easier suture ligation for vessel control rather than stapling has been claimed as well as easier retrohepatic dissection thus facilitating access to the hepatic veins^[260,261]. Intracorporeal suturing and tying in difficult locations can also be facilitated by the robotic technology.

Among the disadvantages of robotic hepatectomy are the longer times required to dock the robot, to exchange instruments, and to reposition or redock the instruments if the viewing field has to be changed. Also, the lack of tactile sensation when suturing and knot tying might lead to injury due to uncontrolled tissue overstretching or suture disruption^[262].

Although the published experience on robotic hepatectomy is scanty, Ho *et al*^[262] published a systematic review in 2013 comprising data of more than 200 patients. The procedures performed included wedge resection, segmentectomy, right and left hepatectomy and left lateral segmentectomy for both benign and malignant diseases. A right live donor hepatectomy was also done. The conversion rate was 4.6%. Mean operation time was variable, ranging 200 to 507 min. The morbidity rate was 20.3%, with bile collections and abdominal abscess being the most frequent complications. No mortality has been reported. It can be concluded that robotic liver resection is safe and feasible when performed by experienced surgeons.

Because of the relatively short follow-up, results concerning cancer-specific survival are scanty and most papers only report some recurrence cases. As a result it is too early to draw any conclusion concerning oncological results.

Some studies comparing robotic vs laparoscopic hepatectomy in both benign and malignant lesions have been published.

Ji *et al*^[261] showed that robotic hepatectomy is safe and feasible, with slightly lower complication and conversion rates than traditional laparoscopic and open resections. However, longer operative times and hospital stays were found by comparison with laparoscopic procedures, as well as increased costs. On the other hand, Berber *et al*^[263] did not find significant differences in operative time in a comparison between a small series of robotic and laparoscopic resections.

Tsung *et al*^[260] recently reported 51 robotic resections. Importantly, liver mobilization and adhesiolysis were carried out by conventional laparoscopy prior to robotic docking for transection. When compared with matched laparoscopic resections, no significant differences in blood losses, postoperative complication rates, mortality rates, postoperative intensive care unit admission rate, length of stay or margin involvement were found. Robotic resections used up more operation room time, although leading to less conversions than laparoscopic resections did.

In summary, although robotic liver resection is feasible in skilled hands, experience is very short. Indeed, the Second International Consensus Conference considers that there is insufficient data for evaluation^[224].

PANCREATIC SURGERY

Different types of laparoscopic pancreatic resection are performed: tumor enucleation, distal pancreatectomy, central pancreatectomy, pancreatico-duodenectomy and total pancreatectomy, although the latter is anecdotal. These techniques have been used for both benign (chronic pancreatitis, cystic tumors) and malignant diseases.

Tumor enucleation

Although in general formal pancreatic resection is recommended for most tumors, enucleation can be performed for neuroendocrine neoplasms if tumor size does not exceed 2 cm and if no findings of malignancy are detected on preoperative staging^[264]. Pancreatic tumor enucleation can be easily performed by laparoscopy with excellent morbidity and mortality outcomes^[265].

Distal pancreatectomy (pancreatic left resection)

This is the most frequently performed type of laparoscopic pancreatectomy for both benign and malignant diseases^[266,267].

Distal pancreatectomy can be accomplished with or without splenic preservation. Splenectomy could adversely influence oncologic long-term outcomes, in addition to predispose to infectious complications. As a result, efforts to preserve the spleen should be done in case of benign or low grade malignancies, provided that splenic vessels are not involved with the tumor. More controversial is splenic preservation in case of adenocarcinoma. Whatever the case, if the spleen is to be preserved, two techniques are used^[264,268]: in the first, section of the splenic vessels both at the level of transection of the pancreas and at the splenic hilum is performed, leaving the short gastric vessels as the only blood flow supply. In the second technique, the splenic vessels are preserved by meticulous ligation of all the branches reaching the pancreas. However, patency of splenic vein - although not that of the artery - can be compromised after a laparoscopic DP in as high as 35%^[269]. The frequency of splenic infarction and appearance of gastric varices is higher in case of splenic vessel section^[268].

Blood losses have been reported to be significantly lower in case of a laparoscopic approach by comparison with open procedures^[270-273]. Localizing small tumors by laparoscopy or laparoscopic ultrasonography can be difficult and this leads to conversion in many cases. Other causes of conversion can be bleeding or difficult structure identification which lead to conversion in

17%-30% of cases^[270-272]. Obesity is significantly associated to conversion^[271].

The complication rate has been reported significantly lower in laparoscopic cases^[271,274], although the severity grade was similar than in open procedures^[263]. Other papers showed similar rates^[272,273]. Tran Cao *et al*^[267] studied a nationwide database and compared the short-term outcomes of 382 minimally invasive distal pancreatectomy (mainly laparoscopic and some robotic) with those of open distal pancreatectomy. They found a significant reduction in overall perioperative morbidity among patients undergoing minimally invasive surgery, including a significant decrease in hemorrhagic complications and postoperative infections in laparoscopically treated patients. Results of five meta-analyses^[266] support these findings concerning overall perioperative morbidity, although clinically relevant pancreatic fistula frequency was significantly lower in laparoscopic cases only in one of the analyzed studies.

Patients converted from laparoscopic to open surgery have significantly more severe complications than those with not converted^[271] which reflects the need of proper selection for a laparoscopic approach. The reported mortality ranges 0%-1%^[267,271,273].

Some have reported operation times significantly longer in laparoscopic than in open procedures^[271], but others find similar duration^[273].

The hospital stay has also been reported to be significantly lower with a laparoscopic approach^[27,270,271,273].

A recently published meta-analyses^[275], comprising data of 3701 patients, all of them from non-randomized studies, confirmed most of the above mentioned findings: superiority of laparoscopic over open DP in terms of blood loss, time to first oral intake, and hospital stay. Mean operation time, mortality -0.4% in DP-morbidity and safety showed no difference. However, data concerning oncologic radicality and effectiveness are limited.

Distal pancreatectomy for adenocarcinoma

Most articles report a mixture of benign and malignant diseases as indication of distal pancreatectomy (DP). As a result, to reach conclusions concerning safety and oncological outcomes when dealing with adenocarcinomas is difficult. A few papers compare open and laparoscopic approaches only in case of pancreatic adenocarcinoma. Kooby *et al*^[270] performed a multicenter matched analysis of 23 laparoscopic procedures compared with 189 open procedures and found no significant differences in positive margin rates, number of nodes examined or overall survival. The median follow-up was only 10 mo and, thus, it is premature to conclude that the long-term results are as safe as in open procedures. Magge *et al*^[272] compared 28 patients with 34 operated on by an open approach. They found not significant differences in margin-negative resection (open, 88%; laparoscopic,

86%) and median lymph node clearance. Also, no significant differences were found for overall survival or risk of local recurrence.

Distal pancreatectomy for neuroendocrine tumors:

A recent paper compared laparoscopic an open DP performed for neuroendocrine tumors^[274]. The laparoscopic procedure showed no mortality, a significant lower complication rate and shorter hospital stay. No significant difference was found concerning margin involvement, long-term survival, and overall costs.

In summary, DP is feasible, safe and reproducible for most laparoscopy skilled surgeons. Concerning its use in case of adenocarcinoma, it also appears as safe as open DP but more studies analyzing long-term results are needed.

Robotic distal pancreatectomy

Theoretical advantages and disadvantages of robotic surgery have been discussed earlier. The published papers dealing with robotic DP are still scanty.

Zureikat *et al*^[276] report 83 robotic DP with no mortality and a low rate of severe complications, although with 43% cases of pancreatic fistula. The average operative time was 256 min and 2% required conversion.

Waters *et al*^[277] compared 17 robotic against 32 open and 28 laparoscopic DP. Cystic tumors predominated among the indications of robotic resection. In this group, longer operative times, similar blood losses, no mortality, shorter hospital stay and lower hospital costs were found. Higher rates of splenic preservation were achieved in comparison with the open and laparoscopic approaches. Daouadi *et al*^[278] compared 30 robotic DP and 94 laparoscopic DP patients. The robotic DP group included more adenocarcinomas. Significant differences in blood loss, hospital stay, or morbidity were not found. However, there was a statistically significant 36% increase in R0 resection with robotic surgery, as well as more lymph nodes harvested. Also the conversion rate was lower.

The study by Kang *et al*^[279] included 20 robotic and 25 laparoscopic DP for benign and borderline malignant lesions. Although the intent was to preserve the spleen, the overall rate of splenic preservation was very low. A higher splenic preservation rate was achieved with the robotic approach. This took longer operative times and had increased costs compared to laparoscopic cases.

In summary, although feasible and safe, robotic DP has not yet shown clear advantages over laparoscopic DP.

Central pancreatectomy

This technique can be applied for the treatment of benign or borderline lesions of the pancreas situated in the pancreas neck. Although uncommonly performed,

this technique is feasible by laparoscopy^[265-280].

A review from 2013^[281] collected 51 published cases operated on through total laparoscopy or robotic assistance. Pancreatic reconstruction was done with a Roux-en-Y pancreato-jejunostomy, or pancreato-gastrostomy. The procedure was long with a mean time of 356 min. Blood loss was minimal in most cases. Mortality was nil, but morbidity was high, mainly due to pancreatic fistula (46%).

When performed by a robotic approach, the procedure is associated with long operative times (mean: 394 min) and a 23% of severe complications, although without mortality^[276].

Pancreaticoduodenectomy

Three techniques are currently employed for LPD: pure laparoscopy (PL), hand-assisted (HA) laparoscopy, and laparoscopic-assisted (LA) surgery. In contrast with DP, where only resection is to be performed, the pancreaticoduodenectomy (PD) requires a complex resection as well as pancreato-jejunal, hepato-jejunal and gastro-jejunal anastomoses. As a result, although feasible, the difficulty of the procedure makes that experience with laparoscopic pancreaticoduodenectomy is limited to a few case series.

Gumbs *et al*^[282] analyzed most reported cases until 2011 comprising 285 patients, although only 32% had pancreatic adenocarcinoma. The most important findings for the entire cohort were a 9% conversion rate, 2% mortality, 48% morbidity and a length of stay of 12 d. Margin involvement was found in 0.4% with an average of retrieved lymph nodes of 15. The mean disadvantage was a long operation time ranging from 263 to 750 min.

Direct comparison between laparoscopic and open PD has been performed by some groups. The first^[273], which included 60% of patients with malignant tumors - pancreatic, biliary, ampullary - found significantly lower blood losses, and hospital stay as well as more lymph node harvested in the laparoscopic group. However, the average operative time was significantly increased. There were no differences in overall complications, pancreatic fistula, delayed gastric emptying, and resection margin involvement. The main finding of the second study^[283] was a significant reduction in blood losses and, thus, the transfusion need in the laparoscopic patients although it did not show relevant differences in morbidity.

Asbun and Stauffer^[284] compared 53 laparoscopic and 215 open PD. They found significant differences favoring laparoscopic PD blood losses, transfusions, length of hospital stay, length of ICU stay ($P < 0.001$), and number of lymph nodes retrieved although the operative time was significantly longer. The rates of overall complications, pancreas fistula, delayed gastric emptying, margin involvement were not significantly different.

Kuroki *et al*^[283] compared 20 laparoscopic and 31

open PD. They also report a significant reduction of blood losses and transfusion need but they did not find significant differences in morbidity.

Song *et al.*^[285] report a comparison between 137 laparoscopic and 2055 open pylorus-preserving PD for periampullary tumors. A shorter hospital stay and a lower analgesic consumption was seen in the laparoscopic cases. No other difference was found including complications, blood losses margin involvement or lymph nodes retrieved.

In a recent systematic review of several series^[286], the operative time averaged 464.3 min (338-710 min) with conversion in 9.1% of patients. The mean estimated blood loss was 575 mL. Average mortality was 1.9% and morbidity ranged between 18.1% and 64.2%, with pancreatic fistula ranging between 4.5% and 52.3% of patients. An average of 14.4 lymph nodes were retrieved and 4.4% of cases had marginal involvement.

In summary, laparoscopic PD is feasible and as safe as the open procedure in highly skilled hands and high-volume centers. Long operative times are needed. Decreased blood losses seem to be the main advantage. Concerning its use in case of adenocarcinoma, more studies are needed.

Robotic PD

Published experiences concerning robotic PD are even less common. Zureikat *et al.*^[276] report 132 cases with a 90 d mortality rate of 3.8% and a frequency of severe complications (III-IV) of 21%. The frequency of pancreatic fistula was 17%, although only 3.7% were of grade C. Conversion was needed in 8%. The procedure took long operative times (mean 527 min), and although decreased with improved experience, the last cases of the series lasted over 400 min.

In the systematic review by Boggi *et al.*^[286], the robotic PD used up more operative time and had higher pancreatic fistula rate by comparison with pure laparoscopic PD (but not laparoscopically assisted PD). However, significant differences were not found concerning overall morbidity or mortality (2.7%, 1.1%, and 2.4% for pure laparoscopic, laparoscopically assisted, and robotic PD, respectively).

Another systematic review analyzed several non-randomized studies comparing open and robotic pancreatectomies, most of them PD^[287]. It included 137 cases of robotic PD and 203 open PD. The median conversion rate was 10%. Overall complications, reoperation rate, and margin involvement were significantly lower in robotic group, with no significant difference in postoperative pancreatic fistula, and mortality. In one study included in this review^[288] involving only PD, a significant increase in the R0 resection rate, with 100 % of patients in the robotic group compared with 87% in the open group.

A recently published paper^[289] comparing robotic and open PD, report similar findings such as lower

blood losses, shorter hospital stay, longer operative times with no significant difference in morbidity, mortality, as well as R0 resection rate and number of lymph nodes retrieved in case of malignancy. Patients with pancreatic adenocarcinoma of both groups had similar overall and disease-free survival.

In summary, the same as laparoscopic PD, robotic PD is feasible in highly skilled hands and high-volume centers. Although the very short published experience makes to reach firm conclusions difficult, it suggests that robotic PD is as safe as laparoscopic PD with some advantages over open PD. However, the former is more time-consuming.

TRAINING IN LAPAROSCOPIC SURGERY

In 1889 Halsted established at the Johns Hopkins Hospital the need of a new training system in surgery. Nowadays, it is rewarding to see how surgeons can be proficient in several techniques when they repeat them in a simulator^[290].

Today, traditional methods are not enough to teach and learn surgical skills because of the reductions in training hours during the residency programs and the lack of time of the surgeons to adequately teach these techniques. On the other hand, laparoscopic surgery learning curve and the risk of severe complications when inexperienced surgeons perform these procedures, make it more difficult for residents to learn minimally invasive techniques.

Costs per procedure have increased with minimally invasive procedures and technology is making the surgical environment more complex. A surgeon does not learn alone anymore, and they depend on a complex team that has to be trained and work together. As a result, surgical training models are evolving to serve as a complement to the standard surgical training in the theatre^[291].

Current situation of surgical residents training

With the arrival of laparoscopic surgery to the surgical departments in the 90s, the surgical resident training has suffered a huge transformation with a decrease in its autonomy. This has resulted in the need of additional training to obtain the confidence and maturity of judgment. As a result, the number of fellowships has dramatically increased during the last ten years. This change has enlarged the surgical training^[290].

Laparoscopic surgery training programs have evolved in several ways in the different countries. Sweden or The Netherlands have national programs limited to basic procedures (cholecystectomy) using training techniques as virtual reality^[292]. In the United States and Canada, the surgical training method is structured and the program of the American College of Surgeons (ACS) demands the inclusion of basic training in the Fundamentals of Laparoscopic Surgery

(FLS) in the residency programs^[293]. Spain has a limited training of two 3-d courses for the residents, one basic and another for advanced training^[294]. In Latin America, the project of Laparoscopia Avanzada Práctica (LAP), pretends to bring near a big number of countries to basic programs in laparoscopic training^[295].

How does the resident get trained in laparoscopic surgery? The curricular model

One of the most important aspects of the residency program is that training has to be based in a curricular model. Based on the premises that experts indicate^[296], these curricula have to be: (1) Endorsed by an accredited training institution (ACS) with a clear message regarding how this surgical training will be; (2) If simulation is used as a training tool, this demands a new and different approach of the instructors; (3) Training has to be integrated with clinical practice; (4) Adequate simulators have to be used in the correct timing and they should be validated for training; (5) The features and different types of surgical simulators are continuously changing. This has to be taken into account in order to plan the update of these simulators; (6) Residents must have reserved time to use simulators in their training; and (7) Financing has to be sustainable using business principles.

How do surgical simulators contribute to laparoscopic training?

Virtual reality (VR) simulators provide surgical training without supervision in a safe environment for both patient and trainees. Skills obtained in the VR simulator can be transferred to the theatre. However, evidence is only limited to basic surgical skills and laparoscopic cholecystectomy. There is no evidence yet of the effect of VR simulators on advanced laparoscopic procedures^[297]. The introduction of haptic feedback in these VR simulators has not increased the validation of laparoscopic surgical competences^[298]. In summary, all the trails that compare the training using VR simulators and standard laparoscopic training in the theatre observe a higher performance after training with VR, confirming that current training using structured simulation is more efficient than traditional training^[291]. Supported by these findings, countries such as Sweden or The Netherlands have established a structured training for laparoscopic cholecystectomy based in the use of VR simulators. This has allowed these countries validate resident competency before being trained in the theatre^[299,300].

Animal models for resident training in advanced laparoscopic techniques (Nissen, Colectomy) are the more realistic models, but they are limited in some countries because of religious beliefs or laws. All these reasons are behind the substitution of these animals by synthetic models or even by simulated models with

"ex vivo" viscera^[301]. Without any doubt, the animal model most frequently used is the porcine model, mainly in colorectal surgery training^[302]. Despite this, it is not possible to define nowadays when competency is reached with an animal model, if this model is the best one for laparoscopic training and if what is learned in this model is translated into clinical practice.

There are few studies about the use of cadaveric human models^[303]. These models have been used for training in laparoscopic skills, such as cholecystectomy, and have demonstrated the increase of capacitation by comparison with VR simulators. We believe that this model has to be reserved for advanced laparoscopic procedures (colorectal or bariatric surgery) that require a huge degree of realism. Leblanc *et al*^[304] assessed the use of fresh human cadavers for surgical training in laparoscopic sigmoidectomy. They observed that the use of this model improved clinical practice in terms of dissection, traction/counter traction, eye-hand coordination, suture, bleeding control and theatre time comparing with the use of a VR simulator. Palter *et al*^[305] studied the sequencing of VR simulators with human cadaver models for training in colorectal laparoscopic surgery. He added a cognitive module in order to help the participants to understand the procedure and how to plan and execute a right colectomy or a sigmoidectomy. He observed that this training approach improved the technical knowledge and the performance in the theatre in comparison with the traditional training during the residency program.

Hybrid models are those that use a complex robotized mannequin together with the abdomen/thorax of a live animal. These models allow us a high laparoscopic realism simulation while we adjust the cardiologic/respiratory parameters of the robotized mannequin. This way we are able to simulate for example a coronary event during the simulated laparoscopic procedure.

In other opportunities^[306], scenarios can be created to train the laparoscopic surgery team in a simulated theatre with a hybrid patient (SimMan 3G; Laerdal) and a laparoscopic VR simulator (Lap Mentor Express, Simbionix). These authors observed that the global assessment of the team showed a high qualification. Powers *et al*^[307] observed that these simulation models let us discriminate between the technical and non-technical abilities in residents and experienced surgeons. The target of this innovative multidisciplinary simulation is to identify the problem and to start with the adequate solution by the surgical team.

Zendejas *et al*^[308] observed in a recent review that whenever simulator is used, it has huge advantages in laparoscopic or open surgery training comparing with no simulator use. These authors also observed that adding the use of simulators to the traditional training is more effective than the use of traditional training

alone.

Our model for laparoscopic surgery training for residents

Following the experience of authors such as Haluck *et al.*^[296], we think that the University Hospital Marqués de Valdecilla resident training meets a number of requirements^[309]: (1) Our curriculum in laparoscopic procedures is developed during the full residency period. We think like Sadideen *et al.*^[310] that the first steps in surgical skill training have to be done outside the theatre and that practice is the most important thing to achieve automaticity in surgical skills. In the clinical environment, the needs of the patient prevail over the needs of the trainee; (2) The curriculum is compound by 19 laparoscopic modules; this allows the trainee to progressively gain competence as he learns. At the same time, each year modules are more technically advanced; (3) We support training in a simulated environment and advance in the learning curve during simulation. We have observed that doing gastrointestinal anastomosis and colonic resections this curve is shorter and increases patient safety^[311]; (4) Working with the most realistic models in each training phase, we observed that an important part of the initial training can be performed with low cost animal "ex vivo" models. Some examples are gastrointestinal anastomosis, cholecystectomy or gastric bypass; (5) The modular curriculum covers not only technical skills competencies but also teamwork competencies during crisis in laparoscopic surgery procedures. Teamwork competencies are trained in hybrid simulators; (6) The training process of the resident is assessed with Global Rating Scales of Operative Performance. Even though we also apply this assessment to the clinical practice, it is very difficult to move this assessment to the professional competence^[312]; and (7) The ACS accredits this training program and the center where it is developed.

We think that progress is very difficult and it may be necessary a trial/error system that let us advance. Learning from other programs that have tried, failed or succeeded may be a key point^[313].

Current challenges of resident training in laparoscopic surgery

For most of the groups around the world, the most important challenge is defining how simulation based training can be implemented and improve the training system. There are many questions to be answered, as we cannot say with scientific evidence in which degree simulated training improves results or quality in clinical practice and patient safety.

Simulators have to improve their benefits and design, and objective measures have to be developed so we can say in which degree the trainee acquires the adequate clinical competencies that can be translated in to the theatre^[314].

How can we keep and improve the acquired training during residency?

Mattar *et al.*^[315] observed that general surgery residency inadequately prepares trainees for fellowship results of a survey of fellowship program directors.

Fellowship programs are well established in countries such as the United States, Canada, United Kingdom or Australia. On the other hand, they have not had a good degree of implementation in the rest of Europe or in Eastern countries. These fellowship programs are advanced laparoscopic surgery training programs that have their own problems. Fellowships coexist with residency programs in the same institution and the continuous advance of new surgical techniques and the complexity of the surgical equipment make that some of the training is short in time or objectives. These reasons are making fellowships more and more specific as the recent fellowships in robotic colorectal surgery or rectal surgery is^[316].

The development of national training programs in advanced laparoscopic surgery, such as the one developed in the United Kingdom^[317] in laparoscopic colorectal surgery (Lapco) have shown good results in terms of short- and long-term training and have had a positive impact on the trainee learning curves^[318,319].

There are an increasing number of short length training courses in advanced laparoscopic surgery. They are usually limited to 3-5 d and they include live procedures performed by expert surgeons in most of the cases. Some theoretical knowledge is also given either during the course or on-line and, in some cases, the trainees have the opportunity to practice the technique on an animal or cadaver model. These courses have been mostly developed in colorectal, bariatric and upper gastro-intestinal surgery.

The impact of these courses on the training of the participant will depend on his previous experience in laparoscopic or open surgery. This way, we see that surgeons that come to a course with previous laparoscopic experience posteriorly implement the acquired knowledge during the course in a 60%-70%. Without this experience the degree of implementation is under 25%^[320,321]. Kinoshita *et al.*^[322] have demonstrated in Japan that after a training course in gastric laparoscopic surgery the number of laparoscopic gastrectomies increased in a 50% in the participating institutions. Participants answered to the survey saying that they felt improvement in their surgical skills in 100% of the initial procedures. On the other hand, Brunckhorst *et al.*^[323] say that there is very poor evidence concerning the training value of live operations and that very few high quality studies have been performed in this field.

Our point of view is that these 3-5 d courses provide knowledge and skills that help the trainee in starting laparoscopic surgery in an already established unit. These courses also help surgeons in sharing experience with experts.

In summary, we can say that current literature consistently proves the positive impact of simulation in theatre time and the scores in predefined performance but, however, this is not enough to ensure the transfer of these lab acquired skills to the theatre.

Which is the future of surgical training?

We are living a huge technological advance in all social scopes and also in surgical training^[324]. In this context, the acquisition of knowledge is progressively moving to e-learning platforms that reduce classroom hours. This system includes interactive feedback with the instructor, assessment of the procedure and interaction with other participants. This system may also be combined with VR training^[325].

VR simulators for laparoscopic surgery have been importantly improved with haptic technologies. In the near future, it may even be possible to import real 3D images to VR software. This may allow the trainees to perform real operations in virtual surgical fields that look like the real ones. Modelling this imported images, the trainee may even be able to work in fields with anatomical variations^[326].

"Virtual cadavers" based on 3D images reconstructed from computerized tomographies will replace human cadaver and animal models. It will be possible to create huge libraries with this "virtual cadavers". The exposure to multiple scenarios during the same basic procedure will make easier the trainee progress from competency to expertise.

Tele-simulation will be possible thanks to this libraries allowing tele-training. Virtual environments as second life (SL) will be used to completely represent a training centre or meeting room. These environments are already available in the market and can make possible the interaction of scenarios, patients, VR simulators, lectures or videoclips. Surgeons will be able to build their own virtual clinic or whatever they may need to simulate according to the level of competency that is trained^[327].

Tele-mentoring^[328] using robots as RP-7 (RP-7, Intouch Health, Santa Barbara, California) makes possible active mentoring of the trainees with verbal instructions or changing position of the instruments/camera when needed. It also makes possible a passive mentoring just with verbal instructions. This tele-mentoring seems to be a valuable tool for training minimally invasive procedures.

Now is the moment when all this separate tools, laparoscopic surgery, tele-presence, VR, digital image, networking... join together making tele-surgery possible. A surgeon is able to be miles away from the theatre and assist a surgical procedure.

Robotic surgery has been progressively incorporated to advanced laparoscopic procedures and has made those procedures easier. Robotics will facilitate the training of those surgical procedures. Mixing VR or virtual libraries with the surgical console of the robotic

surgical systems will make it possible to train the procedure before doing it in the real world. It will make training a particular procedure in a particular patient possible.

In summary, we should imagine a surgeon being trained in his work environment in complex minimally invasive procedures by another surgeon that is "on-line". Robotic surgical systems will be present in daily work and training.

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2016 Liver Transplantation: Global view

Coagulopathy and transfusion therapy in pediatric liver transplantation

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Abstract

Bleeding and coagulopathy are critical issues complicating pediatric liver transplantation and contributing to morbidity and mortality in the cirrhotic child. The complexity of coagulopathy in the pediatric patient is illustrated by the interaction between three basic models. The first model, "developmental hemostasis", demonstrates how a different balance between pro- and anticoagulation factors leads to a normal hemostatic capacity in the pediatric patient at various ages. The second, the "cell based model of coagulation", takes into account the interaction between plasma proteins and cells. In the last, the concept of "rebalanced coagulation" highlights how the reduction of both pro- and anticoagulation factors leads to a normal, although unstable, coagulation profile. This new concept has led to the development of novel techniques used to analyze the coagulation capacity of whole blood for all patients. For example, viscoelastic methodologies are increasingly used on adult patients to test hemostatic capacity and to guide transfusion protocols. However, results are often confounding or have limited impact on morbidity and mortality. Moreover, data from pediatric patients remain inadequate. In addition, several interventions have been proposed to limit blood loss during transplantation, including the use of antifibrinolytic drugs and surgical techniques, such as the piggyback and lowering the central venous pressure during the hepatic dissection phase. The rationale for the use of these interventions is quite solid and has led to their incorporation into clinical practice; yet few of them have been rigorously tested in adults, let alone in children. Finally, the postoperative period in pediatric cohorts of patients has been characterized by an enhanced risk of hepatic vessel thrombosis. Thrombosis in fact remains the primary cause of early graft failure and re-transplantation within the first 30

d following surgery, and it occurs despite prolongation of standard coagulation assays. Data, however, are currently lacking regarding the use of anti-aggregation/anticoagulation therapies and how to best monitor for thrombosis in the early postoperative period in pediatric patients. Therefore, further studies are necessary to elucidate the interaction between the development of the coagulation system and cirrhosis in children. Moreover, strategies to optimize blood transfusion and anticoagulation must be tested specifically in pediatric patients. In conclusion, data from the adult world can be translated with difficulty into the pediatric field as indication for transplantation, baseline pathologies and levels of pro- and anticoagulation factors are not comparable between the two populations.

Key words: Children; Coagulation; Thrombosis; Liver disease; Transfusion; Transplantation; Point of care coagulation

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Core tip: In the last two decades, extensive investigation of abnormalities in hemostasis in adult cirrhotic patients and improvements in both surgical and anesthetic management has enhanced outcome following liver transplantation. Unfortunately, such knowledge cannot be directly applied to pediatric patients, as major differences exist between adults and children undergoing liver transplantation. In this review, we discuss the pattern of hemostatic abnormalities in children with end-stage liver disease, point-of-care coagulation monitoring, and clinical strategies designed to reduce bleeding and thrombosis in pediatric liver transplantation. In conclusion, we propose a prioritized research agenda for this pediatric subspecialty.

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INTRODUCTION

Pediatric liver transplantation (PLTx) is the treatment of choice for children suffering from end-stage liver disease^[1,2]. The reported 5-year survival rate after PLTx is over 85%^[3-5]. In the last two decades, a growing body of research has provided insight into the donor and recipient factors affecting graft and patient survival^[6-13], and incorporation of these findings into clinical practice has improved graft and patient survival following PLTx. Red blood cell (RBC) transfusion, for example, is one factor which has a negative impact on outcome following liver transplantation in adult patients^[14-17]. A similar result was recently reported

for PLTx^[18]. One of the critical challenges in liver transplantation is the maintenance of hemostasis perioperatively^[19,20] as liver disease already leads to decreases in the hemostatic reserve^[21]. Furthermore, conventional coagulation tests are inappropriate for clinical decision-making^[22,23]. Finally, even though general rules valid in transfusion medicine can be applied to pediatric patients^[24], children have a number of important differences compared to adults, especially with respect to the hemostatic system^[25-31]. Here, we review the pattern of hemostatic abnormalities observed in pediatric patients with end-stage liver disease, point-of-care coagulation (POC) monitoring during PLTx, and the transfusional and non-transfusional agents clinically useful to prevent or reduce bleeding and thrombosis in PLTx. The review ends with a discussion concerning future areas of investigation for PLTx.

HEMOSTASIS DEFECTS IN PEDIATRIC LIVER DISEASE

Normal hemostatic mechanism in the pediatric population

"Developmental hemostasis" and the "cell based model of coagulation" are the two basic concepts required to evaluate hemostatic disorders in children with coagulopathy. "Developmental hemostasis" was coined in the late 1980s by Andrew *et al*^[32] to describe hemostasis as an evolving process that is age dependent, from fetal to geriatric periods. Accordingly, the functional levels of proteins change in a predictable way with age. Vitamin K-dependent factors, the contact factors and eight inhibitors [antithrombin III (AT-III), heparin cofactor II, α₂-antiplasmin, tissue factor pathway inhibitor (TFPI), α₁-antitrypsin, C1 esterase inhibitor, protein C (PC) and protein S (PS)] are at minimum levels at birth but gradually increase approaching adult levels by 6 mo of life. Conversely, factor VIII and von Willebrand factor (vWF, the main platelet-vessel wall adhesive protein) are the only two procoagulant proteins that exhibit markedly elevated levels at birth when compared to adult values. Moreover, the protein levels of α₂-macroglobulin (α₂M), a thrombin inhibitor of secondary importance in adults, are extremely high in neonates and often reach twice those measured in adults^[25,32-34]. Both quantitative and qualitative differences of the coagulation protein have been reported. Post-translational modifications (PTMs) may in particular affect the structure of hemostatic proteins and alter their function. For example, mean fibrinogen values are comparable between neonates and adults, but results indicate that neonatal fibrinogen is dysfunctional, and it is this form which persists until 1 year of age^[34].

However, despite differences in the quantity and quality of hemostatic proteins relative to the adult (Table 1), hemostasis is intact in neonates and young

Table 1 Hemostatic differences in the pediatric population

| Parameters | Defects | Activity | Time to normalization |
|--|------------------------------|----------|-----------------------|
| Procoagulant factors | | | |
| Factors II, VII, IX, X (vitamin K-dependent) | ↓ quantity | ↓ | 6 mo |
| Fibrinogen | Dysfunctional | ↓ | 1 yr |
| Platelet | Hyporesponsiveness | ↓ | 2-4 wk |
| vWF | ↑ quantity/ Hyperfunction | ↑ | / |
| Factor VIII | ↑ quantity | ↑ | / |
| Anticoagulant factors | | | |
| AT-III | ↓ quantity/ dysfunctional | ↓ | 6 mo |
| Heparin Cofactor II | ↓ quantity | ↓ | 6 mo |
| TFPI | ↓ quantity | ↓ | 6 mo |
| α2-antiplasmin | ↓ quantity | ↓ | 6 mo |
| α1-antitrypsin | ↓ quantity | ↓ | 6 mo |
| C1 esterase inhibitor | ↓ quantity | ↓ | 6 mo |
| Protein C | ↓ quantity | ↓ | 6 mo |
| Protein S | ↓ quantity | ↓ | 6 mo |
| α2-macroglobulin | ↑ quantity | ↑ | 6 mo |

infants. However, this “restored balance” can only be demonstrated using an assay, such as endogenous potential thrombin generation (EPT)^[35], which reflects the interaction of pro- and anticoagulants. Consequently, traditional tests, such as prothrombin time (PT) and activated partial prothrombin time (APTT), might be inadequate to assess coagulation capacity in neonates^[27,36].

This discrepancy may be explained by the complexity of the hemostatic system. It is not a simple cascade model with an “intrinsic” and an “extrinsic” component^[37,38], but rather an interaction between the blood vessel wall, coagulation proteins within the plasma and cellular components of the blood which are predominantly platelets. Hoffman and Monroe in 2000^[39] proposed a new concept of hemostasis that integrates both the humoral cascade and cellular factors, which is now widely accepted as representative of actual hemostasis *in vivo*. These investigators conceptualized a cell-based model of coagulation in which the three overlapping phases of coagulation (initiation, amplification, and propagation) occur on different cell surfaces. The initiation phase takes place on tissue factor-bearing cells such as fibroblasts. If the procoagulant stimulus is strong enough to produce adequate levels of the factors Xa, IXa and thrombin, then the amplification phase is triggered. Small amounts of thrombin generated on TF-bearing cells amplify the initial procoagulant signal by enhancing adhesion and activation of platelets and activating factors V, VIII and XI. In the final stage, the propagation phase, the active proteases combine with their cofactors on the surface of platelets producing thrombin on a large scale which results in the polymerization of fibrin.

According to this model, TF-bearing cells and

platelets have specialized procoagulant functions, while vascular endothelial cells have specialized anticoagulant features. The endothelial cells express the thrombomodulin (TM) which binds the thrombin released into the circulation from the site of injury, and converts it from a procoagulant to an antithrombotic. Thrombin in complex with TM activates Protein C on the endothelial cell surface, and this activated Protein C (APC) forms a complex with Protein S. The APC/Protein S complex cleaves and inactivates any factor Va and factor VIIIa that has been activated on endothelial cell surfaces and thus, prevents further formation of additional procoagulant enzymes where an intact endothelium is present. In addition to TM, endothelial cells also express other important antithrombotic surface proteins, such as antithrombin III (ATIII) and tissue factor pathway inhibitor (TFPI). These plasma protease inhibitors prevent inappropriate intravascular coagulation by inhibiting active proteases which have been released from the cell surface into the fluid phase.

This cell-based model may explain some aspects of pediatric hemostasis that a protein-centric model does not. For example, while platelet number and volume are similar between neonates and adult, neonatal platelets clearly exhibit hyporesponsiveness during the first 2 to 4 wk after birth^[40]. However, in most *in vivo* assays used to detect platelet function, platelet dysfunction is not observed in neonates. This result is probably due to the fact that vWF plays a more prominent role in neonatal hemostasis^[41], so that a higher concentration of circulating vWF and a greater percentage of large vWF multimers, the molecules most effective in promoting platelet vessel wall adhesiveness, are present. This model also potentially provides an explanation for the regulation of the plasma levels of hemostatic proteins. Lisman *et al.*^[30] reported that children transplanted with an adult liver graft maintain a pediatric hemostatic profile after transplantation despite receiving the adult graft. This result indicates that the liver graft does not control the plasma levels of hemostatic proteins. Instead, regulatory mechanisms may involve the hormonal system^[42,43] or alternatively, an extra-hepatic sensor which might be present on the vascular endothelium.

Why does “developmental hemostasis” even exist? For this answer, we have to look at hemostasis as a system working within a network of physiological systems, such as wound repair, inflammation and angiogenesis. Recently, ATIII has also been shown to possess potent anti-angiogenic properties^[44]. Interestingly, the levels of ATIII are low in the neonate, a time at which angiogenesis is extremely active. It is therefore possible that the levels of ATIII modulate angiogenesis, making AT replacement therapy potentially deleterious during neonatal life^[45].

Table 2 Liver transplantation phases and coagulation status

| Liver transplantation phases | Coagulation status |
|-------------------------------------|---|
| Pre-existing coagulation disorders | Developmental hemostasis Rebalanced hemostasis in chronic disease Unpredictable effect in acute liver failure |
| Liver transplantation surgery | Coagulopathy of surgical trauma |
| Pre-anhepatic phase | Surgical challenge |
| Anhepatic phase | Hyperfibrinolysis |
| Reperfusion phase | (lack tPa clearance) Ischemic-reperfusion syndrome Heparin-like effect tPa release |
| After liver transplantation surgery | Tendency to thrombotic state (days 1-14 after liver transplantation) Recovery of coagulation activity in 1 mo |

COAGULAPATHY IN PEDIATRIC LIVER DISEASE AND DURING LIVER TRANSPLANTATION

The mantra of pediatricians that “children are not little adults” is especially true for pediatric patients with end-stage liver disease because of differences in the natural history of their disease, responses to medical therapy and overall nutritional status^[31]. Although research focused on coagulopathy in the context of PLTx is lacking, data from adult patients indicates that derangements of the hemostatic system occur in three phases: pre-operatively (pre-existing), intraoperatively, or postoperatively (Table 2).

Pre-existing coagulation disorders

The first variable to consider is age of the pediatric patient, which ranges widely from the newborn to the adolescent. As discussed above, the entire coagulation system is normally subject to substantial changes in the early years of life^[45], especially in the first months, and it is unclear how these changes influence the development of coagulopathy in liver disease. While no scientific studies have specifically addressed this topic, patient weight, a proxy of age in the pediatric population, has been related to blood loss in some case series^[46-48], yielding a threshold value of 10 kg^[49]. This finding however was not confirmed in a more recent study^[50]. The relevance of other factors, such as indication for PLTx or technical difficulty of the surgery, was also not defined. What is certain, is that our current knowledge and ability to monitor and interpret the hemostatic system in young children, especially in the newborn, is limited^[27,36].

The second major variable to consider is the heterogeneity in the indications for PLTx. There are four major indications: cholestatic cirrhotic disease, acute liver failure, metabolic disease and cancer. The clinical features of the pediatric patient with chronic

and acute liver failure are completely different, and it remains unknown as to whether or not alterations in hemostasis influence outcome in these patients^[22,51-54].

Indication for PLTx in 30%-50% of cases is cholestatic disease, such as biliary atresia or familial intrahepatic cholestasis^[1,7,8,10]. These patients exhibit a normal hemostatic profile^[55]. It remains uncertain however as to whether one of the features of their disease, as in their adult counterparts, is a hypercoagulable state^[56]. It is a reasonable possibility as these pediatric patients have a higher incidence of hepatic vessel thrombosis following transplantation.

Acute liver failure^[51] is the indication for PLTx in about 10% to 15% of cases, but it is associated with a higher mortality rate^[57-59]. In two studies, coagulation impairment during acute liver failure in adult patients has been evaluated^[60,61]. Patients were assessed simultaneously using the PT test and the thromboelastogram (TEG). In both studies, the two methods did not generate comparable results. PT was elevated in most patients with acute liver failure, but TEG results were very different; in most cases, coagulation appeared to be normal, but when altered (either hypo- or hypercoagulability), coagulation status was not predictable with classical laboratory tests. Similar evaluations have not yet been performed on pediatric patients so it remains unclear as to how these results and methodologies can be applied to different patient populations.

Exceptions to this argument are metabolic disease and cancer. In these cases, patients do not experience classic “global” liver failure from a functional point of view. Deficient synthesis for metabolic disease is mostly associated with specific metabolic pathways, but such changes do not affect the hemostatic system. Although data is lacking, it can be inferred that alterations of coagulation during PLTx in patients with metabolic disease and cancer can be entirely attributed to the surgery and not to a pre-existing coagulopathy.

The last variable to consider is the concept of rebalanced coagulation in liver disease.

The liver is the site where procoagulation, anticoagulation and fibrinolytic factors are produced. As liver failure progresses, production of these factors is generally reduced^[21,22,62-67] which subsequently drives the establishment of a “rebalanced system”. This rebalanced system includes multiple alterations in the clotting cascade, clot lysis, and the number and function of platelets (Table 3).

However, rebalanced hemostasis is typically less stable with the possibility of a rapid switch from a hypo- to a hypercoagulable state along with clinical complications such as renal failure, infection and/or trauma. Furthermore, coagulopathy, as detected by PT/PTT, does not protect from thrombotic events. Indeed, cirrhotic patients exhibit an incidence of peripheral deep vein thrombosis and pulmonary embolism that is two-fold higher than in controls (0.5%-1.0%)^[68,69].

Table 3 Factors implicated in rebalancing

| System | Increases | Decreases |
|------------------|--|---|
| Clotting cascade | Factor VIII | Procoagulation factors V, VII, IX, X, XI, prothrombin Vitamin K-dependent procoagulation factors II, VII, IX, X Vitamin K-dependent anticoagulation factors protein C and protein S; anticoagulant proteins synthesized by the liver such as protein Z, protein Z-dependent protease inhibitor, antithrombin, heparin cofactor II, and alpha-2-macroglobulin |
| Clot lysis | Tissue plasminogen activator (tPA) (due to enhanced release by the activated endothelium and/or by reduced hepatic clearance) Levels of plasminogen activator inhibitor (PAI-1) | Fibrinogen and dysfibrinogenemia Plasminogen, antiplasmin (alpha-2 plasmin inhibitor or alpha-2 PI), thrombin-activatable fibrinolysis inhibitor (TAFI), and factor XIII |
| Platelet | Plasma von Willebrand factor (vWF; main platelet vessel wall adhesive protein) | Thrombocytopenia and thrombocytopathy (usually from hypersplenism, altered levels of thrombopoietin metabolism, antiplatelet antibodies and defective platelet aggregation) ADAMTS-13 (vWF cleaving protease) |

The incidence of portal vein thrombosis in children in end-stage liver disease has been reported to be about 10%^[70].

PT and APTT fail to detect this rebalanced state because these tests are insensitive to plasma levels of anticoagulant proteins (protein C pathway, antithrombin and tissue factor pathway inhibitor) and cannot take into account the role of the endothelium and cells in the hemostatic process^[22]. By contrast, global assays, such as EPT, which test for both procoagulant and anticoagulant components, will detect this rebalanced state^[65,66].

A recent study^[71] performed on children and adolescents with chronic liver disease seems to contradict the concept of "rebalanced hemostasis" observed in cirrhotic adults: EPT data was found to be in agreement with routine coagulation tests in this pediatric cohort. However, the reference values for TF and TM concentrations chosen to evaluate EPT were not based on those used in other studies involving adult patients^[65,66]. This conflicting result represents only one of the many pitfalls and dilemmas emerging from the research agenda to evaluate hemostasis in neonates and children^[27]. To our knowledge, no other significant studies have undertaken the issue of hemostasis impairment in pediatric chronic liver disease. However, several studies promoted by the Biliary Atresia Research Consortium (BARC) and the Cholestatic Liver Disease Consortium (CLIC) are currently in progress.

Coagulation disorders during liver transplantation

Liver transplant surgery is academically divided into three phases^[72]: pre-anhepatic, anhepatic and post-reperfusion phases.

The pre-anhepatic phase is characterized by the presence of pre-existing coagulopathy superimposed with other factors, including coagulopathy due to the trauma of surgery, intraoperative bleeding related to surgical challenge, such as lysis of adhesions in the case of reoperations, bleeding due to the development of collateral circulation and portal hypertension,

increased capillary fragility, and dilution coagulopathy secondary to fluid replacement^[72,73].

The anhepatic phase includes by definition procedures from the occlusion of hepatic vasculature to revascularization of the transplanted-liver. During this phase, the production of coagulation factors and hepatic clearance is reduced. Hyperfibrinolysis may be the major problem during this phase due to lack of tissue plasminogen activator (tPA) clearance while levels of PAI-1 remain relatively unchanged^[74].

Reperfusion is the most delicate of the three phases because several imbalances, as a part of a larger ischemia/reperfusion injury (IRI) syndrome, may affect both anticoagulant and procoagulant pathways. Thrombocytopenia is readily apparent, mostly due to the trapping of platelets in the liver sinusoids, but platelet activation is also occurring^[23]. Additional complications originate from the so-called heparin-like effect (HLE) due to release of heparinoids from the endothelium of the donor tissue^[75] and an enhanced release of t-PA causing hyperfibrinolysis^[74].

In a recent retrospective study, analysis of a series of TEG (native and heparinase) collected during each surgical phase of liver transplantation was performed and demonstrated that alterations in coagulation were occurring during liver transplantation^[76]. Apart from a transient period of HLE, a significant number of patients presented with hypercoagulable TEGs during the procedure. In addition, a prospective study using trans-esophageal monitoring detected incidental intracardiac thromboemboli in 1.9% of the patients during liver transplantation^[77]. Thus, hypercoagulability during liver transplantation deserves a closer look as thromboembolic events are associated with high morbidity and mortality rates^[78].

Only one study describing the alterations of coagulation during liver transplantation in pediatric patients was found^[55]. The study was well conducted but included only 8 children. TEG data was collected for these pediatric patients in each phase of surgery. Coagulation changes were found to be similar to

those recorded in adults^[76] but less severe. Authors speculated that the reason for this difference was the preponderance of cholestatic disease in children compared to hepatocellular disease in adults.

In another pediatric study^[79], platelet function during PLTx was evaluated using aggregometry, with the assumption that platelet function could be implicated in the development of the IRI syndrome. Analysis of platelet function revealed that a reduction in aggregation occurred after surgery began, reaching a nadir during anhepatic and post-reperfusion phases. A slow normalization followed in the first 6 d after surgery. Interestingly, ADP triggered platelet aggregation levels were characterized by a strong linear correlation with markers of liver injury and IRI.

In summary, a tendency towards a prothrombotic state with an initial worsening of platelet function during the dissection and anhepatic phase of surgery has been observed. During the reperfusion phase, the heparin-like effect predominates but appears to be generally a transient effect. At this stage, there is, on average, a slight deterioration of coagulation and platelet functions, in terms of strength of the clot.

Coagulation disorders after liver transplantation

Early surgical complications after liver transplantation include primary nonfunction (PNF) of the graft, bleeding, hepatic artery thrombosis (HAT) and portal vein thrombosis (PVT)^[1,4,5,11,80,81]. PNF is a rare but catastrophic event of unknown etiology that is most likely related to IRI syndrome. It is characterized by high transaminase levels, coagulopathy and progression to multiple organ failure^[1]. HAT occurs in 5%-18% of pediatric recipients, which is three to four times more frequent than in adult transplant patients^[4,5,80,82]. HAT occurs most often within the first 30 d after transplantation and leads to massive graft failure in early onset cases. PVT occurs in 5%-10% of recipients^[4,5,80,82] and may lead to progressive portal hypertensive complications. It is more frequent in children transplanted for biliary atresia, because of pre-existing portal vein hypoplasia, which requires a complex surgical anastomosis^[5].

Avoidance of vascular complications is critical in liver transplantation, especially with today's paucity of liver donors. Etiology of this apparent prothrombotic state after PLTx has been explored in the last year. Lisman *et al.*^[30] showed that the hemostatic profile of the pediatric group receiving a left split from an adult donor liver was remarkably different from the adult group receiving the right split for several months after liver transplantation. In a recent paper, Chen *et al.*^[81] monitored the biochemical markers of coagulation impairment (PT, APTT, thrombin time, fibrinogen and platelet count) for the first 7 d after surgery in 20 children undergoing liver transplant from a living donor. Post-operative tests did not detect a deficit in coagulation function. Mimuro *et al.*^[83] studied the

coagulation and fibrinolysis system in 63 pediatric patients following liver transplantation by measuring PAI-1, TM, ADAMTS-13, soluble E-selectin, protein C and plasminogen activity, fibrin and PT. Blood samples were obtained from day 0 to day 28 after liver transplantation in order to develop a complete postoperative profile. Results showed a rapid recovery of coagulation activity from day 1 with a full recovery of the coagulation and fibrinolysis system to normal levels by days 21-28. Soluble fibrin levels, a marker of the thrombogenic state, increased significantly on day 1 and then gradually decreased, normalizing by day 14. These data indicate that the prothrombotic state may continue for a timeframe of up to 14 d after liver transplantation, and that appropriate antithrombotic therapy may therefore be required during this period. Therapy to counteract these imbalances has been tested. Hardikar *et al.*^[80] treated 41 children after PLTx with a standardized hemostatic replacement therapy protocol to reduce hemorrhagic and thrombotic complications. Plasmatic antithrombin levels were measured daily, and replacement therapy was given to maintain levels between 70% and 100%. Fresh frozen plasma (FFP; 15 mL/kg) was given daily to supplement native protein C and protein S levels and ceased once native protein C levels were maintained/stabilized. Intravenous unfractionated heparin was started 24 h postoperatively at 10 UI/kg per hour, and monitored to maintain Anti-factor Xa levels between 0.1 and 0.3 UI/mL. As a result, both thrombotic and bleeding complications were reduced. While the use of FFP in the period after PLTx is questionable because of impact on graft survival^[18], this study raises the importance of the development of clinical guidelines addressing perioperative management of coagulation for this particular pediatric group.

In summary, analysis of the key data available in the literature indicates that the post-operative period after PLTx is not characterized by significant bleeding disorders, but rather by prothrombotic activity which can last for up to at least 14 d following surgery.

POINT-OF-CARE COAGULATION MONITORING DURING PEDIATRIC LIVER TRANSPLANTATION

POC devices for coagulation have been developed in order to provide the clinician with immediate and reliable results near the patients without the need for a central laboratory. Long turnaround times of standard coagulation tests from a central laboratory (about 45-60 min) stimulated POC device development. The main goal is to avoid blind transfusion of blood product in massively bleeding patients. Blind massive transfusion is still advocated in resuscitation algorithms^[84] when POC devices are not available. A determined amount of blood products is often

used in these cases, maintaining a defined ratio between platelets, red blood cells and plasma^[85,86]. The second goal is to spare blood products in order to reduce costs and to avoid inappropriate use of them. Growing evidence indicates that transfusion *per se* is an independent risk factor for mortality, respiratory failure, sepsis and graft survival^[16,18,87,88]. Moreover, every blood product may have a detrimental effect on morbidity and mortality, at least in liver transplant patients^[16].

Three families of POC devices developed for monitoring coagulation are commercially available. The first family is made of POC, such as the CoaguChek XS (Roche Diagnostics, West Sussex, United Kingdom) and i-STAT (Abbott Point of Care, Abbott Park, IL, United States), that gives as an output the PT/INR ratio. Although fast and reliable^[89], they are useless in the setting of cirrhotic patients as the standard coagulation tests are sufficient. PT and APTT do not predict bleeding or thrombotic events and are not useful in determining the type of blood product to be transfused. This reflects the fact that these tests are not sensitive to the reduction of antithrombotic factors^[21,22] and give no insight into the process of the generation of thrombin^[65], interaction between platelets and fibrinogen or fibrinolysis. The role of these POC devices in the treatment of the cirrhotic patient is thus limited to risk stratification for post-transplant morbidity and mortality^[90].

The second family of devices is made up of a large group of POCs that have been developed to study the functional activity of the platelets. Platelets lead the process of clot formation as they initiate the plug process adhering to the vascular injuries, activate the aggregation process and are eventually responsible for the propagation phase. The number and function of platelets is decreased in cirrhotic patients. However, POCs for coagulation function have been developed to assess platelet function impairment mostly due to anti-aggregation therapies, and they are therefore mainly useful in two therapeutic scenarios^[91]: stable patients to achieve an anti-aggregation profile in order to reduce their atherothrombotic risk; and bleeding patients to rule out a possible pharmacologic impairment of platelet function^[92].

Among the POCs marketed to evaluate platelet function, only three have been implemented in the pediatric population. The Verify Now Rapid platelet Function analyzer (Accumetrics, San Diego, CA, United States) is a POC that measures the degree of aggregation of platelets in whole blood over fibrinogen-coated polystyrene microparticles in the presence of a platelet agonist. The agonists specifically test for inhibition due to the aspirin-like effect (agents active over the arachidonic acid pathway), P2Y₁₂ inhibitors and GP II b/IIIa inhibitors. The main indications for the use of this device are to evaluate the efficacy of anti-aggregation therapies^[93] and to guide the intervention in order to counteract the platelet inhibitors in the case

of bleeding. Two POCs measure platelet function under high shear stress conditions in whole blood. These tests have the advantage of assessing the ability of the platelet to initiate the process of primary hemostasis in whole blood. The high shear stress condition closely simulates that of flow in blood vessels. The first, the Platelet Function Analyzer-100 (PFA-100; Siemens Healthcare Diagnostics, Deerfield, IL, United States) measures the time a plug takes to occlude the aperture of a capillary. Platelet aggregation is enhanced by the capillary membrane, which is coated with either collagen/epinephrine or collagen/ADP, and by the high shear stress condition. PFA-100 has been investigated in the pediatric population, and reference values from healthy subjects are available^[94]. This test is sensitive to a multitude of different factors: platelet number, hematocrit, drug and dietary effects, platelet receptor defects, vWF deficit, release and granule defect, and anti-aggregation therapies^[95]. However, this global test of hemostasis is not able to discriminate between different types of primary defects in hemostasis^[95], and its main indication is in monitoring aspirin and desmopressin therapy^[96]. Although tested in the pediatric population, mainly in cardiac patients, neither PFA-100 nor Verify Now was entered into the guidelines of antithrombotic therapy for pediatric patients in 2012^[97].

The Cone and Platelet Analyzer (CPA) is the second device that analyzes primary hemostasis in whole blood under high shear conditions^[98]. It has been used to test platelet function in newborns^[99] and in the cardiac pediatric patients^[100], but the available data for pediatric patients remain limited. These POCs in fact have little utility in the setting of a transplant or when the patient is bleeding as they are designed largely to evaluate platelet function which is mainly a characteristic of cirrhotic coagulopathy. Their clinical utility for pediatric as well as adult patients can be related to their ability to monitor anti-aggregation therapy in those patients needing long term therapy following a thrombotic event. Their utility in the setting of PLTx is potentially more appropriate for the postoperative period in order to reduce the incidence of HAT/PVT while maintaining a targeted anti-aggregation profile.

Devices that measure the viscoelastic properties of a clot in whole blood make up the third group of POCs. Described for the first time in the 1940s by Hartert^[101], these devices assess clot formation and dissolution kinetics. They measure the force transmitted to a pin that is immersed in the blood by the rotation of the cup in the case of the thromboelastography (TEG; Haemonetics Corporation, Braintree, MA, United States) or by the rotation of the pin itself in the case of the rotational thromboelastography (ROTEM; Tem International GmbH, Munich, Germany).

These tests have been extensively studied as monitor devices to guide transfusion during surgery and in particular, in trauma, cardiac or liver transplant

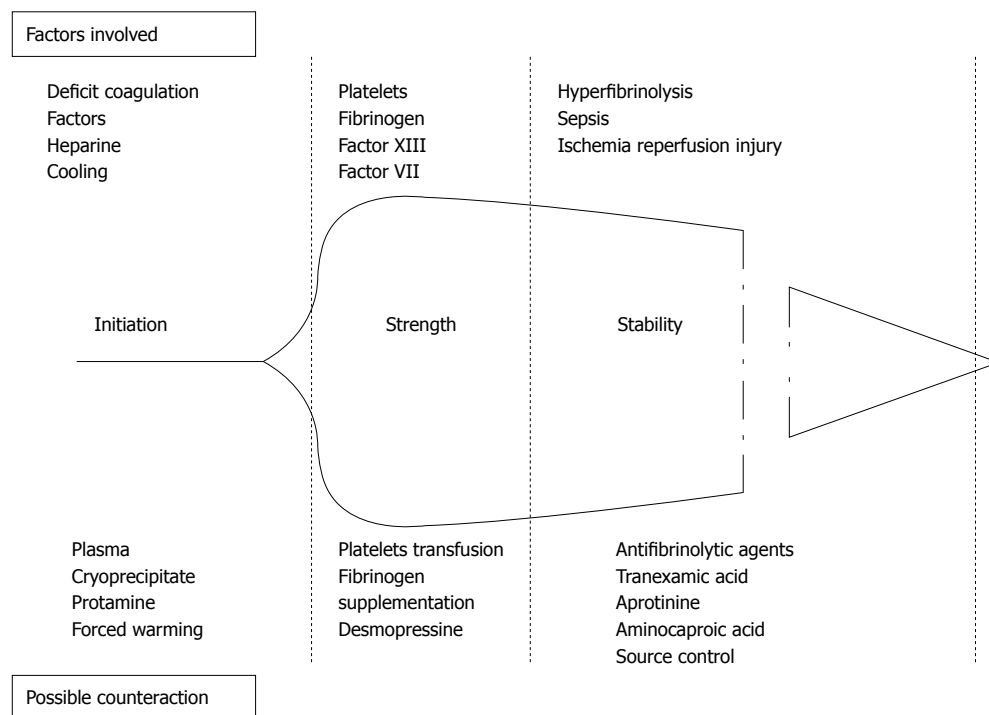


Figure 1 Standard thromboelastographic trace and liver transplant. A thromboelastographic trace superimposed with the surgical phases, affected factors, and counteractive measures.

patients. The standard tests have been modified with the inclusion of a particular activator or inhibitor to detect specific drug effects on coagulation, as in the case of the platelet mapping panel for TEG, or to define a specific factor effect such as FIBTEM for functional fibrinogen levels. These tests were entered into the guidelines of the European Society of Anaesthesiology^[102] for application with massively bleeding patients in the perioperative period and into the Task Force for Advanced Bleeding Care in Trauma in 2007^[84,103]. TEG and ROTEM generate basically the same information on clot kinetics through a slightly different technique. However, the two outputs are not directly interchangeable. TEG and ROTEM are particularly appealing because of their ability to obtain information at each stage of clot formation and dissolution. The tests collect information far beyond the initiation of clot information, which is typically evaluated in standard coagulation testing with PT and APTT. TEG and ROTEM provide information about the strength of the clot and fibrinolysis. These two data seem to be of the utmost importance because a reduction in clot strength is an ominous sign in a bleeding patient, and it is strongly and directly correlated with mortality^[104,105].

Hyperfibrinolysis is a well-known complication in the setting of liver failure. There is strong evidence that by counteracting it with antifibrinolytic drugs, such as aprotinine or tranexamic acid, at least blood product transfusions can be reduced^[106]. Moreover, a reduction in mortality was observed in massive bleeding trauma treated with tranexamic acid in the CRASH-2 trial^[107]. These results can potentially be applied to cirrhotic

patients with massive bleeding, where consumption coagulopathy is viewed as a common feature and in PLTx patients experiencing HAT because a hypercoagulative state can be detected^[82]. In Figure 1, a standard thromboelastographic trace is shown, along with the factors that can alter the trace and the possible action used to counteract the disorder.

TEG/ROTEM tests and their clinical application have been extensively discussed in several reviews in recent years^[108]. Enthusiasm for these techniques must be balanced against weak evidence for their utility and the need for better studies to sustain their wide spread use in clinical practice. To date, TEG/ROTEM tests have been included in three reviews from the Cochrane Collaboration. The first review demonstrated that a thromboelastographic guided algorithm reduced the amount of blood product transfused but had little, if any, impact on mortality and morbidity^[109]. The second review failed to detect a reduction in blood product transfusion with a TEG guided transfusion algorithm, but the number of studies was limited and of poor quality^[106]. In the most recent study, thromboelastographic technique failed to identify trauma induced coagulopathy. However, the analysis was performed on the data from a small number of poor quality trials so that the authors suggested that a greater research effort be put forth in this field^[110].

TEG/ROTEM tests share some drawbacks that have slowed their entry into routine clinical practice. The first is that these test are perceived as awkward to use by doctors. Trained personnel are needed, as well as daily calibration and a standardized technique.

Table 4 Reference parameters for Thromboelastogram in healthy and pathologic pediatric populations

| Ref. | Type of patient | Age | n | R, min | k, min | Alpha, ° | MA, mm | Lys30, % | Note |
|---|----------------------|--------------|----|----------------|---------------|------------------|------------------|---------------|---------------------|
| Rajwal <i>et al</i> ^[113] | Healthy | 1-15 yr | 14 | 17.4 ± 3.6 | 7.6 ± 1.4 | 31.2 ± 4.8 | 52.6 ± 6.4 | 0.8 ± 1.0 | Native blood |
| Pivalizza <i>et al</i> ^[114] | Healthy | < 13 mo | 25 | 7.7 ± 2.6 | 3.1 ± 1.4 | 55 ± 10.5 | 57.6 ± 3.7 | 1.9 ± 1.2 | recalcified blood |
| | | 13-24 mo | 33 | 10.1 ± 3.1 | 2.4 ± 0.5 | 74.2 ± 3.5 | 70.2 ± 6.1 | 3.4 ± 2.9 | Celite as activator |
| | | 25-48 mo | 24 | 9.8 ± 3.3 | 2.5 ± 0.6 | 73.2 ± 3.1 | 70.2 ± 4.7 | 2.1 ± 1.3 | |
| | | 49 mo-9 yr | 29 | 10.5 ± 3.0 | 2.7 ± 0.7 | 71.2 ± 4.2 | 68.4 ± 5.2 | 2.1 ± 1.4 | |
| Edwards <i>et al</i> ^[112] | Healthy | Newborn | 59 | 9.6 ± 2.5 | 2.8 ± 0.6 | 72.9 ± 4.2 | 70.5 ± 3.3 | 2.0 ± 1.1 | |
| | | | | 5.3 ± 1.3 | 1.6 ± 0.4 | 67.2 ± 4.5 | 61.8 ± 4.6 | 0.7 ± 0.7 | Cord blood, |
| Chan <i>et al</i> ^[29] | Healthy | | | | | | | | recalcified blood |
| | | 8.7 ± 2.6 yr | 44 | 8.7 ± 2.6 | 2.1 ± 0.7 | 61.6 ± 7.1 | 59.6 ± 4.2 | 0.2 ± 1.3 | Recalcified blood |
| | | < 1 yr | 24 | 7.7 (4.5-11.6) | 1.8 (1.2-2.3) | 66.5 (58.8-73.4) | 67.2 (60.7-73.2) | 3.8 (0.3-8.4) | Mean and |
| | | 1-5 yr | 24 | 8.3 (5.7-10.9) | 2.0 (1.4-3.3) | 63.6 (53.8-70.3) | 65.2 (57.6-71.3) | 3.0 (0.2-7.8) | 2.5%-97.5% |
| | | 6-10 yr | 26 | 7.8 (5.3-11.0) | 2.0 (1.4-2.8) | 63.9 (54.3-70.7) | 65.0 (57.3-72.8) | 3.3 (0.2-6.2) | |
| Brenn <i>et al</i> ^[115] | Cerebral palsy | 11-16 yr | 26 | 6.9 (3.8-11.1) | 1.9 (1.2-2.9) | 65.1 (54.9-73.2) | 66.5 (56.8-74.4) | 3.7 (0.5-8.0) | |
| | | 15 ± 3 yr | 15 | 4.8 ± 1.0 | 1.6 ± 0.6 | 68 ± 8 | 65 ± 8 | | Patients |
| | | 14 ± 1.5 yr | 15 | 5.0 ± 0.6 | 1.3 ± 0.4 | 71 ± 5 | 70 ± 4 | | undergoing spinal |
| Kang <i>et al</i> ^[55] | Idiopathic scoliosis | | | | | | | | fusion surgery |
| | | 9 mo-7 yr | 8 | 9.6 ± 6.2 | | | 46.9 ± 11.5 | | Baseline |
| | | | | 7.4 ± 2.8 | | | 47.3 ± 7.9 | | Anhepatic phase |
| | | | | 10.1 ± 2.4 | | | 46.1 ± 9.7 | | 30' after |
| | | | | | | | | | reperfusion |
| | | | | 9.2 ± 3.2 | | | 49.9 ± 8.8 | | 90' after |
| | | | | | | | | | reperfusion |

R: Reaction time for the time from placement of blood in the cup until the clot formation; k: Coagulation time, the time between TEG trace elevation from 2 to 20 mm; Alpha: Alpha angle, the slope of the TEG trace describing the kinetics of clot formation; MA: Maximum amplitude as an indicator of clot stability and firmness; Lys30: Clot lysis expressed as an amplitude reduction at 30 min after MA. TEG: Thromboelastogram.

This last issue is of utmost importance because without standardized technique, it is difficult to directly compare data across studies. For example, reference values vary between cohorts of patients with differences in age^[29,111], pathology, type of activator used (kaolin vs tissue factor), and the use of native or citrated whole blood, or plasma. Moreover, pre-analytical factors, such as the type of phlebotomy, the mean time before starting the assay, the methods in which the different factors are added to the blood sample, or even the way they are mixed, may profoundly influence the results of the test. The need for standardization is illustrated by the fact that in the last few years, three societies were founded with this focus: the TEG-ROTEM Working Group, the Working Party for the Standardization of Thromboelastography, and a subcommittee of the International Society on Thrombosis and Hemostasis.

Experience with these two methodologies on pediatric patients is increasing, but they were applied to pathologic patients long before reference values were defined for different age ranges. One of the first reports of thromboelastographic parameters in children were from patients undergoing liver transplantation in 1989^[55] while the first report on reference values from healthy individuals was in 2007^[29]. One of the most significant findings was with neonates. These tests revealed a nearly normal pattern of clotting kinetics despite profound alterations detected in standard coagulation assays^[112]. This result seems to reinforce the concept of restored coagulation in neonates, where concomitant reduction of pro- and anticoagulants has occurred.

Restored balance in neonates was confirmed *in vitro* with the ETP test, which includes thrombomodulin^[35], but not by conventional coagulation tests, such as PT or APTT^[27,36].

In Tables 4 and 5, the thromboelastometric parameters published for the different pediatric populations considering the underlying pathology/disease and the technical characteristics of the exam are shown.

MEASURES TO REDUCE BLEEDING AND TRANSFUSION

Strong data indicate that the use of blood products during liver transplantation in adults is associated with morbidity and mortality^[14-17,87]. Our group found similar results with PLTx exploring both intraoperative and the post-transplant phase^[18]. In our study, perioperative transfusion of FFP and RBCs was found to be an independent risk factor for predicting one-year patient and graft survival. The effect on one-year survival was dose-related. All of these studies were retrospective, and the main criticism is that the transfusion requirements may be considered as a surrogate marker for sicker patients. Prospective randomized trials analyzing different transfusion strategies are needed, but they are very difficult to design as levels of hemoglobin and coagulation factors are highly variable during surgery. The mechanism linking transfusion and poor outcomes after liver transplantation is unknown. Transfusion related immunomodulation, transfusion

Table 5 Reference parameters for rotational thromboelastogram in healthy and pathologic pediatric populations

| Ref. | Type of patient | Age | n | CT, s | CFT, s | MCF, mm | CLI60, % | Note |
|---|--------------------|-------------|-----|---------------|-------------|------------|------------|-------------------|
| Strauss <i>et al</i> ^[28] | Pre-term | Newborn | 47 | 185 (108-357) | 80 (52-183) | 57 (42-66) | | Median (MIN-MAX) |
| | Full term | | 184 | 194 (98-588) | 76 (34-208) | 60 (39-71) | | |
| Oswald <i>et al</i> ^[111] | Healthy | 0-3 mo | 51 | 184 (105-285) | 44 (27-88) | 66 (54-73) | | InTEM median |
| | | 4-12 mo | 55 | 172 (76-239) | 60 (37-100) | 63 (52-73) | | (2.5%-97.5%) |
| | | 13-24 mo | 54 | 161 (99-207) | 61 (42-112) | 64 (50-72) | | |
| | | 2-5 yr | 70 | 170 (99-239) | 60 (40-94) | 63 (53-73) | | |
| | | 6-10 yr | 79 | 168 (97-212) | 64 (48-93) | 62 (53-69) | | |
| | | 11-16 yr | 50 | 171 (128-206) | 68 (45-106) | 62 (54-71) | | |
| | | 0-3 mo | 51 | 48 (38-65) | 57 (30-105) | 62 (54-74) | 87 (71-94) | ExTEM median |
| | | 4-12 mo | 55 | 53 (37-77) | 72 (44-146) | 60 (46-71) | 86 (71-95) | (2.5%-97.5%) |
| | | 13-24 mo | 54 | 55 (37-73) | 75 (46-139) | 60 (46-72) | 88 (77-94) | |
| | | 2-5 yr | 70 | 56 (46-97) | 72 (41-109) | 61 (52-70) | 86 (74-93) | |
| | | 6-10 yr | 79 | 57 (43-74) | 77 (49-114) | 60 (53-68) | 87 (70-97) | |
| | | 11-16 yr | 50 | 59 (44-91) | 81 (53-115) | 62 (53-72) | 88 (76-94) | |
| Oasthaus <i>et al</i> ^[116] | Normal | 211 ± 116 d | 17 | 177 ± 28 | 60 ± 21 | 64 ± 6 | | InTEM (mean ± SD) |
| | Acyanotic | 134 ± 61 d | 17 | 178 ± 41 | 70 ± 16 | 61 ± 4 | | |
| | Cyanotic | 135 ± 132 d | 17 | 194 ± 43 | 105 ± 68 | 56 ± 6 | | |
| | Normal | 211 ± 116 d | 17 | 51 ± 6 | 71 ± 25 | 62 ± 6 | 94 ± 2 | ExTEM (mean ± SD) |
| | Acyanotic | 134 ± 61 d | 17 | 50 ± 5 | 88 ± 22 | 59 ± 6 | 93 ± 2 | |
| | Cyanotic | 135 ± 132 d | 17 | 68 ± 40 | 141 ± 99 | 54 ± 9 | 91 ± 4 | |
| Haizinger <i>et al</i> ^[117] | ASA I | 0-1 mo | 6 | 179 ± 17 | 56 ± 23 | 68 ± 7 | 91 ± 2 | InTEM (mean ± SD) |
| | ASA III-IV cardiac | | 17 | 332 ± 207 | 127 ± 184 | 62 ± 10 | 93 ± 3 | |
| | ASA I | 1-3 mo | 6 | 166 ± 25 | 45 ± 9 | 69 ± 3 | 89 ± 2 | |
| | ASA III-IV cardiac | | 6 | 257 ± 95 | 78 ± 47 | 61 ± 6 | 91 ± 2 | |
| | ASA I | 3-6 mo | 6 | 183 ± 22 | 49 ± 22 | 67 ± 7 | 90 ± 3 | |
| | ASA III-IV cardiac | | 6 | 187 ± 29 | 53 ± 11 | 69 ± 4 | 94 ± 3 | |
| | ASA I | 6-12 mo | 6 | 172 ± 11 | 60 ± 17 | 63 ± 8 | 89 ± 2 | |
| | ASA III-IV cardiac | | 6 | 196 ± 55 | 61 ± 11 | 66 ± 3 | 92 ± 4 | |
| | ASA I | 0-1 mo | 6 | 35 ± 12 | 65 ± 31 | 65 ± 9 | 91 ± 2 | ExTEM (mean ± SD) |
| | ASA III-IV cardiac | | 17 | 55 ± 62 | 119 ± 119 | 54 ± 10 | 92 ± 4 | |
| | ASA I | 1-3 mo | 6 | 35 ± 9 | 65 ± 12 | 64 ± 2 | 90 ± 4 | |
| | ASA III-IV cardiac | | 6 | 35 ± 7 | 98 ± 43 | 54 ± 7 | 91 ± 2 | |
| | ASA I | 3-6 mo | 6 | 33 ± 9 | 75 ± 33 | 65 ± 9 | 89 ± 3 | |
| | ASA III-IV cardiac | | 6 | 34 ± 15 | 79 ± 29 | 63 ± 5 | 94 ± 4 | |
| | ASA I | 6-12 mo | 6 | 45 ± 19 | 96 ± 39 | 59 ± 9 | 89 ± 2 | |
| | ASA III-IV cardiac | | 6 | 36 ± 11 | 85 ± 17 | 58 ± 5 | 92 ± 3 | |

CT: Clotting time, "r" time in TEG; CFT: Clot formation time, "k" time in TEG; MCF: Maximum clot firmness, MA in TEG; CLI60: Final clot lysis index at 60 min = LY A60 in TEG; INTEM and EXTEM are two different activated baseline analyses. ROTEM: Rotational thromboelastogram; TEG: Thromboelastogram.

associated circulatory overload, transfusion associated acute lung injury, hemolytic transfusion reactions, acute non-hemolytic transfusion reactions, transfusion-associated graft versus host disease and transfusion transmitted infection have all been proposed as possible mechanisms^[118-121].

A significant decrease in blood loss and blood product requirement has been observed during liver transplantation over the past 10 years, even if a wide range of blood product transfusion rates still exist between organ transplantation centers^[86]. This decrease can be explained by improvements in surgical and anesthetic techniques, and by a better understanding of the rebalanced hemostatic system in cirrhotic adult patients^[21,22]. Pediatric studies in this field are often observational studies or case reports. Based on data from adult patients, the strategies to reduce bleeding and transfusion should be analyzed in three different phases: pre-operative, intraoperative, and postoperative (Table 6).

PRE-OPERATIVE MANAGEMENT

In some pediatric and adult series, the value of pre-operative hemoglobin was found to be one of the factors most strongly correlated to the need for transfusion during surgery^[122,123]. It has been shown that platelet activation, as well as RBCs, have an active role in the generation of thrombin^[21]. It therefore seems reasonable to treat children with erythropoietin, supplemental iron and folic acid when feasible, in order to achieve higher hemoglobin levels before surgery^[124,125]. Although portal hypertension and hypersplenism can slow the rise of the pre-operative mass of red cells, bone marrow normally responds to stimulation with erythropoietin. The time available before transplantation appears to be the determining factor since this type of therapy requires several weeks to be effective. In adults, the transjugular intrahepatic portal-systemic shunt (TIPS) may be considered to optimize the pre-operative state of the patient. TIPS

Table 6 Measures to reduce bleeding complications and transfusions during liver transplantation

| Surgical phase | Procedure |
|----------------|--|
| Pre-operative | Erythropoietin Supplemental iron and folic acid |
| Intraoperative | Low CVP (fluid restriction, phlebotomy, vasopressors, Pringle maneuver) Acute intraoperative hemodilution Low transfusional trigger Drugs (rFVIIa, antifibrinolytics) Blood salvage Surgical technique TEG/ROTEM |
| Postoperative | Low transfusional trigger Minimize blood sampling Erythropoietin Supplemental iron and folic acid |

CVP: Central venous pressure; rFVIIa: Activated recombinant factor VII; TEG: Thromboelastogram; ROTEM: Rotational thromboelastogram.

is mainly used as therapy for upper gastrointestinal bleeding but also less frequently as a treatment to reduce portal hypertension in patients with pronounced varicose veins^[126]. Some authors have speculated that this second effect could reduce bleeding during surgery, especially during hepatectomy^[84]. A recent study indicated that TIPS is a feasible and effective technique also in children with ascites or gastrointestinal bleeding who are unresponsive to medical and endoscopic treatment^[127].

Given the lack of scientific studies of high quality, none of these strategies has been rigorously validated statistically, although they have already entered into clinical medical practice.

INTRAOPERATIVE MANAGEMENT

Blood volume management

Different strategies in the management of blood volume can influence the amount of blood loss in the course of liver transplantation, especially during the dissection phase of surgery. Some studies have reported a lower amount of blood loss when strategies to reduce central venous pressure (CVP) are utilized^[122,128-130]. These strategies are realized mostly through intraoperative fluid restriction, phlebotomy, vasodilators and the Pringle maneuver. The rationale is that low CVP should favor the venous return from the liver, thereby reducing the portal venous pressure and blood loss from surgical tranche. In addition, in case of injury to the vena cava, this strategy would facilitate surgical repair maneuvers, although with a possible increased risk of venous air embolism. All of these studies are prospective, nonrandomized, underpowered, and on adults. For these reasons, they cannot be considered conclusive, although almost all reported a reduction of blood loss when a policy of CVP reduction was implemented. The only pediatric

study^[131] reporting a reduction of blood loss with low CVP management is of limited scientific value because bleeding was not defined as the primary outcome. However, the possible effects of such a strategy cause concern. A low CVP strategy may reduce cardiac output and, ultimately, oxygen delivery to peripheral tissues which could lead to secondary damage, especially to the kidney, and impact mortality, as reported by Schroeder *et al.*^[132].

Based on the data available, it is not currently possible to state any clear indications for or against strategies for lowering the CVP during liver dissection, for either pediatric or adult patients.

Acute intraoperative hemodilution and transfusion trigger

Another strategy for the reduction of perioperative blood transfusions in the course of liver transplant is acute intraoperative hemodilution^[125]. It is generally performed by the removal of a certain amount of the circulating blood mass of the patient, up to 30%, with immediate reinfusion of the same amount of crystalloid or colloid solutions. The blood collected is then reinfused as needed, based on blood loss or after implantation of the liver. So far, there is no precise information concerning the target hemoglobin or hematocrit to reach or effect on outcome.

Some studies in PLTx and in the craniosynostosis repair field have reported a safe transfusion trigger as up to 8 g/L of hemoglobin and a 25% hematocrit^[123,133]. In the pediatric critical care area, it has been reported that in a stable child, a transfusion trigger of 7 g/L of hemoglobin appears to be safe^[134].

Further research is necessary to define the transfusion trigger based on absolute values of hemoglobin rather than on other pathophysiological parameters^[24].

Pharmacological strategies

Drugs that have been used during liver transplantation surgery to reduce blood losses are essentially activated recombinant factor VII (rFVIIa) and antifibrinolytics.

Activated recombinant factor VII

rFVIIa acts by directly binding to tissue factor (TF). This complex activates the common pathway of coagulation in turn by activating factor X, which precipitates the conversion of prothrombin to thrombin to form a hemostatic clot. In addition, rFVIIa binds the activated platelets at the site of tissue damage^[135]. There are two case series which describe the use of rFVIIa within the liver transplant. In the first^[125], two cases are described as part of overall strategies for saving blood components in patients who were Jehovah's Witnesses. In this case, the drug, which has a half-life of 2-3 h, was administered prophylactically before skin incision, in order to reduce bleeding during the liver dissection phase. In the second series^[136], the rFVIIa was used as a treatment for post-reperfusion

coagulopathy with apparently good results in terms of clinical and laboratory data. However, antifibrinolytic drugs were simultaneously used, so they could be an important source of bias. Systematic reviews^[137,138] of the use of rFVIIa in the adult population (including liver transplantation) failed to show a benefit in terms of the number of blood transfusions, and it was associated with a significant increase in the incidence of arterial thrombotic events. For this reason, rFVIIa is recommended only as rescue therapy for uncontrolled bleeding^[102].

Antifibrinolytics

As discussed in the previous section, hyperfibrinolysis may be enhanced during anhepatic and post-reperfusion phases. There are two families of antifibrinolytics: serine protease inhibitors (aprotinin) and lysine analogs (tranexamic acid and epsilon aminocaproic acid). Aprotinin is a non-specific Kunitz-type protease inhibitor. In addition to plasmin, it also inhibits trypsin, kallikrein, elastase, urokinase and thrombin. Lysine analogs competitively inhibit the activation of plasminogen to plasmin, preventing plasmin from degrading fibrin^[135].

Several systematic reviews have shown that all antifibrinolytic drugs safely reduce blood loss and transfusion, in both adult liver transplantations^[106,139,140] and in major pediatric surgeries^[141-143]. No randomized controlled trials have been performed in the PLTx field. Aprotinine is no longer available, having been withdrawn from the market due to safety concerns^[144]. With regard to the use of antifibrinolytic drugs, it is important to understand whether they should be used extensively prophylactically or only in a documented hyperfibrinolysis state, because pediatric patients show an increased prothrombotic state^[4,5,80,82].

Other pharmacological methods, such as fibrinogen and prothrombin complex concentrates (PCC), have not been investigated in PLTx. Fibrinogen use in adult patients is recommended by the European Society of Anaesthesiology according to FIBTEM tracing^[102]. A randomized controlled trial (the PROTON trial) studying the effect of PCC on RBC transfusion requirements in adult liver transplantation is currently in progress^[145]. Use of protamine at the time of reperfusion to reverse the HLE^[72] does not seem useful because HLE is usually a temporary phenomenon unless graft function is poor^[75,146].

Blood salvage

The use of the cell saver for blood salvage within the surgical field is now considered standard in the management strategies of liver transplantation in patients who are Jehovah's Witnesses^[124]. With the cell saver technique, shed blood is suctioned from the surgical field, centrifuged, washed, mixed with an additive/anticoagulant solution and then reinfused as required^[147].

A few case reports suggest that disseminated intravascular coagulopathy, acute respiratory distress syndrome and renal failure can arise from the reintroduction of fat microemboli, denatured protein, free hemoglobin, cell fragments, and platelet-leukocyte aggregates into the blood stream. More carefully designed studies have failed to show a significant increase in these complications. The washing phase after centrifugation eliminates these products as well as heparin, plasma elastases and soluble cytokines such as TNF-alpha. By now, the safety of this procedure has been well demonstrated in adults^[148,149]. In the pediatric field, there are some studies which have evaluated the effectiveness of the cell saver, especially in cardiac surgery and surgery for scoliosis^[150,151]. Traditionally, patients undergoing bowel resection and cancer surgery are not considered as suitable candidates for the use of intraoperative blood scavenging because of the fear of retransfusing bowel flora and exfoliated cancer cells. At present, the discussion is still open as to whether these are contraindications for the use of the cell saver^[102,147]. The use of these techniques in liver transplantation still remains controversial^[152,153]. Moreover, these techniques are relatively expensive and require sophisticated equipment and trained personnel^[20].

Surgical techniques

There are essentially two surgical techniques used to decrease blood loss: the application of a veno-venous bypass and the piggyback technique^[20,154]. In the first, the bypass allows decompression of the splanchnic venous congestion allowing less loss and enhancing hemodynamic stability. In the second technique, a direct anastomosis between the retrohepatic vena cava of the donor with the inferior vena cava of the recipient is performed with tangential clamping. There are several variants of this technique, but all enable a less challenging retroperitoneal dissection and avoid a vascular anastomosis between the graft and the recipient cava. A recent Cochrane review^[154] has shown no significant difference in postoperative mortality and morbidity between the two techniques. The warm ischemic time was significantly shorter in the piggyback method. No scientific studies investigate this issue in pediatrics. One last issue discussed in the literature is the possible difference in bleeding between transplants from living or deceased donors. Two case series compared the intraoperative bleeding trend of liver transplants from living and deceased donors. In the first study of 157 pediatric patients^[155], a significant increase in blood transfusions was observed in liver transplants from living donor relative to deceased donors. The second study ($n = 46$ pediatric cases)^[156] confirmed the trend although statistical significance was not achieved because of sample size. These data should be further validated because of limitations in

terms of number and homogeneity in the cohorts.

POSTOPERATIVE MANAGEMENT

The main strategy to decrease transfusion requirements without increasing adverse outcomes in a pediatric intensive care unit is to maintain a hemoglobin threshold of 7 g/dL for red cell transfusion^[134]. Another feasible strategy to reduce the possibility of transfusion in the postoperative period is to minimize the amount of blood sampling. In addition, the early use of erythropoietin, early supplementation with iron, multivitamins and folic acid have been described in some case series^[124,125].

UP-TO-DATE CLINICAL PRACTICE GUIDELINES

In the previous paragraphs, we briefly went through the possible clinical strategies aimed at reducing the use and need of blood products during PLTx. Those strategies have been extrapolated from different population settings, such as healthy individuals, adult or pediatric cardiac patients, or from very small and biased trials. Even though some evidence exists, standardized protocols have not yet been established, so that local clinical practice currently prevails. However, the European Society of Anaesthesiology^[102] has published some clinical guidelines for the treatment of perioperative bleeding in patients in 2013.

The recommendations suggested for pediatric surgery patients to reduce blood loss and transfusion requirements are the following: (1) perioperative antifibrinolytic therapy (2A); (2) TEG/ROTEM or timely detection of coagulation defects, including dilutional coagulopathy and hyperfibrinolysis (2C); (3) critical hemoglobin threshold of 8 mg/dL for RBC transfusion (2C); (4) transfusion of PLTs concentrates if PLTs count < 50000/ μ L (2C); (5) fibrinogen concentrate (30-50 mg/kg) or cryoprecipitate (5 mL/kg) may be used to increase plasma fibrinogen concentrations above trigger values of 1.5-2.0 g/L or FIBTEM MCF > 7 mm (2C); (6) no clear recommendation can be made regarding the indication for FFP transfusion, PCC, or FXIII; and (7) recommendation against the use of rFVIIa (1C) and routine use of desmopressin in the absence of hemophilia (2C).

Other recommendations from adult liver transplant patients that may be translated into the pediatric cirrhotic field but are waiting for pediatric derived evidence are the following: (1) use of a viscoelastic test to evaluate the coagulation profile and recommendation against the use of standard laboratory tests, such as PT/APTT for cirrhotic patients (C); (2) fluid restriction, phlebotomy, vasopressors, transfusion protocols (C) and low central venous pressure (B) may be associated with low transfusion rates during liver transplantation; (3)

antifibrinolytic drugs for treatment of fibrinolysis (evident from microvascular oozing or TEG/ROTEM clot lysis measurements) but not for routine prophylaxis (1C); and (4) recommendation against the routine use of rFVIIa (1A) and against the use of FFP for preprocedural correction of mild to moderately elevated INR (1C).

SUMMARY AND FUTURE DIRECTIONS

The first international conference on Coagulopathy of Liver Disease was held in 1995 in Charlottesville, VA, United States. This event stimulated the creation of a stable multidisciplinary group with a focus on coagulation disorders in liver disease where the two fields of hematology and hepatology converge^[22]. Unfortunately, new knowledge in this area cannot be simply applied to pediatric patients as emphasized by Lisman *et al.*^[30]. Few pediatric data have been published, and clinical algorithms used in adult practice must be specifically tested in pediatric settings before they can be applied to neonates and children for many reasons. First of all, hemostasis is still in development in children, and they suffer from different liver disease pathologies than adults. Thus, the age of onset and etiology of the end-stage liver disease may influence the hemostatic system in unpredictable ways. For example, hemostasis in healthy neonates is already considered to be rebalanced^[35,118]. Second, laboratory tests for the evaluation of coagulation require standardization, including maintenance of sample integrity, equipment/analyzers, reagents and age dependent reference levels, in order to avoid erroneous or conflicting results^[27]. Third, the impact of the use of the blood products on morbidity and mortality of children with end-stage liver disease must be further investigated both before and after liver transplantation. Lastly, more randomized controlled trials analyzing the medical strategies to reduce bleeding and thrombotic complications should be performed specifically in PLTx patients. In conclusion, a multidisciplinary effort involving hematologists, hepatologists, pediatricians, surgeons, and anesthesiologists is needed to fill the gap.

RESEARCH AGENDA

The end-stage liver disease responsible for the observed age and disease related changes in coagulation proteins must be elucidated because it may have important biological ramifications. The concept of rebalanced hemostasis should be evaluated in cirrhotic pediatric patients.

New laboratory assays to evaluate the coagulation system need to be validated in children, in light of our understanding of developmental hemostasis.

The development of pediatric specific algorithms to guide clinical management of bleeding disorders and transfusion therapy during liver transplantation requires specific clinical outcome studies in children.

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2016 Liver Transplantation: Global view

Portopulmonary hypertension in liver transplant candidates

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Abstract

Pulmonary vascular disorders including portopulmonary hypertension (PoPHT) are among the common complications of liver disease and are prognostically

significant. Survival is very low without medical treatment and liver transplantation. With advances in medical therapy for elevated pulmonary artery pressure (PAP) and liver transplant surgery, survival of patients with PoPHT and advanced liver disease is significantly improved. Because of the prognostic significance of PoPHT and the limited donor pool, a comprehensive preoperative cardio-pulmonary assessment is of great importance in cirrhotic patients prior to transplant surgery. Therefore, a detailed transthoracic Doppler echocardiographic examination must be an essential component of this evaluation. Patients with mild PoPHT can safely undergo liver transplant surgery. In cases of moderate to severe PoPHT, right heart catheterization (RHC) should be performed. In patients with moderate to severe PoPHT on RHC (mean PAP 35-45 mmHg), vasodilator therapy should be attempted. Liver transplantation should be encouraged in cases that demonstrate a positive response. Bridging therapy with specific pulmonary arterial hypertension treatment agents should be considered until the transplant surgery and should be continued during the peri- and post-operative periods as needed.

Key words: Portopulmonary hypertension; Pulmonary arterial hypertension; Liver disease; Liver transplantation; Portal hypertension

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Core tip: Portopulmonary hypertension (PoPHT) is one of the most common findings on preoperative assessment of cirrhotic patients prior to liver transplant surgery. Since it has prognostic significance, diagnosis of PoPHT by Doppler echocardiography and further characterization by right heart catheterization is critical in classifying these patients. Therapy with pulmonary arterial hypertension (PAH)-specific agents should be started when PoPHT is moderate to severe. Patients with a positive response should be encouraged to undergo liver transplant surgery. Bridging therapy with

these agents should be considered until the time of transplant surgery and continued during the peri and postoperative periods as needed.

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INTRODUCTION

Patients with advanced liver disease display a variety of pulmonary abnormalities. Pulmonary vascular disorders - including portopulmonary hypertension (PoPHT) - are among the common complications of liver disease. Pulmonary hypertension in these patients is also defined as PoPHT when pulmonary arterial hypertension (PAH) is associated with portal hypertension^[1-3]. Although it is commonly seen in cirrhotic patients, it may also occur in the absence of liver disease. The Nice World Pulmonary Hypertension Symposium classified PoPHT as one of the associated forms of PAH^[4]. In this paper we review the literature about PoPHT in liver transplant candidates.

DIAGNOSIS

The diagnosis of PoPHT requires the following 2 conditions: (1) Elevated pulmonary arterial pressure (PAP) and other hemodynamic variables obtained by right heart catheterization (RHC). Mean PAP > 25 mmHg, pulmonary capillary wedge pressure (PCWP) < 15 mmHg and pulmonary vascular resistance > 240 dynes/s per cm⁻⁵; and (2) presence of portal hypertension (with or without liver disease). Documentation of splenomegaly, portosystemic shunts, thrombocytopenia or varices - these are accepted as signs of portal hypertension.

Assessment and staging severity of PoPHT based on transthoracic Doppler echocardiography (TTDE) and RHC are provided in Table 1^[1].

EPIDEMIOLOGY AND PATHOPHYSIOLOGY

Although evidence varies widely, PoPHT is seen in as many as quarter of patients with advanced liver disease^[5]. Krowka *et al*^[6] demonstrated that 5% of patients with chronic liver disease have hemodynamic criteria for PoPHT. Data from a French PAH registry showed a prevalence of 9.4%^[7]. A report from the multicenter United States-based REVEAL registry involving more than 3500 patients showed that 5% had PoPHT^[8]. The exact pathogenesis of PoPHT is not well defined. Arterial hyperdynamic circulation, increased

Table 1 Summary on assessment, grading and therapy for portopulmonary hypertension

| |
|--|
| TTDE in all patients prior to transplant surgery, systolic PAP ≥ 30 mmHg |
| RHC in selected cases, confirmation and grading of PoPHT: |
| Mild PoPHT: mPAP > 25 mmHg, PVR > 240 dynes/s per cm ⁻⁵ , PCWP ≤ 15 mmHg, allow LT |
| Moderate PoPHT: mPAP 35-45 mmHg, PVR > 240 dynes/s per cm ⁻⁵ , PCWP ≤ 15 mmHg if responder to vasodilator therapy, allow LT |
| Severe PoPHT: mPAP > 45 mmHg, PVR > 240 dynes/s per cm ⁻⁵ , PCWP ≤ 15 mmHg, medical therapy |

PoPHT: Portopulmonary hypertension; TTDE: Transthoracic Doppler echocardiography; PAP: Pulmonary artery pressure; RHC: Right heart catheterization; PVR: Pulmonary vascular resistance; LT: Liver transplantation.

venous blood volume and pressure, pulmonary artery vasoconstriction and neurohumoral activation are responsible for the development of PoPHT in cirrhotic patients^[9]. It has similar histopathological features as other types of PAH^[10,11]. Obstruction of pulmonary arterial blood flow, proliferation of periarteriole smooth muscle cells, vasoconstriction, increased endothelin concentrations, platelet aggregation/in situ thrombosis are characteristic findings in PoPHT and other forms of PAH^[11-14]. The severity of liver disease did not correlate with the severity of PoPHT^[15].

SYMPTOMS AND SIGNS

Symptoms of PoPHT may not be overt and may be attributed to underlying liver disease or associated conditions. Therefore, the recognition of PoPHT requires high clinical suspicion. Symptoms are similar to those in other forms of PAH. Dyspnea is the most common symptom in patients with PoPHT. Weakness, easy fatigue, edema, orthopnea, palpitation and chest pain on exertion are other common symptoms. Syncope may occur in patients with severe PoPHT.

On physical examination, there may be jugular venous distension, a loud S2, left parasternal systolic murmur due to tricuspid regurgitation, peripheral edema, and ascites.

Chest X-ray findings are non-diagnostic and may show cardiomegaly and dilation of main pulmonary arteries. Electrocardiographic findings include right bundle branch block, right axis deviation and precordial T wave inversions. Mild-moderate hypoxemia and hypocapnia are common in arterial blood gases analyses.

ASSESSMENT

TTDE is an accurate, reliable, and reproducible tool to estimate systolic PAP in the absence of pulmonary stenosis or right ventricular outflow tract obstruction^[8]. It has high sensitivity as a screening test. Its non-invasive nature makes it a recommended method

to screen for PoPHT in patients with cirrhosis^[7]. Pulmonary artery pressure should be carefully evaluated. For this purpose, the maximum tricuspid regurgitant flow velocity (*v*) should be obtained using continuous wave Doppler examination followed by systolic PAP calculations using the modified Bernoulli equation. [Systolic PAP (mmHg) = $4v^2$ + estimated mean right atrial pressure]. Patients with a systolic PAP value ≥ 30 mmHg are usually diagnosed with PoPHT^[16]. With this cutoff (30 mmHg), positive and negative predictive values of 59% and 100% respectively were reached^[17].

TTDE also provides additional valuable information about the indirect signs of PAH such as right ventricular dilatation, right ventricular hypertrophy and abnormal septal motion^[18]. TTDE has a pivotal role in the initial evaluation of PoPHT as recommended by both European and the American guidelines^[19,20].

Because PoPHT has prognostic significance it is common belief that TTDE is mandatory in the evaluation of cirrhotic patients prior to liver transplant even when the patient is asymptomatic.

However, the exact timing of RHC and systolic PAP cutoff values have yet to be identified. Different centers report varying approaches. A systolic PAP value > 50 mmHg *via* TTDE gave a sensitivity of 97% and a specificity of 77% *via* RHC^[21]. This suggests that RHC should be considered when systolic PAP > 50 mmHg and in situations where systolic PAP is 30-50 mmHg with other signs of PoPHT such as right ventricular dilation or dysfunction as well as signs of right ventricular failure.

RHC is the gold standard method for the diagnosis of PoPHT^[3,22]. Not only does it confirm PoPHT but it also provides additional data to exclude other causes of PAH in liver transplant candidates. By obtaining pulmonary vascular resistance (PVR), cardiac output, mean PAP and PCWP, the nature and severity of PoPHT can be highlighted (Table 1). For example, in cases of left heart disease, the cardiac output (CO) is reduced and PVR and PCWP are increased. In patients with PoPHT, CO and PCWP are low while PVR is elevated. In the hyperdynamic state the CO increases, PCWP is normal and PVR decreases. Because the treatment and prognosis of these clinical conditions are entirely different, their documentation is very important. Patients with elevated mean PAP but normal PVR (hyperdynamic state) benefit from liver transplantation. These patients should proceed to liver transplant surgery without further characterization and treatment. The trans-pulmonary gradient can be calculated as a mean PAP-PCWP. Values above 12 mmHg have been shown to correlate with increased PVR^[23,24].

PROGNOSIS AND OUTCOME

Without liver transplantation and medical therapy,

cirrhotic patients with PoPHT have very poor prognosis^[13,25,26]. Before the availability of PAH-specific therapies, the survival of patients with PoPHT is very poor^[27]. Swanson *et al.*^[28] from the Mayo Clinic reported a 14% survival at 5 years in PoPHT patients without liver transplantation and PAH-specific therapies. Complications of advanced liver disease and right ventricular failure due to progressive elevation in PAP are responsible for poor survival rates.

A study by Kia *et al.*^[29] demonstrated that elevated PAP determined by TTDE was associated with increased mortality and morbidity following liver transplant surgery. Data from the Multicenter Liver Transplant Database demonstrated a mortality rate of 36% in patients with PoPHT undergoing liver transplantation^[30]. The exact mechanism of how the risk increases in cirrhotic patients with PoPHT following liver transplant surgery is unclear. However, multisystemic involvement, pleural effusion, right-sided heart failure, peri- and post-operative fluid shift, hypoxia and surgical trauma are among the possible mechanisms.

The REVEAL registry provides us with valuable information regarding treatment and outcome of PoPHT^[8]. Initiation of PAH-specific therapies were delayed in PoPHT vs idiopathic PAH. Baseline hemodynamics were better in PoPHT than those in idiopathic PAH; survival was worse in PoPHT.

TREATMENT

Drug therapy

Calcium channel blockers are not advised in patients with PoPHT. As a consequence of mesenteric vasodilation, they have the potential to worsen portal hypertension. Beta-blockers are not suggested because they may reduce exercise capacity. Warfarin anticoagulation is not recommended because of the risk of hemorrhage in cirrhotic patients. Diuretics are useful agents for symptomatic benefits in patients with volume overload and fluid retention. Both loop and potassium sparing diuretics can be used for this purpose. Oxygen therapy is recommended in hypoxic patients ($\text{PaO}_2 < 60$ mmHg, O_2 saturation $< 90\%$).

Data regarding the use of PAH-specific therapies are limited in patients with PoPHT. This is because most studies excluded patients with PoPHT. Table 2 provides a short summary on the new PAH specific agents in use. However, these agents were used in small studies that enrolled a limited number of patients with PoPHT^[31-35]. In general, these agents provided significant hemodynamic improvement.

Liver transplantation

Innovation in surgical techniques, improvements in peri-operative care and developments in immune suppressant drugs have made organ transplantation the preferred therapy for patients with end stage

Table 2 Pulmonary arterial hypertension-specific therapy: A summary of agents used

| |
|--|
| Endothelin receptor antagonists |
| Agents used in PoPHT: bosentan, ambrisentan |
| Mechanism of action: blockade of endothelin receptors |
| Route of administration: oral |
| Effects: vasodilation, decrease in PVR and portal pressure |
| Adverse effects: hepatotoxicity |
| PDE-5 inhibitors |
| Agents used in PoPHT: sildenafil, tadalafil, vardenafil |
| Mechanism of action: inhibition of PDE-5 enzyme |
| Route of administration: oral |
| Effects: vasodilation, decrease in portal pressure |
| Adverse effects: hypotension |
| Prostacyclins |
| Agents used in PoPHT: epoprostenol, iloprost |
| Mechanism of action: prostaglandin analogue, increase in cAMP |
| Route of administration: epoprostenol IV, iloprost inhalation |
| Effects: vasodilation, antiaggregation |
| Adverse effects: flushing, headache, nausea, diarrhea. Problems with IV epoprostenol use: cost, infection, catheter thrombosis, and thrombocytopenia |

PAH: Pulmonary arterial hypertension; PoPHT: Portopulmonary hypertension; PDE-5: Phosphodiesterase type 5 enzyme; PVR: Pulmonary vascular resistance; IV: Intravenous.

organ failure. Liver transplantation is the best available therapeutic option for the treatment of patients with end stage liver disease. Advances in medical therapy for elevated PAP and liver transplant surgery, survival of patients with PoPHT and advanced liver disease are thus significantly improved^[28]. Treatment strategies for PoPHT in cirrhotic patients are mainly based on recommendations for idiopathic PAH management and the treatment of underlying liver disease. Thus, by definition, a functioning transplanted liver graft emerges as a therapeutic option for PoPHT in such cases. As surgical techniques, hospital care, and PAH-specific therapies have improved, the stabilization and improvement and even cure of PoPHT in cirrhotic patients is achievable with liver transplantation. In a previous study we determined that liver transplantation improves PoPHT^[36].

Patients with mild PoPHT can safely undergo liver transplant surgery. In cases of moderate to severe PoPHT, the response to vasodilator therapy should be evaluated. When mean PAP is lowered to < 35 mmHg, patients benefit from liver transplant surgery and liver transplantation should be encouraged.

Swanson *et al.*^[28] demonstrated that a combination of PAH-specific therapy and liver transplantation provided the best outcome vs those who did not receive any therapy and those who only received medical therapy. Survival at 5 years were 76%, 14% and 45%, respectively. These findings are valuable and clinically relevant. Therefore it is of great value to evaluate patients with moderate to severe PoPHT whether they respond or not. Unfortunately, there is no way to predict long-term response to vasodilator therapy. The evaluation of PAP should be repeated

at 3-mo intervals because response to vasodilator therapy cannot be obtained with short-term use. Thus, the determination of acute responders during RHC is not recommended^[37].

When PoPHT is severe it usually contraindicates liver transplantation^[38,39]. Conversely, liver transplantation is a therapeutic option in patients with PoPHT and advanced liver disease. A functioning liver graft decreases pulmonary artery pressure in these patients^[28,36].

In a study by Starkel *et al.*^[5] PoPHT was identified in 38 of 145 patients (26%). It was mild in 82% of cases. The duration of mechanical ventilation and intensive care unit stay was similar between patients with and without PoPHT. One of the 5 patients who had severe PoPHT (mean PAP > 40 mmHg) died, but the remaining 4 patients were alive and in excellent clinical condition during the 3 years of follow up.

In the United States, transplant programs prioritize if the hemodynamics can be improved with PAH-specific therapies and meet standardized MELD exception guidelines^[40]. Goals of treatment for this approach are as follows: (1) Documentation of moderate to severe PoPHT by RHC (mean PAP \geq 35 mmHg, PCWP \leq 15 mmHg, and PVR > 400 dynes/s per cm⁻⁵); (2) Improvement in hemodynamics with PAH-specific therapy (mean PAP < 35 mmHg, PVR < 400 dynes/s per cm⁻⁵ and satisfactory right ventricular function on TTDE, as well as improvement in right ventricular dilation and function); and (3) MELD exception update every 3 mo.

The aim of this policy is to perform liver transplantation before irreversible changes related to PoPHT occur. When liver transplantation is to be performed in patients with severe PoPHT demonstrating a positive response to vasodilator therapy, perioperative use of inhaled nitric oxide and intravenous epoprostenol should be considered. Adequate right ventricular function is very significant with regard to a successful transplant operation in patients with PoPHT. Prolonged PoPHT and progressively increasing PAP may predispose subjects to right ventricular hypertrophy and eventually right ventricular dysfunction. In such cases an acute rise in cardiac output to more than 15 L/min during the reperfusion of the liver graft may precipitate acute right ventricular failure. Liver transplant surgery itself induces marked hemodynamic alterations that can adversely affect the peri-operative course in patients with pulmonary vascular disease. Acute right ventricular failure and right ventricular dysfunction may develop in patients with severe PoPHT that may be fatal. Therefore right ventricular function should also be evaluated during the peri- and post-operative periods. In case of right ventricular failure, milrinone can be used, and PAH specific agents should be continued following transplant surgery as necessary.

Growing evidence suggests that unless severe and associated with right ventricular dysfunction, PoPHT should no longer be considered to be an

absolute contraindication to liver transplantation. The current data indicate that PoPHT improves and even normalizes in some cases following successful liver transplantation^[5,36,41]. Therefore, this suggests that liver transplantation should not be denied in all patients with severe PoPHT. When patients are in good clinical condition, young, and without severe right ventricular dysfunction or high pulmonary vascular resistance, improvements in PAP with vasodilator therapy liver transplantation should still be considered even when PoPHT is severe.

Khaderi *et al.*^[42] reported the long-term follow up results of patients with severe PoPHT who underwent orthotopic liver transplantation. Of the 488 liver transplant patients, 7 had severe PoPHT. All 7 of these patients received vasodilator therapy (6 patients IV epoprostenol, 1 oral sildenafil) and their mean PAP reduced to ≤ 35 mmHg. They also received IV or inhaled epoprostenol during the perioperative period. The survival rate was 85.7% after a median follow up of 7.8 years. Furthermore, all the surviving patients were in good functional status (NYHA I or II). Although the number of patients enrolled is small, the findings of this study are valuable and clinically relevant. This demonstrates that liver transplantation improves PoPHT even when severe. The authors concluded that moderate to severe PoPHT responsive to vasodilator therapy does well with liver transplantation, and such patients should be accepted as good candidates for transplant surgery.

CONCLUSION

Given the prognostic significance of PoPHT and the limited donor pool, a comprehensive preoperative cardio-pulmonary assessment is of paramount importance in cirrhotic patients prior to transplant surgery. A detailed Doppler echocardiographic examination must be the essential component of this evaluation. Pulmonary artery pressure should be calculated and right ventricular function should also be assessed. The RHC should be considered when PoPHT is moderate to severe.

PAH-specific agents - either alone or in combination - are critical when deciding on liver transplant surgery in patients with moderate to severe PoPHT. Liver transplantation should be encouraged in cases displaying a positive response. Bridging therapy with specific PAH treatment agents should be considered until transplant surgery. This should be continued during the perioperative period and following surgery as needed.

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Cytomegalovirus and ulcerative colitis: Place of antiviral therapy

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Abstract

The link between cytomegalovirus (CMV) infection and inflammatory bowel diseases remains an important subject of debate. CMV infection is frequent in ulcerative colitis (UC) and has been shown to be potentially harmful. CMV reactivation needs to be diagnosed using methods that include *in situ* detection of viral markers by immunohistochemistry or by nucleic acid amplification techniques. Determination of the density of infection using quantitative tools (numbers of infected cells or copies of the genome) is particularly important. Although CMV reactivation can be considered as an innocent bystander in active flare-ups of refractory UC, an increasing number of studies suggest a deleterious role of CMV in this situation. The presence of colonic CMV infection is possibly linked to a decreased response to steroids and other immunosuppressive agents. Some treatments, notably steroids and cyclosporine A, have been shown to favor CMV reactivation, which seems not to be the case for therapies using anti-tumor necrosis factor drugs. According to these findings, in flare-ups of refractory UC, it is now recommended to look for the presence of CMV reactivation by using quantitative tools in colonic biopsies and to treat them with ganciclovir in cases of high viral load or severe disease.

Key words: Human cytomegalovirus; Ulcerative colitis; Inflammatory bowel disease; Ganciclovir; Viral load; Flare-up; Inflammation; Intestinal mucosa; Quantitative polymerase chain reaction

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Core tip: There is increasing evidence for the deleterious effect of *in situ* cytomegalovirus (CMV) reactivation in flare-ups of refractory ulcerative colitis. In patients aged > 30 years with a high density of infection in the colonic tissue, or with stigmata of

severe disease associated with colonic markers of CMV reactivation (whatever the density of infection), treatment with ganciclovir is highly recommended, together with anti-tumor necrosis factor monoclonal antibody therapy in the absence of any contraindication to these drugs. For validating the present strategy based on our experience and the in-depth analysis of the available literature presented in this review, prospective randomized controlled studies are urgently needed.

Pillet S, Pozzetto B, Roblin X. Cytomegalovirus and ulcerative colitis: Place of antiviral therapy. *World J Gastroenterol* 2016; 22(6): 2030-2045 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i6/2030.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i6.2030>

INTRODUCTION

Cytomegalovirus (CMV) belongs to the *Herpesviridae* family. The viral genome consists of linear double-stranded DNA protected by a capsid and an envelope. After primary infection, which may or may not be symptomatic, the virus is known to maintain a persistent, life-long infection of the host, often as a latent form that can be found in several cell types. These cells are mainly myeloid progenitors, monocytes and endothelial cells, meaning that CMV could be latent in several organs or tissues, and especially in the colon^[1,2]. During the latent stage, the CMV genome is present as an episomal circular form in the cell nucleus, with minimal viral expression and without viral particle production. CMV can reactivate from the latent stage, leading to the production of new viral particles. CMV reactivation is triggered by inflammation or immunosuppression. Beside reactivation from an endogenous latent virus, reinfection can be induced by an exogenous strain present in a tissue/organ graft or blood transfusion.

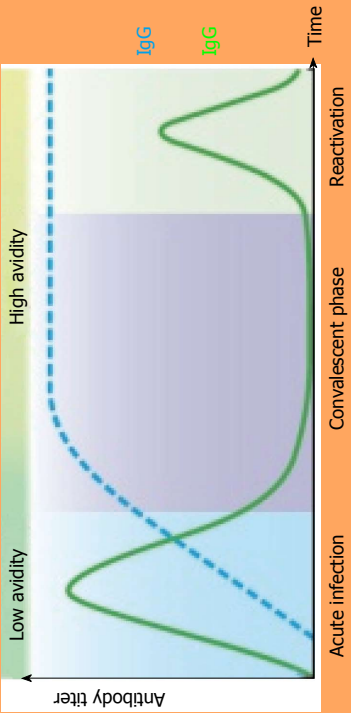
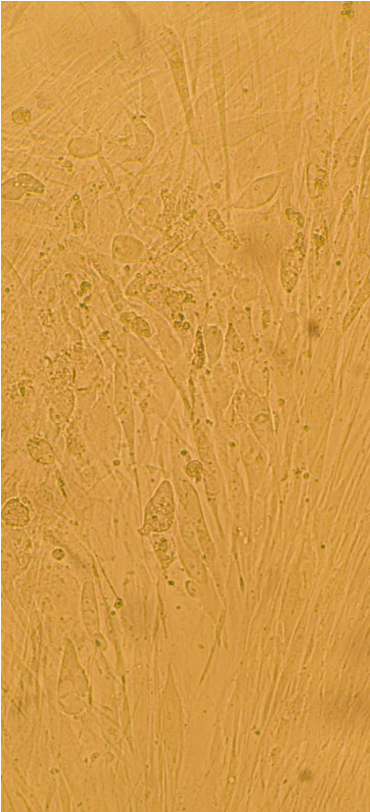
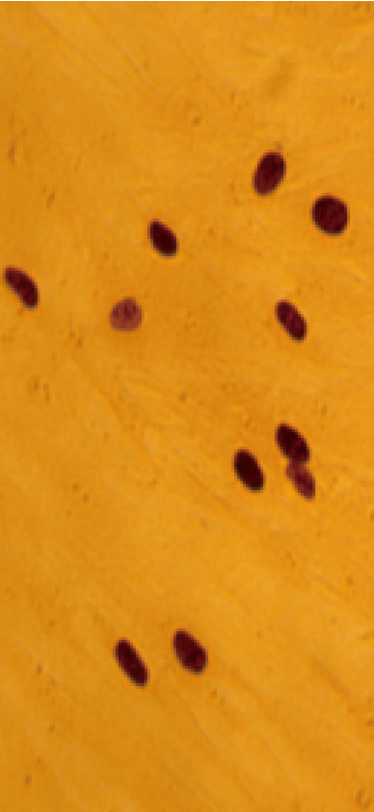
The host immune response is critical in controlling CMV infection. Cellular immunity, especially natural killer cells, and interferons play a major role at the stage of primary infection and in long-term control of the infection. Consequently, the clinical expression of CMV infection is generally absent in an immunocompetent host, even if some severe infections, especially colitis^[1-5], have been reported in the literature. In contrast, the most preoccupant manifestations of CMV infection are observed in immunocompromised patients with altered cellular immunity, that is, after transplantation of solid organ grafts or hematopoietic stem cells, in cases of HIV infection, in patients undergoing chemotherapy or immunotherapy, and during pregnancy. Clinical manifestations may vary from acute febrile illness to organ disease (*e.g.*, retinitis, pneumonitis, encephalitis, colitis and hepatitis)^[6].


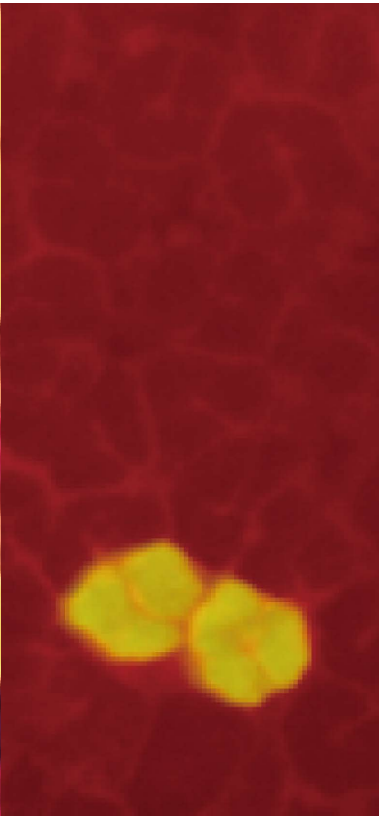

CMV infection is of particular interest in inflammatory bowel diseases (IBD) that combine inflammation in the colon and the long-term maintenance of immunosuppressive therapy; both of which can reactivate latent CMV^[7]. Local inflammation in the bowel wall leads to the secretion of proinflammatory cytokines, including tumor necrosis factor (TNF)- α . As a consequence, these cytokines are able to activate CMV replication and the migration of CMV-infected monocytes and macrophages in the inflamed tissue to propagate infection further, generating a vicious cycle of pathology^[2]. However, IBD is a complex entity that involves different clinical situations dominated by Crohn's disease (CD) and ulcerative colitis (UC). In CD, severe CMV primary infections have been reported; some of them being complicated further by hemophagocytic lymphohistiocytosis^[8]. The administration of ganciclovir was shown to contribute to clinical remission^[9]. However, and although the seroprevalence to CMV is similar in CD and UC patients^[10], CMV reactivation was shown to be much less frequent in CD than in UC patients, with no significant impact on clinical evolution^[10-20]. This observation can be attributed to the different cytokine profiles observed in these two IBDs: CD is most likely attributed to T helper (Th)1 and Th17 CD4⁺ T-cell differentiation with secretion of interferon- γ that exerts an inhibiting effect on CMV replication. In contrast, UC exhibits a Th2 profile with limited secretion of antiviral cytokines, which could favor viral reactivation or tolerance^[21]. Consequently, CD will be excluded from the scope of this review.

The use of various virological methods for diagnosing CMV reactivation affects the results obtained when exploring the role of this agent in UC and has led to controversial theories. Once the role of CMV is established in the evolution of UC, several predictive factors can be selected in order to identify those patients who are more likely at risk of developing CMV reactivation in the colon, and who may therefore benefit from antiviral therapy. Accordingly, the aim of this review is to answer four successive questions: (1) how to diagnose CMV reactivation accurately in colonic tissue of UC patients; (2) what is the impact of colonic CMV infection in the evolution of UC; (3) what are the predictive factors that may help to identify those patients at risk of unfavorable evolution; and (4) in this population, can antiviral therapy be of any use in improving the long-term evolution of UC?

HOW TO DIAGNOSE CMV REACTIVATION ACCURATELY IN COLONIC TISSUE OF UC PATIENTS

Figure 1 shows the different techniques that are presently used for the diagnosis of CMV infection. Only a few techniques are indicated for the current diagnosis of CMV reactivation in the colonic tissue of

| Markers | Biological specimens | Technique(s) | Delay to result | Advantages | Disadvantages | Illustration |
|------------------------|---|--|-----------------|---|--|---|
| IgG antibodies to CMV | Peripheral blood | ELISA | Few hours | Marker of previous infection with CMV | - |  |
| | | | | Possible dating of primary infection by testing IgG avidity | | |
| IgM antibodies to CMV | Peripheral blood | ELISA | Few hours | Marker of recent infection with CMV | False positive results Possible persistence for several weeks or months | |
| Classical cell culture | Peripheral blood, saliva, urines, tissues, fluids | Detection of cytopathic effect in tissue culture | Days or weeks | Isolation of infectious viruses and of clinical strains | Fastidious |  |
| | | | | | Poorly sensitive | |
| | | | | | Long lasting | |
| 'Rapid' cell culture | Urines | Centrifugation of clinical specimen on tissue culture and detection of viral proteins expressed at early stages of infection | 2 d | Screening test for CMV detection in urine | Fastidious |  |
| | | | | Faster than classical cell culture | No strain isolation | |

| | | | | | | |
|--|------------------|---|----------|--|---------------|---|
| Histological examination after HE staining | Tissue | Detection of infected cells with characteristic aspects (i.e., "owl's eye", intracellular inclusion bodies) in tissue specimens | Few days | No specific reagents required | Fastidious |  |
| | | | | | | |
| | | | | | | |
| Detection of antigens | Peripheral blood | pp65 antigenemia (detection of viral inclusions in polymorphonuclear cells) | 24 h | Presence of active blood infection (viremia) | Not automated |  |
| | | | | | | |
| | | | | | | |
| Detection of antigens | Tissue | IHC | Few days | Presence of active tissue infection | Not automated |  |
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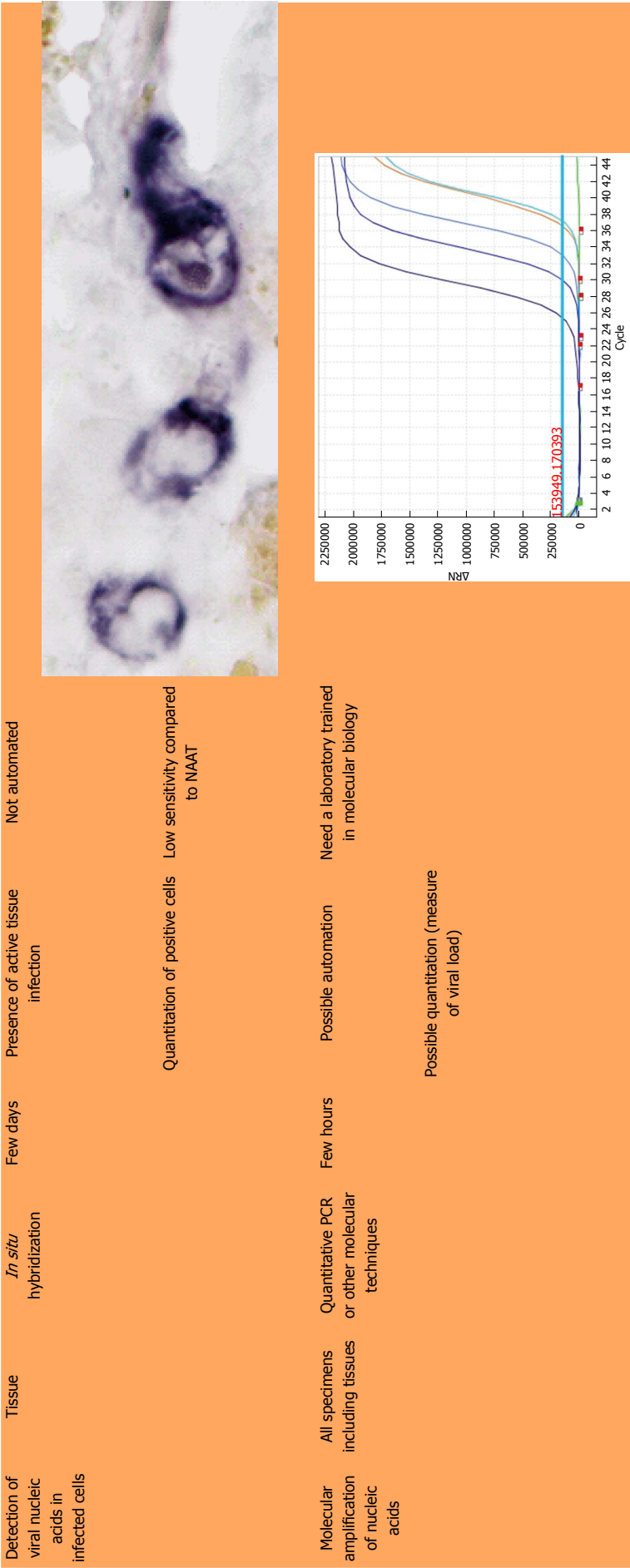


Figure 1 Techniques currently used for the detection of markers of cytomegalovirus infection. Analyses highlighted in orange are very useful, those highlighted in yellow of little use, and those without color of no use, for the diagnosis of CMV reactivation in inflammatory bowel diseases. ELISA: Enzyme-linked immunosorbent assay; IHC: Immunohistochemistry; CMV: Cytomegalovirus; HE staining: Hematoxylin and eosin staining; NAAT: Nucleic acid amplification test.

UC patients.

Specific IgG serology, usually performed by ELISA, is necessary to identify those patients who have already been in contact with CMV and consequently could be at risk of an endogenous reactivation at the colonic level. In seropositive patients, two kinds of techniques can identify CMV reactivation at this level.

The first group of techniques relies on histological examination of colonic tissue. The direct examination of colon biopsies after hematoxylin and eosin (HE) staining can show typical aspects of "owl's eye" images (Figure 1) but this technique is poorly sensitive and frequently leads to false-negative results. Immunohistochemistry (IHC) on colonic tissue is much more sensitive and can be quantitative by numbering the infected cells^[22-25]. However, a recent paper from McCurdy *et al*^[26] indicated that a great number of biopsy samples must be examined in order to achieve adequate sensitivity.

The second group of techniques is based on the detection of CMV DNA in colonic tissue. *In situ* hybridization can be used for this purpose but, as for HE staining, this technique lacks sensitivity and can only identify severe CMV reactivation episodes. It has largely been replaced by molecular techniques based on nucleic acid amplification tests (NAATs). Although very sensitive, qualitative PCR with two rounds of amplification (nested PCR) should be avoided because of the risk of cross-contamination and false-positive results^[27,28]. In contrast, real-time quantitative PCR (qPCR) assays are very sensitive, allowing the detection of low-level reactivation,

and accurate determination of the viral load, and can be automated. In contrast with IHC, they give no information on the infectious potential of the detected genome, nor on the stage (latent or productive) of CMV infection. To optimize the predictive value of these tests, it is necessary to determine the thresholds of CMV DNA load that would require initiating antiviral therapy^[6,29]. One of the main difficulties with NAATs is the inter-laboratory standardization of quantitative data^[6,30,31], together with the harmonization of viral load expression in tissue specimens (copies^[10,25,29,32-34] or international units^[6], per mg of tissue^[10,25], µg of DNA^[14,34] or number of cells^[29,32,33]). This lack of standardization makes the comparison of results between studies difficult and universally accepted cut-off values of CMV DNA load for assessing CMV disease have still to be defined^[24,32,33]. Another important feature with NAATs is the risk of a false-negative result if the biopsy is performed at a distance from an inflamed focus; indeed, CMV markers are detected in inflamed tissue only^[10,14,25,34] and inflammation^[35-40] is present in the mucosa as foci that are sometimes difficult to identify during colonoscopy. To minimize this risk, it is our experience to measure CMV DNA on a couple of biopsies taken at the same time and to use the result exhibiting the highest viral load (manuscript in press). As detailed below, the presence of ulcers is correlated with that of viral stigmata^[20,25,41,42], which indicates that these areas must be privileged in performing the biopsies. As an alternative to colonic biopsies, some authors have proposed the determination of viral load in feces^[43-45]; however, this technique was recently shown to be poorly sensitive for the detection of CMV colitis in immunosuppressed patients^[46].

IHC is still considered to be the gold standard for the identification of CMV in tissue sections^[26,47,48]. However, the choice between IHC and NAAT (mainly qPCR) for detecting CMV reactivation in colon biopsy of UC patients is a matter of ongoing debate^[25], even in current international recommendations^[49], although an increasing number of laboratories are switching from histology-based techniques to qPCR assays for the quantification of CMV load in colonic tissue, due to the simplicity and rapidity of the latter tests. Indeed, with current NAATs, the results of viral load can be recovered within one working day. Due to the absence of any indication on the infectivity of a detected genome, the use of viral load thresholds avoids the useless treatment of latent infection.

WHAT IS THE IMPACT OF COLONIC CMV INFECTION ON THE EVOLUTION OF UC?

General considerations

The implication of CMV reactivation in colonic tissue

on the clinical evolution of UC has been highly debated^[22,27,50]. Table 1^[10,12-17,20,22,41,42,51-85] lists, in chronological order, the main studies that have tried to explore this relationship. Some of them have reported CMV markers in patients without an impact on IBD evolution, which has led to the idea that CMV infection could be considered as an "innocent bystander"^[27] or byproduct of the pathology. Many others have shown a negative impact of CMV infection in UC evolution and, in some of them, an improvement of clinical status when antiviral therapy was initiated, suggesting an active role of CMV.

In our opinion, many of these discrepancies are related to misleading definitions of the populations of patients included in the studies or to the use of inadequate tools for the evaluation CMV reactivation in the gut. First, patients with CMV primary infection exhibiting CMV colitis are sometimes mixed with patients with CMV reactivation, notably in historic studies, which introduced a bias in the evaluation of prognosis^[53,58,86-90]. Second, several studies, including some recent ones^[41,74,78,84], have evaluated CMV markers in both UC and CD patients, although these two IBDs are very different in terms of the risks of CMV reactivation, as discussed above. Third, a few studies used peripheral blood markers, and notably pp65 antigenemia (Figure 1), to evaluate CMV reactivation in UC patients; positive antigenemia was associated with steroid refractoriness and UC exacerbation in one study^[71], corticoreistance in another^[5], and the presence of ulcer and risk of colectomy in a third^[76]. However, viremia is poorly sensitive^[10,14,19,64,77,78,82], no threshold has been established for starting therapy; and the search for CMV should be performed in colonic biopsy in order to evaluate the risk of reactivation at an early stage of infection, corresponding to an increased chance of successful antiviral treatment. Finally, as stated in the previous section, the comparison of clinical results between studies is rendered difficult by the diversity of techniques that are used to determine CMV reactivation at the colonic level (IHC vs NAATs) and the lack of standardization of the different tests used for quantifying the viral load.

Despite these discrepancies, there is an increasing consensus for considering CMV reactivation as a marker of poor prognosis in UC patients, as illustrated by the results of the studies listed in Table 1 and by the recommendations of international guidelines^[47,49,91] for the systematic detection of CMV reactivation in flare-ups of UC patients, and in using antiviral drugs in particular circumstances that will be detailed later in this review.

Factors implicated in the occurrence of CMV reactivation in UC patients

Role of immunotherapy: Administration of steroids is a known predisposing factor for CMV reactivation by suppressing anti-CMV T-cell specific function^[92]

Table 1 Main studies recording the impact of cytomegalovirus on inflammatory bowel diseases course

| Studies by chronological order | No. of studied patients by type of IBD | Method used for CMV detection | Main results of the study | Impact of CMV |
|--|---|---|--|---------------|
| Vega <i>et al</i> ^[51] , 1999 | 7 UC and 2 CD | Histology and IHC | Ganciclovir allowed clinical remission in 5/7 patients, with absence of CMV markers after antiviral therapy | Unfavorable |
| Cottone <i>et al</i> ^[52] , 2001 | 55 UC and 7 CD | Histology and IHC PCR in PBMC | Antiviral treatment (3 with ganciclovir and 2 with foscarnet) allowed clinical remission in 5/7 patients | Unfavorable |
| Papadakis <i>et al</i> ^[53] , 2001 | 5 UC, 3 CD, 2 indeterminate colitis; all medically refractory | Heterogeneous (serology, histology, IHC, ISH, PCR, cell culture) | Ganciclovir improved clinical outcome in 8/9 patients | Unfavorable |
| Wada <i>et al</i> ^[54] , 2003 | 47 moderate to severe UC | pp65 antigenemia and IHC | Association of CMV infection with steroid resistance [13/16 (81.3%) <i>vs</i> 9/31 (29%), $P = 0.001$] and severe endoscopic score ($P < 0.05$); ganciclovir effective in 8/12 patients (66.7%) | Unfavorable |
| Criscuoli <i>et al</i> ^[55] , 2004 | 38 UC and 4 CD with severe disease | pp65 antigenemia, qualitative PCR in leucocytes, histology and IHC | No clear association with steroid resistance, no need for antiviral therapy | None |
| Kambham <i>et al</i> ^[56] , 2004 | 80 UC | IHC | CMV detected in 10 of 40 (25%) patients with refractory UC <i>vs</i> 1 of 40 (2.5%) patients with nonrefractory UC | Unfavorable |
| Kishore <i>et al</i> ^[57] , 2004 | 61 UC and 2 CD | Serology (IgM), qualitative PCR in biopsy | CMV infection associated with poor outcome, with surgical treatment (4/10 <i>vs</i> 4/53, $P < 0.05$) and death (3/10 <i>vs</i> 0/53, $P < 0.005$) | Unfavorable |
| Alain <i>et al</i> ^[58] , 2005 | 63 CD and 28 UC | Serology (IgM), viruria, pp65 antigenemia, detection of mRNA in blood, tissue cell culture of blood and tissue, histology and IHC | 8/14 patients with CMV infection experienced high dose steroid or azathioprine; ganciclovir improved 4/4 treated patients | Unfavorable |
| Maconi <i>et al</i> ^[59] , 2005 | 77 UC with colectomy | Histology and IHC | Trend for an association between CMV reactivation and corticoreistance (15/55, 27.3% <i>vs</i> 2/22, 9.1%, $P = 0.123$) | Unfavorable |
| Dimitroulia <i>et al</i> ^[112] , 2006 | 58 UC and 27 CD | PCR in blood and IHC | No association with disease severity | None |
| Kojima <i>et al</i> ^[60] , 2006 | 126 UC with colectomy | Histology and IHC | CMV markers in surgical specimens more frequently detected in patients with severe or refractory disease | Unfavorable |
| Lavagna <i>et al</i> ^[61] , 2006 | 24 refractory UC leading to colectomy | IHC and PCR in tissue | No pouchitis in CMV positive patients (compared to 3/21 of CMV negative ones) | None |
| Kuwabara <i>et al</i> ^[133] , 2007 | 34 UC and 16 CD | IHC | CMV positive cell density associated with steroid resistance and colectomy rate | Unfavorable |
| Minami <i>et al</i> ^[62] , 2007 | 23 severe UC | Heterogeneous (serology or histology or IHC or PCR in blood) | 18 out 23 patients receiving CyA exhibited CMV infection; 15/18 (83.3%) CMV positive required colectomy; colectomy could be avoided in the 3 remaining patients by administration of ganciclovir | Unfavorable |
| Matsuoka <i>et al</i> ^[63] , 2007 | 69 moderate to severe UC | pp65 antigenemia and qPCR in plasma, histology | Low peripheral viral load observed in 25/48 patients; none exhibited CMV markers in tissue. No impact on clinical outcome and spontaneous clearance of CMV markers in blood without ganciclovir | None |
| Yoshino <i>et al</i> ^[141] , 2007 | 30 UC refractory to immunosuppressive therapies | qPCR in tissue | Clinical remission after ganciclovir alone in 4/12 treated, the remaining 8 required additional anti-inflammatory treatment | Unfavorable |
| Domènech <i>et al</i> ^[64] , 2008 | 114 active UC | pp65 antigenemia tissue: histology, IHC and detection of pp67 mRNA | Steroid and CyA treatment predisposes to CMV reactivation in colon (6/19); ganciclovir associated to remission in 3/6 patients; CMV markers detected in 2 surgical specimens | Unfavorable |
| Maher <i>et al</i> ^[65] , 2009 | 49 UC and 23 CD with active disease | Serology, histology and IHC | CMV infection more frequent in steroid resistant patients (8/23, 34.8% <i>vs</i> 1/31, 3.2%) | Unfavorable |
| Kim <i>et al</i> ^[17] , 2010 | 122 UC | IHC | CMV-positive patients required hospitalization (OR = 4.9; 95%CI: 1.2-19.0) and were hospitalized ≥ 7 d (OR = 5.0; 95%CI: 1.6-21.3) | Unfavorable |
| Lévêque <i>et al</i> ^[16] , 2010 | 33 CD and 20 UC | qPCR in tissue | CMV infection more frequent after corticoid or azathioprine therapy; no relation with disease severity; no need of antiviral therapy | None |
| Omiya <i>et al</i> ^[142] , 2010 | 20 UC | PCR in tissue | Absence of large ulcer in case of CMV infection | None |
| Suzuki <i>et al</i> ^[66] , 2010 | 73 UC | pp65 antigenemia | Irregular ulceration associated to 100% of CMV infection | Unfavorable |
| Criscuoli <i>et al</i> ^[67] , 2011 | 28 UC with CMV reactivation | Histology, IHC and nested PCR in tissue | Persistence of CMV markers in colon after acute colitis flare-up despite remission | None |

| | | | | |
|---|--|---|--|-------------|
| Nguyen <i>et al</i> ^[22] , 2011 | 26 UC and 17 CD | Histology and IHC | Higher colectomy rate in patients exhibiting high grade infection; decreased colectomy rate with ganciclovir use | Unfavorable |
| Roblin <i>et al</i> ^[10] , 2011 | 42 moderate to severe UC | qPCR in tissue | The tissue CMV DNA load is predictive of resistance to immunosuppressive therapy; ganciclovir treatment cleared CMV DNA in tissue and improved outcome in 7/8 patients | Unfavorable |
| Al-Zafiri <i>et al</i> ^[20] , 2012 | 13 CD and 18 UC with CMV reactivation | IHC | Colectomy rate higher (9/31, 29%) in CMV positive than in CMV negative (65/581, 11.2%) IBD patients | Unfavorable |
| Kim <i>et al</i> ^[68] , 2012 | 72 moderate to severe UC treated with IV steroids | PCR in tissue | Association of CMV infection with steroid resistance; clinical improvement after ganciclovir (11/14) | Unfavorable |
| Yoshino <i>et al</i> ^[69] , 2012 | 17 UC refractory to tacrolimus | qPCR in tissue | Colectomy-free time lower in CMV positive patients than in CMV-negative ones (35.7% at 17.7 mo <i>vs</i> 88.9% at 45.9 mo respectively, log-rank test $P < 0.005$) | Unfavorable |
| Fukuchi <i>et al</i> ^[70] , 2013 | 51 active UC | IHC or qPCR in tissue | CMV DNA became negative after GMAA in patients with clinical remission | Unfavorable |
| Ilda <i>et al</i> ^[71] , 2013 | 187 active UC | pp65 antigenemia | CMV infection more frequent in steroid refractory patients (27/82, 32.9% <i>vs</i> 6/105, 5.7%) | Unfavorable |
| Kopylov <i>et al</i> ^[72] , 2013 | 13 UC with CMV reactivation | IHC | The disease was more severe in the 7 patients requiring ganciclovir therapy, including 1 death and 3 colectomies | Unfavorable |
| Delvincourt <i>et al</i> ^[73] , 2014 | 26 UC and 110 IBD hospitalized | qPCR in blood or tissue | No alteration of the course of IBD flare | None |
| Do Carmo <i>et al</i> ^[74] , 2014 | 249 CD+151 UC | Qualitative PCR in stools | CMV infection is rare (only 9 patients) and is not associated with IBD disease activity | None |
| Inokuchi <i>et al</i> ^[75] , 2014 | 118 UC | pp65 antigenemia | Delay to clinical remission higher in CMV positive patients (21 d <i>vs</i> 16 d, $P < 0.01$); ganciclovir decreased the rate of colectomy in multivariate analysis | Unfavorable |
| Kim <i>et al</i> ^[76] , 2014 | 72 moderate to severe UC | Heterogeneous (serology or histology or IHC or PCR) | Cumulative colectomy (log rank, $P = 0.025$) and disease flare-up rates (log-rank, $P = 0.048$) higher in CMV positive patients | Unfavorable |
| Kim <i>et al</i> ^[77] , 2014 | 229 moderate to severe UC | IHC and pp65 antigenemia | Association between positive pp65 antigenemia and rate of colectomy (13/39, 33.3% <i>vs</i> 5/44, 11.4%, $P < 0.05$) | Unfavorable |
| Maconi <i>et al</i> ^[78] , 2014 | 30 UC and 8 CD with active colitis and CMV infection | Histology/IHC | Antiviral therapy associated with a higher clinical remission rate at 12 mo (77.8% <i>vs</i> 45%, $P < 0.05$, and 77.8% <i>vs</i> 19.4%, $P < 0.05$) in UC patients and patients with steroid-dependent/refractory disease, respectively | Unfavorable |
| Matsumoto <i>et al</i> ^[79] , 2014 | 222 UC | Antigenemia, histology, PCR | CMV infection as a risk factor for hospitalization because of UC aggravation (OR = 8.2, 95%CI: 1.91-35.33, $P < 0.005$) | Unfavorable |
| Olaisen <i>et al</i> ^[80] , 2014 | 77 patients undergoing colectomy | IHC | CMV positive patients received higher doses of corticoids and were at higher risk of postoperative complications | Unfavorable |
| Yamada <i>et al</i> ^[81] , 2014 | 33 refractory UC | qPCR in tissue | Induction remission rate by infliximab lower (54.5%) in CMV-positive patients than in CMV-negative ones (81.8%) although not statistically significant | Unfavorable |
| Chun <i>et al</i> ^[82] , 2015 | 43 moderate to severe UC | pp65 antigenemia | Positive antigenemia associated with steroid refractoriness (11/12, 91.7% <i>vs</i> 12/31, 38.7%, $P < 0.005$); ganciclovir improved outcome: colectomy in 2/8 (25%) <i>vs</i> 2/4 (50%) | Unfavorable |
| Ciccocioppo <i>et al</i> ^[32] , 2015 | 24 UC and 16 CD | qPCR in tissue | In refractory patients, more frequent CMV infection and higher viral load; efficacy of ganciclovir in all refractory patients | Unfavorable |
| Jones <i>et al</i> ^[83] , 2015 | 1111 IBD patients | Histology, IHC, ISH | Antiviral therapy improved surgery-free survival outcome | Unfavorable |
| Gauss <i>et al</i> ^[84] , 2015 | 166 UC and 131 CD | IHC and PCR in tissue | CMV reactivation associated to longer hospital stay ($P < 0.001$) | Unfavorable |
| McCurdy <i>et al</i> ^[41] , 2015 | 45 UC, 21 CD and 2 indeterminate IBD colitis | Histology, ISH, IHC | CMV reactivation associated to medically refractory disease (OR = 3.69, $P < 0.001$) and endoscopic ulcers (OR = 2.95, $P < 0.001$) | Unfavorable |

| | | | | |
|--|---|----------------|---|-------------|
| Minami <i>et al</i> ^[85] , 2015 | 29 severe UC treated either with tacrolimus or infliximab | qPCR in tissue | Colectomy rate higher in patients with CMV infection (5/6, 83.3% vs 8/23, 34.8%, $P < 0.05$) | Unfavorable |
|--|---|----------------|---|-------------|

GMAA: Granulocyte/monocyte adsorptive apheresis; IHC: Immunohistochemistry; ISH: *In situ* hybridization; NAAT: Nucleic acid amplification test; PBMC: Peripheral blood mononuclear cells; PCR: Polymerase chain reaction; qPCR: Quantitative real-time PCR; IBD: Inflammatory bowel diseases; CD: Crohn's disease; UC: Ulcerative colitis; CMV: Cytomegalovirus.

and by directly activating viral replication^[93,94]. Indeed, many studies have documented this risk in UC patients^[14,17,32,52,59,80]. It has been shown that administration of steroids over a period of at least 3 mo at a dose of at least 10 mg is associated with a risk of CMV reactivation, without any effect of cumulative doses^[52]. The prevalence of CMV reactivation increased with the exposition of high-dose steroid therapy for 7-14 d^[17].

With regards to immunomodulatory therapy other than steroids, cyclosporine (CyA) is also associated with the risk of active CMV infection^[62,64,83]. In a study including 23 patients with severe UC undergoing CyA treatment, 18 of them developed CMV infection, as illustrated by the presence of IgM antibody, CMV DNA or inclusion bodies by histology after approximately 8 d of treatment^[62]. In a prospective study, CMV infection was observed in five of six UC patients after 7-10 d CyA treatment^[64]. Consequently, the risk of CMV infection should be carefully monitored when this drug is used as an alternative to other contraindicated immunomodulatory agents. In contrast, the use of azathioprine or anti-TNF monoclonal antibodies (mAbs) was not associated with an increased risk of CMV reactivation^[10,41,52,64,95-99]. We recently reported 109 consecutive flares-up of UC in patients undergoing anti-TNF maintenance therapy; these patients were not at a higher risk of CMV reactivation and, reciprocally, the occurrence of CMV reactivation had no effect on the further evolution of UC. These results plead for the preferential use of these molecules in cases of refractory flare-up associated with CMV reactivation^[100]. However, in a recent study combining CD and UC patients, the use of immunomodulators, including thiopurines or methotrexate, was significantly associated with occurrence of CMV disease^[41]. Tacrolimus was recently proposed as an alternative to previous treatments, especially in cases of refractory flare-up^[85,98]; further studies are needed to appreciate the risk of developing CMV reactivation in this context^[101].

Age > 30 years: Two recent studies have documented the risk of CMV reactivation in IBD patients older than 30 years. In a retrospective case-control study performed on 68 IBD patients (66% with UC) exhibiting CMV infection by tissue analysis, who were each matched to three controls without stigmata of CMV infection, McCurdy *et al*^[41] showed that CMV disease was significantly associated with age > 30 years. No stratification was performed by type of IBD

(CD or UC). In another retrospective study, Gauss *et al*^[84] recorded positive CMV markers in 21 IBD patients - 18 with CMV DNA in colonic biopsy and three with positive blood antigenemia (the PCR assay was not done) - out of 100 patients, and most of them (17/21) exhibited UC. The presence of CMV markers was significantly associated with age ≥ 30 years (OR = 14.26; 95%CI: 2.89-118.57). Despite the high significance of these data, they relied only on two studies with a low number of patients, which implies that further trials are required to consolidate these observations.

Other predictive factors of CMV infection in IBD patients:

The two retrospective studies mentioned in the above paragraph also documented other predictive factors of CMV infection in IBD patients. In addition to age > 30 years, McCurdy *et al*^[41] identified four additional risk factors: medically refractory IBD; the presence of ulcers at endoscopy; treatment with corticosteroids; and treatment with immunomodulators (with the exception of anti-TNF mAb). After adjustment in a multivariate model, refractory disease, treatment with immunomodulators and age > 30 years remained independently associated with CMV infection. The authors propose a CMV risk score based on these criteria for the prediction of CMV infection in IBD patients. Furthermore, in addition to age > 30 years, the case-control study of Gauss *et al*^[84] identified a blood leukocyte count < 11000/mL, disease duration at admission < 60 mo, and the presence of immunosuppressive therapy at admission as significant predictors of CMV infection in IBD patients. As no stratification was done by type of IBD in these two retrospective studies, it would be interesting to re-evaluate specifically these predictors in UC patients who are most at risk of CMV infection among IBD patients.

WHAT ARE THE PREDICTIVE FACTORS OF UNFAVORABLE EVOLUTION IN UC PATIENTS WITH CMV INFECTION?

Resistance to steroids and other immunosuppressive agents

CMV reactivation was recorded as one of the most important risk factors for steroid-refractory UC. A retrospective study that investigated CMV infection by IHC in 77 surgical specimens reported a rate of CMV

infection of 27.3% in samples from steroid-refractory UC patients compared to 9.1% in those from steroid-sensitive ones^[102]. In the prospective study that we conducted on 42 consecutive patients hospitalized for moderate to severe UC and treated with IV steroids, the only factor associated by multivariate analysis with CMV DNA in inflammatory tissue was resistance to steroids (OR = 4.7; 95%CI: 1.2-22.5)^[10]. Two other prospective studies reported the same association between resistance to steroids and CMV reactivation^[52,64]. Recent studies^[71,78] including two multivariate analyses^[41,84] confirmed the link between CMV reactivation and steroid resistance. In a meta-analysis published last year and summarizing 11 studies involving 867 IBD patients, the relative risk for steroid resistance was significantly higher in CMV-positive patients (OR = 2.07; 95%CI: 1.80-2.39)^[103].

As shown in our work on flare-ups of refractory UC, CMV reactivation affects the response to immunosuppressive therapy, including anti-TNF mAbs^[10]. In a similar context, Yamada *et al*^[81] showed that the induction remission rate by infliximab was lower (54.5%) in CMV-positive than in CMV-negative patients (81.8%), although the difference was not statistically significant.

Acute severe colitis and requirement of colectomy

Since the first description of CMV markers in surgical specimens^[104], a higher rate of colectomy has been observed in cases of CMV reactivation vs CMV-negative groups^[20,69,76,85]. In the prospective study published by Domènech *et al*^[64], colectomy was performed in 3/6 patients exhibiting CMV reactivation compared to 2/12 patients without markers of CMV infection. The prevalence of CMV markers detected using IHC in surgical specimens was also shown to be higher in severe UC than in refractory UC (25% vs 8.3% and 25% vs 2.5%^[56,60], respectively). In a recent report, Yoshino *et al*^[69] showed that the colectomy-free time was higher in patients without CMV colitis. Finally, Matsumoto and Yoshida reported recently that CMV infection and steroid use were independent risk factors for hospitalization because of UC aggravation and the need for surgery^[79]. By retrospective analysis of a surgery database including 1100 patients, Uchino *et al*^[105] recorded seven cases exhibiting UC-related lesions in the stomach and small intestine after colectomy; six of seven exhibited CMV infection either with positive antigenemia or CMV markers in tissue (IHC or PCR). These severe CMV infections were all refractory to ganciclovir treatment.

Presence of ulcers with endoscopic examination

Several studies have argued for a link between the presence of ulcers after endoscopic examination, CMV reactivation and unfavorable evolution. In a study of UC patients hospitalized due to exacerbation of symptoms,

colonoscopic findings were compared between 15 CMV-positive and 58 CMV-negative patients, as determined by blood antigenemia: more abnormalities (irregular ulceration, wide mucosal defect) were observed in patients with UC complicated by CMV infection^[66]. More recently, the retrospective study mentioned previously^[41] reported a trend towards severe endoscopic disease in CMV-infected IBD patients (OR = 1.67; 95%CI: 0.85-3.32). In the subgroup of UC patients, the presence of endoscopic ulcers was significantly associated with CMV disease (OR = 3.0; 95%CI: 1.38-6.51). In another study, the absence of large ulcers was predictive of non-active CMV infection in UC patients positive for the presence of colonic CMV DNA: the 10 patients exhibiting this profile attained remission without antiviral therapy at 2 mo and maintained remission^[42]. However, other studies, including ours^[10,54], did not identify stigmata of tissue injury as a marker of CMV infection. It may depend upon the severity of UC in the studied populations that may have been lower in the latter studies.

Density of viral infection

Using either molecular or histological assays to evaluate the density of viral infection, this quantitative or semi-quantitative marker was shown to be related to the severity of colonic lesions in UC patients. Using histopathology, Nguyen *et al*^[22] distinguished low-grade CMV infection (when IHC was positive only) from high-grade infection (detected by HE staining): colectomy rates were 29% and 83%, respectively, in untreated patients. Jones *et al*^[83] defined high-grade CMV density by the presence of more than four typical inclusions in biopsy specimens. Similarly, Kuwabara *et al*^[13] proposed that dense CMV disease, defined as > 10 inclusions per histological section, was shown to be predictive of significantly higher final daily doses of steroids before surgery, and showed increased steroid resistance. In addition, the frequency of emergency surgery was higher and postoperative hospital stay was significantly longer in the dense CMV group.

By using qPCR in colon biopsies, we performed a random sensitivity analysis for correlating the presence of CMV in tissue with the occurrence of resistance to the successive lines of treatment^[10]. A positive colonic CMV load was associated with an increased risk of steroid resistance [likelihood ratio (LR+) of 3.0], with a sensitivity of 50% and a specificity of 100% [area under the receiver operating characteristic curve (AUROC) = 0.54, $P < 0.05$]. A viral load of > 250 copies/mg of tissue was predictive of resistance to three successive lines of treatment with a sensitivity of 100% and specificity of 66.6% (LR+ 4.33; AUROC = 0.85, $P < 0.05$). In contrast, the absence of CMV DNA in tissue was predictive of a favorable response to any treatment with a sensitivity of 100% and specificity of 50% (LR+ 2.21; AUROC = 0.65, $P < 0.05$).

WHAT IS THE BENEFIT OF ANTI-CMV THERAPY ON THE EVOLUTION OF UC IN PATIENTS WITH CMV REACTIVATION?

Systematic review of literature

Regarding the management of CMV infection in UC patients, the guidelines of the European Crohn's and Colitis Organization in 2014 are as follows: "Screening for CMV infection is not necessary before starting immunomodulator therapy. In patients with acute steroid-resistant colitis, CMV should be excluded, preferably by tissue PCR or immunohistochemistry, before increasing immunomodulator therapy. In case of severe steroid-resistant colitis with CMV detected in the mucosa during immunomodulator therapy, antiviral therapy should be initiated and discontinuation of immunomodulators considered until colitis symptoms improve. In case of systemic CMV disease, immunomodulator therapy must be discontinued"^[49]. However, randomized controlled trials would be useful in reinforcing the level of evidence supporting these guidelines.

If most of the gastroenterology societies recommend antiviral treatment of severe flare-ups of UC exhibiting CMV markers in inflamed tissue, no recommendations are given on which antiviral drug should be used and for what duration. No study has compared ganciclovir and foscarnet in this indication and no data are available on the pharmacokinetics of antiviral drugs in colonic tissue; notably, regarding the difference between ganciclovir and valganciclovir and the role of possible malabsorption in inflamed tissue. In contrast to transplant recipients^[106], the overall incidence of CMV resistance to ganciclovir in IBD has never been analyzed. In this context, most authors use ganciclovir to treat CMV reactivation in UC patients (reviewed in Shukla *et al.*^[48]). In our clinical practice, we use empirically IV ganciclovir for 1 wk followed by oral valganciclovir for 2 wk but the relevance of this strategy has not been evaluated.

A lot of case reports, as well as punctual prospective studies, have reported a clinical improvement associated with a reduction of colectomy rate when UC patients with CMV reactivation received ganciclovir (or exceptionally, foscarnet). In a previous review paper^[50], we collected seven prospective studies^[14,34,51,64,67,68,95] that analyzed the efficacy of treatment of CMV reactivation by ganciclovir in UC patients: from a total of 58 treated patients, 46 presented a clinical improvement and 11 justified colectomy (18%).

Several studies analyzed the benefit of ganciclovir on colectomy rate according to the density of CMV infection. In the study of Nguyen *et al.*^[22], the antiviral treatment did not change colectomy rate for the patients with low-grade CMV infection (31% vs 29% without CMV treatment) but it significantly decreased the colectomy rate for those with high-

grade CMV infection (44% vs 83% without CMV treatment). Similarly, Jones *et al.*^[83] argued that antiviral treatment significantly reduced the risk of surgery (OR = 0.31; 95%CI: 0.14-0.70); patients with high-grade infection showed a significant benefit of antiviral therapy, whereas those with low-grade infection presented higher rates of colectomy. In a study performed in our hospital^[10], eight patients with a high CMV DNA load in the colon, and who had failed to respond to at least two lines of treatment, were treated with ganciclovir for 15 d in addition to their ongoing immunosuppressive therapy. For seven of them, clinical remission was obtained with a sustained response to the last therapeutic line after a follow-up of 6 mo, which resulted in a step-down therapeutic strategy for all of them^[10].

Recently, a meta-analysis was performed to determine the impact of antiviral therapy on the colectomy rate in UC patients presenting with CMV infection^[48]. Fifteen studies were included in this meta-analysis with a total of 333 patients; 43.2% were treated with antiviral therapy and 56.8% were not. The diagnosis was made primarily by HE and/or IHC in seven studies and by tissue PCR in four. No difference was noticed in terms of colectomy between patients treated with antiviral therapy and those without treatment (OR = 0.92; 95%CI: 0.31-2.76), with moderate heterogeneity ($I^2 = 65\%$). There was no significant difference in the risk of colectomy based on the method of CMV diagnosis. Next, the authors analyzed the risk of colectomy in those patients with corticosteroid-refractory UC related to CMV reactivation; eight studies were available concerning 139 patients, 77 of whom received antiviral therapy. The risk of colectomy was significantly lower in patients with corticosteroid-refractory UC treated with antiviral therapy than in patients not treated with antiviral therapy (OR = 0.20; 95%CI: 0.08-0.49), with no heterogeneity ($I^2 = 0$). When the analysis was limited to studies that defined refractory disease as failure to respond to 1 wk of intravenous corticosteroids, the benefit of antiviral therapy remained significant (OR = 0.23; 95%CI: 0.06-0.82). Finally, when the analysis was further stratified on the method of CMV diagnosis, the risk of colectomy remained significantly lower only when CMV infection was based on histological criteria (3 studies; OR = 0.06; 95%CI: 0.01-0.34) but not on tissue PCR (4 studies; OR = 0.31; 95%CI: 0.09-1.11). The latter observation may be related to the fact that the analysis was not adequately powered and that three of the four studies based on tissue PCR reported only qualitative results.

Place of granulocyte/monocyte adsorptive apheresis in the treatment of CMV-related flare-ups of UC

Granulocyte/monocyte adsorptive apheresis (GMAA) is a biological therapy comprising removal of granulocytes/macrophages producing inflammatory

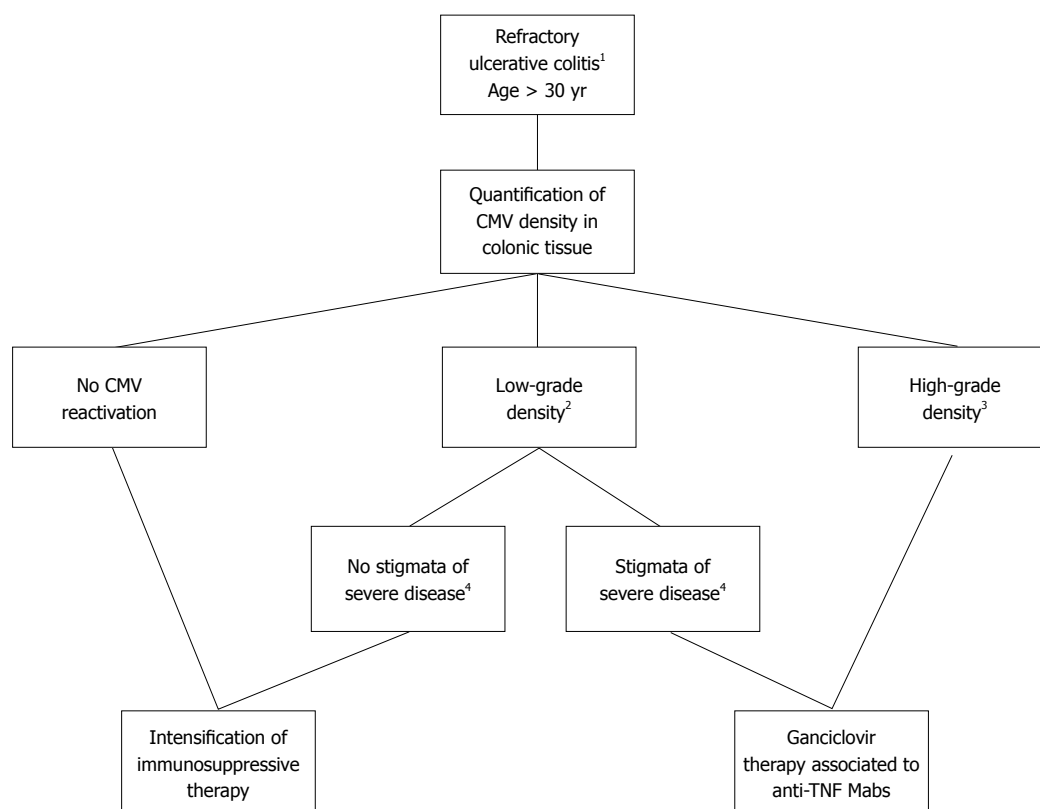


Figure 2 Therapeutic algorithm for the intake of flare-ups of refractory ulcerative colitis in patients aged > 30 years according to the quantification of cytomegalovirus density in colonic tissue. ¹Defined by steroid resistance or immunosuppressive treatment or anti-TNF drugs; ²Defined by quantification of CMV DNA in intestinal tissue of 10-250 copies/mg of inflamed tissue or low-grade CMV density by IHC in biopsy specimens (4 inclusions or less); ³Defined by quantification of CMV DNA in intestinal tissue of > 250 copies/mg of inflamed tissue or high-grade CMV density by IHC in biopsy specimens (more than 4 inclusions); ⁴Defined by a need for hospitalization and a Lichtiger score > 10. CMV: Cytomegalovirus; IHC: immunohistochemistry; TNF: Tumor necrosis factor.

cytokines. This strategy was evaluated in a randomized, double-blind, sham-controlled study for the treatment of UC flare-ups. The treatment was well tolerated but did not demonstrate efficacy for induction of clinical remission or response in patients with moderate-to-severe flare-ups^[107]. More recently, Japanese studies have investigated the efficacy of GMAA in active UC flare-ups associated or not with colonic CMV reactivation. In a retrospective study, 11 UC patients in clinical failure under steroid and immunomodulatory therapy were treated with additional GMAA: nine achieved remission and two underwent colectomy^[108]. Fukuchi *et al.*^[70] tested this strategy in 51 active UC flare-up episodes, and 15 of them were associated with *in situ* CMV infection. In the absence of steroid treatment, the clinical remission rate did not differ between UC patients, whether positive and negative for CMV (73.3% vs 69.4%). CMV DNA became negative in all UC patients positive for CMV who achieved clinical remission 1 wk after completion of intensive GMAA but no data on long-term evolution were reported. Presently GMAA is not recommended in the treatment of UC flare-ups by American and European guidelines. Additional studies are needed to evaluate the benefit of GMAA in UC patients with flare-ups associated with CMV reactivation.

Discussion of therapeutic algorithms

At least three therapeutic algorithms have been proposed for the intake of refractory flare-ups of UC according to the presence of CMV reactivation in the gut^[48,50,109]. These algorithms are all similar but do not take into consideration the risk factors listed above together with the density of CMV infection^[83] and the absence of reciprocal deleterious effects between anti-TNF mAbs and CMV reactivation^[100]. The therapeutic algorithm that we propose in Figure 2 integrates these relatively new concepts. Of note, as recommended by the European guidelines^[49], the antiviral therapy must be initiated after discontinuation of immunomodulators that will be reintroduced at the end of the flare-up.

CONCLUSION

Despite conflicting results, there is increasing evidence, notably in recent studies, for the deleterious effect of *in situ* CMV reactivation in flare-ups of refractory UC. In patients aged > 30 years with a high density of infection in the colonic tissue or with stigmata of severe disease associated with colonic markers of CMV reactivation (whatever the density of infection), treatment with ganciclovir appears to be recommended with anti-TNF mAb therapy in the absence of explicit

contraindications to these drugs. In order to validate the present strategy based on our experience and the in-depth analysis of the available literature presented in this review, prospective randomized controlled studies are urgently needed.

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Glucose metabolism in gastric cancer: The cutting-edge

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Abstract

Glucose metabolism in gastric cancer cells differs from that of normal epithelial cells. Upregulated aerobic glycolysis (Warburg effect) in gastric cancer meeting the demands of cell proliferation is associated with genetic mutations, epigenetic modification and proteomic alteration. Understanding the mechanisms of aerobic glycolysis may contribute to our knowledge of gastric carcinogenesis. Metabolomic studies offer novel, convenient and practical tools in the search for new biomarkers for early detection, diagnosis, prognosis, and chemosensitivity prediction of gastric cancer. Interfering with the process of glycolysis in cancer cells may provide a new and promising therapeutic strategy for gastric cancer. In this article, we present a brief review of recent studies of glucose metabolism in gastric cancer, with primary focus on the clinical applications of new biomarkers and their potential therapeutic role in gastric cancer.

Key words: Glucose metabolism; Warburg effect; Metabolomics; Gastric cancer; Biomarker; Therapy

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Core tip: Increased glucose consumption is a hallmark of cancer cells. Studies focusing on glucose metabolism provide a new perspective on gastric carcinogenesis and a novel approach to exploration of biomarkers and therapeutic targets in gastric cancer.

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INTRODUCTION

Gastric cancer is one of the most common cancers worldwide and ranks second in cancer-related deaths^[1,2]. Recent advances in cancer diagnosis and treatment have resulted in limited improvement in gastric cancer-related mortality^[3]. Estimates even suggest that gastric cancer-related mortality will continue to increase^[4]. To improve the survival rate, several studies have elucidated molecular mechanisms of gastric cancer, and identified biomarkers predicting prognosis and response to treatment^[5,6]. A few biomarkers have been used as therapeutic targets for advanced gastric cancer^[7]. However, the therapeutic results are still unsatisfactory, which may be due to multiple genetic variations and changes in microenvironment, such as altered glucose metabolism promoting gastric carcinogenesis.

Several decades ago, alteration of glucose metabolism in cancer cells, termed "Warburg effect", was described. This discovery has revitalized the interest in the role of glucose metabolism in oncology since the widespread use of ¹⁸F-fluorodeoxyglucose (FDG) positron emission tomography (PET) to evaluate various types of malignant tumors^[8]. Compared with genomics and proteomics, metabolomics is a recent "omic" technique and the last step before phenotype, which provides new insight into pathophysiologic mechanisms in carcinogenesis. In fact, these biological pathways are not independent but co-dependent and co-operative in the progress of carcinogenesis.

The high mortality rate of gastric cancer is due to delayed diagnosis and lack of effective therapies for metastasis. Gastric cancer is generally screened using endoscopy and serum carbohydrate antigens, such as carcinoembryonic antigen (CEA). Clinical application of endoscopy is limited because of its relative invasiveness, cost and technical complexity, even in high-incidence countries of east Asia^[9]. Additionally, the current serum biomarkers have poor sensitivity and specificity for gastric cancer. Therefore, new biomarkers that are non-invasive and enable stratification of patients with high sensitivity and specificity for screening, diagnosis, prognosis, prediction, and monitoring of aggressive and advanced gastric cancer are needed. Metabolomics facilitates the investigation of these biomarkers *via* new and interesting analytical techniques that enable the detection of an array of metabolites in a single assay and open new avenues for diagnostics and drug discovery. By identifying and targeting the key link in altered glucose metabolism, specific and even individualized therapeutic strategies for gastric cancer may be developed.

This article will review the recent studies on glucose metabolism in gastric cancer and particularly the key applications of glucose metabolism in gastric cancer surveillance, diagnosis and therapy.

ALTERED GLUCOSE METABOLISM IN GASTRIC CANCER

In 1956, Otto Warburg initially observed that cancer cells generally undergo glycolysis instead of oxidative phosphorylation for energy, compared with non-neoplastic cells. The metabolic phenomenon is well known as aerobic glycolysis or the "Warburg effect"^[10]. Based on the results of "Warburg effect", increased glucose consumption, increased glycolytic activity and the accumulation of lactic acid are critical hallmarks of cancer cells^[11,12]. Compared with normal cells that mainly generate energy *via* mitochondrial oxidative phosphorylation, cancer cells predominantly obtain energy *via* increased glycolysis even under aerobic conditions. Converting glucose into lactate *via* glycolysis is inefficient in generating ATP, but it produces a large number of intermediate products driving cell proliferation. Therefore, increasing glucose consumption, leading to anaerobic glycolysis, is believed to provide an evolutionary advantage to cancer cells^[13]. The accumulation of lactic acid causes acidic microenvironment, and has a protective effect on tumor cells. Lactic acid induces the expression of glycolytic enzymes in tumor cells, such as 6-phosphofructokinase1 (PFK1) to enhance the supply of ATP, and resist cellular apoptosis and promote metastasis^[14]. In addition, lactic acid promotes tumor angiogenesis, providing a suitable microenvironment for tumor development and metastasis.

A number of studies have confirmed the association between obesity and gastric cancer^[15-19]. An *et al*^[20] confirmed the relationship between glucose metabolism, diabetes and gastric cancer by observing improved glucose metabolism after treatment of gastric cancer. A higher fasting serum glucose level significantly increased the incidence of gastric cancer in *Helicobacter pylori* (*H. pylori*)-seropositive patients nearly 3.5-4.2 fold^[21], suggesting that hyperglycemia may be an important cofactor in *H. pylori*-mediated gastric carcinogenesis^[22,23]. Song *et al*^[24] used gas chromatography/mass spectrometry (GC/MS) to analyze the tissue metabolites of gastric cancer patients and healthy controls. The GC/MS revealed that several intermediate products of aerobic glycolytic pathways, such as fumaric acid and alpha-ketoglutaric acid increase significantly in cancer tissues than in the normal mucosa, suggesting that altered glucose metabolism may be an important parameter in distinguishing gastric cancer cells from normal cells. Similarly, abnormal glucose metabolism was observed by other researchers in gastric cancer tissue^[25-28].

Ikeda *et al*^[29] demonstrated that the serum levels of 3-hydroxypropionic acid and pyruvic acid were upregulated in gastric cancer. Therefore, abnormal glucose metabolism may be related to tumor growth involving aggressive cancer cell proliferation, which requires a lot of energy, possibly causing altered

serum levels of a few intermediate metabolites. Serum metabolic profiling has a great potential role in identifying gastric cancer and the underlying metabolic mechanisms^[30].

Chen *et al.*^[31] used capillary electrophoresis-mass spectrometry based on moving reaction boundary (MRB-CE-MS) to investigate the metabolomics of gastric cancer patients' urinary samples to search for possible tumor biomarkers. They found that lactic acid was remarkably increased, while citric acid, malic acid, and succinate were significantly decreased in patients with gastric cancer compared with controls, suggesting that glycolysis is upregulated while tricarboxylic acid cycle is decreased in gastric cancer^[31]. The results implied that urinary metabolic profiles based on MRB-CE-MS analysis were useful in clinical diagnosis and prognosis of gastric cancer patients, consistent with findings from other studies^[32-36].

POTENTIAL MECHANISMS RESULTING IN ALTERED GLUCOSE METABOLISM IN GASTRIC CANCER

About 80 years after Warburg presented his hypothesis on aberrant glucose metabolism in cancer cells, his viewpoint has been confirmed using positron emission tomography (PET) with the glucose analog tracer in clinical oncology. The potential genetic, epigenetic and proteomic mechanisms underlying the relationship between glucose metabolism and cancer have only been partially elucidated.

Genes and alteration of glucose metabolism

Carcinogenesis is due to proto-oncogene activation and tumor suppressor gene inactivation, which are closely associated with glucose metabolism. As a proto-oncogene, Myc plays an important role in glucose metabolism by enhancing the expression of glycolytic enzymes including glucose transporter 1 (GLUT1)^[37], lactate dehydrogenase A (LDHA)^[38,39] and pyruvate Kinase M2 (PKM2)^[40]. Inactivation of p53, a well-known tumor suppressor, directly mediates the Warburg effect. In many cancers, p53 loss was observed to promote glucose flux *via* glycolytic pathway and reduced oxidative phosphorylation^[40]. The p53 protein increases oxidative phosphorylation and decreases glycolysis *via* downregulation of GLUT1, GLUT3, and GLUT4 expression^[41] and inactivation of glycolytic enzymes, such as phosphoglycerate mutase (PGM)^[42]. Recently, we studied the role of Klotho, an anti-oncogene, in gastric cancer and found that restoration of Klotho gene expression could remarkably inhibit cell proliferation and induce apoptosis in gastric cancer cells by downregulating the phosphorylation levels of IGF-1R, IRS-1, PI3K, Akt, and mTOR proteins. In the process, it may be associated with altered glucose metabolism, which requires further research^[43].

Enzymatic changes in glucose metabolism of cancer cells

The family of glucose transporters (Gluts), which control the glucose transport across the plasma into the cytosol, play a critical role in glucose metabolism^[44-46]. Increasing evidence shows that Gluts, especially the class I Gluts (1-4), play an key role in cancer glucose metabolism and cancer progression, such as in lung tumor^[47], breast cancer^[48], and bladder cancer^[49]. Recently, Shimada *et al.*^[50] reported that Glut-3 and Glut-1 expression were positive in benign gastric schwannoma with a high FDG uptake, but Glut-2 and Glut-4 expression were negative. 18F-FDG uptake in primary gastric lymphoma is also related to GLUT1 expression^[51]. Alakus *et al.*^[52] investigated GLUT-1 expression in 35 patients with gastric cancer, who underwent FDG-PET, and suggested that FDG uptake in gastric cancer is associated with GLUT-1 expression and that low FDG uptake in signet-ring cell carcinoma is due to the low expression of GLUT-1 in this histological subtype. Yamada *et al.*^[53] also observed that GLUT-1 expression occurred from an early cancer stage and was the most influential factor underlying the degree of FDG uptake in gastric carcinoma. FDG uptake correlated with GLUT-1 expression, responding to glucose metabolism, may serve as a prognostic biomarker of gastric cancer^[53]. However, the study of Takebayashi *et al.*^[54] showed no connection between FDG standardized uptake value (SUV) and GLUT-1 expression in gastric cancer. Currently, evidence on the role of Gluts in glucose metabolism in gastric cancer is still limited.

Several other glycolytic enzymes, including glucose-6-phosphate dehydrogenase (G6PD)^[55], hexokinase (HK)-II^[56,57] and pyruvate kinase M2 (PKM2)^[58,59], have been confirmed to participate in the carcinogenesis and predict the progression of gastric cancer. G6PD is involved in the normal processing of carbohydrates by converting glucose into ribose-5-phosphate, which is the first key step in glycolysis. Overexpression of G6PD in gastric cancer tissues is significantly correlated with progression of gastric cancer. Increasing G6PD levels in gastric cancer may enhance the level of NADPH which protects cells from DNA damage induced by reactive oxygen species (ROS)^[55]. Hexokinases catalyze the first phosphorylation step of glycolysis, to produce glucose-6-phosphate. HK-II is upregulated in many human cancers associated with enhanced aerobic glycolysis, the Warburg effect. HK-II was overexpressed in gastric cancer with worse prognosis^[57]. Unlike HK-I that is predominant in gastric polyps and normal mucosa, a significant elevation of HK-II was found in gastric cancer. Changes in the hexokinases isoenzymes composition in gastric mucosa with intestinal metaplasia were expressed to a lesser degree but with similar likelihood of cancer^[60]. HK-II, as a component of survival signaling nexus, integrates glucose metabolism and cell survival through Akt/mTOR pathways. It can positively regulate

glucose starvation-induced autophagy through TORC1 inhibition^[61]. Pyruvate kinase M2 (PKM2) is another glycolytic enzyme, which controls the final rate-limiting step of glycolysis by catalyzing the dephosphorylation of phosphoenolpyruvate (PEP) to pyruvate and is overexpressed in many human cancers^[62]. Recent studies have indicated that PKM2 was overexpressed in gastric cancer and associated with tumor size, depth of invasion and lymph node metastasis^[59,63]. The knockdown of PKM2 partially affected the stability of NF- κ B subunit p65 in gastric cancer cells, suggesting that post-translational regulation of p65 by PKM2 may be a plausible mechanism correlated with cell proliferation^[64]. Another study demonstrated that the PKM2 expression, E-cadherin expression, and ERK1/2 phosphorylation were correlated with each other in gastric cancer cells, which suggested an important connection between PKM2 and E-cadherin in the motility and invasion of gastric cancer cell stimulated by EGFR^[65].

Signaling pathways involved in glycolysis

Hypoxia-inducible factor pathway: One of the common explanations for enhanced glycolysis in cancer cells is cancer tissue hypoxia, attributed to the rapid growth of cancer cells^[66]. Hypoxia is now recognized as a key factor in carcinogenesis, and Hypoxia-inducible factor (HIF)-1 is a critical transcription factor of the HIF pathway involved in both sensing and responding to changes in cellular oxygen, which aids in the survival of cells in hypoxic microenvironment^[67,68]. Increasing evidence shows that HIF-1 and HIF pathway may mediate gastric carcinogenesis. Generally HIF-1 is not or minimally expressed in the normal gastric mucosa from patients with gastric cancer, peptic ulcer or dyspepsia^[69,70]. Recently, Lin *et al.*^[71] conducted a systematic review of the literature and meta-analysis to investigate the role of HIF-1 α in gastric cancer. Of the nine studies including 1103 subjects, HIF-1 α positive expression was observed in half the patients and always indicated poor prognosis for patients with gastric cancer. Activation of the Ras-MAPK signal transduction pathway and PI3K-AKT-mTOR signaling, and loss of tumor suppressor proteins, such as PTEN and p53, elevated HIF-1 α expression. HIF-1 α directly stimulates glycolysis by activating the expression of glucose transporters and several key glycolytic enzymes, such as HK, PKM2 and LDH-A. The HIF-1 α -dependent pathway increases glycolysis and inhibits mitochondrial O₂ consumption, then promoting tumor cell survival^[72,73].

Insulin signaling pathway: Another important signaling pathway involved in glucose metabolism is insulin signaling pathway. Suppressing glucose production in the liver and enhancing glucose uptake in the insulin-sensitive tissues of the human body is well known as the classic action of insulin mediating

glucose homeostasis. Insulin is also implicated in cellular activation and angiogenesis mediated by the activation of signaling of the insulin receptor (IR), insulin growth factor (IGF)-1, IGF2 and the IGF-1R^[74,75]. Increased IGF signaling is associated with many cancers^[74-76]. In addition, downregulation of the IGF-1 receptor expression and reduced signaling have been found to inhibit tumor growth^[77]. Increasing IGF-I expression was observed in gastric tumors progressing from benign proliferative lesions to malignant lesions^[78]. IGF-I can induce epithelial-to-mesenchymal transition (EMT) which is involved in the metastasis of numerous cancers, by activating a PI3K/Akt-GSK-3 β -ZEB2 signaling pathway in gastric cancer BGC-823 cells^[79]. Li *et al.*^[76] and Min *et al.*^[80] found that IGF-IR signaling promoted tumor growth in gastric cancer. IGF-1R blockade reduced gastric tumor growth *in vivo* and *in vitro* by inhibiting both angiogenesis and lymphangiogenesis, attributed to the decreasing activity of both protein kinase B (Akt) and mitogen-activated protein kinase (MAPK). IGF-1R expression in gastric cancer was correlated with lymph node metastasis, poor prognosis and high histological malignancy grade, and may play an important role in tumor growth and metastasis *via* the lymphatic pathway^[81].

PI3K-Akt-mTOR pathway: The PI3K-Akt-mTOR pathway is currently a widely studied intracellular signaling pathway. It is directly associated with cellular quiescence, proliferation, cancer, and longevity. Recent studies showed that the PI3K-Akt-mTOR pathway was activated in gastric cancer and activation of the pathway was correlated with metastasis, poor prognosis and lower survival in gastric cancer^[82,83]. In addition, activation of this pathway promoted glycolysis and inhibited autophagy^[84]. Akt expression directly increases the surface translocation of glucose transporters and enhances aerobic glycolysis by prompting HK- II binding to voltage-dependent anion channel. (VDAC) at the outer mitochondrial membrane^[85]. PI3K/Akt also increased fatty acid synthesis in cancer cells by suppressing mitochondrial acid fatty oxidation and promoting a metabolic phenotype supporting cancer cell growth and proliferation in the absence of glucose by oxidizing fatty acid^[86]. Upregulation of the PI3K-Akt-mTOR pathway and increased glucose consumption *via* glycolysis offer evolutionary advantages to cancer cells in normoxia as well as hypoxia.

In general, the regulation of glucose metabolism in carcinogenesis is a multi-factor, multi-step process. In their review, Smolková *et al.*^[87] presented the wave hypothesis of metabolic regulation during carcinogenesis, which consisted of four waves. First, the fundamental reprogramming of gene expression or initiation by stem cells establishes the conditions conducive to cancer cell proliferation. Second, subsequent responses to microenvironmental conditions cause a typical Warburg

effect. Third, aglycemia and nutrient shortage due to rapid cell growth during malignancy, stimulate glutaminolysis, which may influence restoration of suppressed mitochondrial biogenesis, leading to oxidative phosphorylation (OXPHOS)-dependent cancer cells. The fourth wave of gene reprogramming entails retrograde signaling from revitalized mitochondria.

CLINICAL APPLICATION OF ALTERED GLUCOSE METABOLISM IN GASTRIC CANCER

Role of glucose metabolism in gastric cancer imaging

Based on the increased glucose uptake in cancer cells, PET/CT scan can reflect cancer cell glucose metabolism using ^{18}F -2-fluoro-2-deoxy-D-glucose (^{18}F -FDG) as a tracer and has been widely used in the diagnosis and monitoring of human cancers. ^{18}F -FDG is the most commonly used radiolabeled glucose analog in clinical practice. Currently, gastroscopic biopsy and histopathological examination are the gold standard of diagnosis of gastric cancer. In recent years, PET/CT, integrating images from FDG-PET with CT, have been used to detect gastric cancer. Compared with contrast-enhanced CT (CECT) and endoscopic ultrasonography, PET/CT does not offer the advantages of sensitivity and accuracy, and therefore, FDG-PET/CT scans are not indicated in routine staging of gastric cancer^[88]. Nevertheless, due to its high specificity, PET/CT is useful when CECT findings were equivocal and in the detection of distant lymph node metastasis^[89].

Several factors influence the visibility of PET/CT in gastric carcinoma. PET/CT imaging is based on increased glucose metabolism in gastric cancer. FDG avidity depends on tumor histologic subtype. Low FDG uptake is more often seen in diffuse type histology (mucinous, signet ring and poorly differentiated) compared with the intestinal subtype, which depends on GLUT-1 expression. The low GLUT-1 expression may be lead to low FDG uptake in signet-ring cell carcinoma of gastric cancer^[52,53]. The maximum standardized uptake value [SUV(max)] of PET/CT is significantly correlated with tumor size, and lower FDG uptake is often found in early gastric carcinoma which is likely due to less total cancer cells in the primary lesions^[53,54]. The motility and physiological uptake of ^{18}F -FDG in the stomach also influence the accuracy of PET/CT in diagnosis of gastric cancer. Stomach distension to increase gastric volume with water or milk can reduce physiological gastric FDG uptake, display the lesions more clearly and significantly improve the diagnostic accuracy^[90,91].

Although PET/CT is not recommended in the primary detection of gastric cancer due to its poor sensitivity, FDG-PET shows better results in the evaluation of biological aggressiveness and/or patient prognosis in gastric cancer^[92,93]. ^{18}F -FDG uptake is an independent and significant prognostic indicator of

tumor recurrence in gastric cancer. Lee *et al.*^[92] also investigated the role of ^{18}F -FDG PET in gastric cancer prognosis based on histopathological subtypes and found that patients with negative ^{18}F -FDG tumor uptake showed better recurrence-free survival than those with positive ^{18}F -FDG tumor uptake in the subgroup of patients with gastric adenocarcinoma, while the opposite findings were obtained in the subgroup patients with signet-ring cell carcinoma and mucinous adenocarcinoma.

A higher SUVmax of ^{18}F -FDG PET/CT was linked to the presence of microsatellite instability (MSI) in gastric cancer^[94]. Gastric cancers with MSI tend to show less lymph node metastasis and manifest favorable prognosis^[95]. The high SUVmax of ^{18}F -FDG PET/CT showed poor prognosis in gastric cancer. The SUVmax more than 3.8 indicated increasingly aggressive behavior, elevated postoperative recurrence and shorter relapse-free survival in gastric signet-ring cell carcinoma^[96]. The degree of ^{18}F -FDG uptake in gastric cancer predicts histologically positive lymph nodes and non-curative surgery. The sensitivity, specificity and accuracy of the diagnosis of metastatic lymph node were 73.5%, 74.5% and 74.1%, respectively, using the SUVmax cutoff of 3.75 or greater. When the SUVmax was defined as 4.35 or more for metastatic lymph nodes to predict non-curative surgery, the sensitivity and specificity were 58.8% and 91.6%, respectively, which were higher than those obtained with CT scan. Therefore, pretreatment PET/CT may be helpful in optimizing surgical strategy^[96].

Postoperative routine follow-up of gastric cancer is important to the surveillance of recurrence. The conventional follow-up using computed tomography (CT) and endoscopy, cannot frequently detect recurrence before symptom development. PET/CT shows good specificity for asymptomatic advanced gastric cancer and provides useful information for the clinical management of patients with suspected gastric cancer recurrence^[97]. However, prudence should be exercised with the incidental findings of PET/CT, most of which were benign with additional investigations associated with high cost^[98].

Despite adequate surgery with radical lymphadenectomy, the prognosis of advanced gastric cancer is still poor. Since the early 1990s, neoadjuvant therapy gained importance for the treatment of locally advanced or initially unresectable GC^[99]. Currently, measurement of changes in morphology using different imaging modalities is still the main approach to evaluate the response to neoadjuvant therapy. However, alterations in glucose metabolism always precede changes in morphological changes. The response to neoadjuvant therapy initially manifests in altered glucose uptake, demonstrated with ^{18}F -FDG PET. In fact, a dual modality PET-CT has been recommended for early assessment of therapeutic response in GIST patients treated with imatinib^[100]. In gastric cancer, changes in FDP uptake occur early during the course of neoadjuvant therapy,

which is significantly related with histopathological responses, and a complete metabolic response in FDP-PET always suggest favorable prognosis^[101]. It is still a challenge to distinguish the complete histopathological remission after neoadjuvant therapy by PET/CT. Different histologic subtypes may interfere with the evaluation of PET/CT and the best time to undergo post-treatment PET/CT is still unclear.

PET/CT is a powerful, noninvasive metabolic imaging modality for detecting many human tumors. However, detection of gastric cancer by PET/CT may seem less than ideal, because FDP uptake is strongly related to tumor size and histopathological subtype^[102]. In order to improve the sensitivity and specificity of PET/CT in evaluating gastric cancer, other tracers target more specific biological processes, such as proliferation (¹⁸F-3'-fluoro-3'-deoxy-L-thymidine; ¹⁸F-FLT), tumor hypoxia (¹⁸F-fluoromisonidazol; ¹⁸F-FMISO) and phospholipid metabolism (radioactively labeled choline derivatives)^[103-105]. ¹⁸F-FLT, as a substrate for thymidine kinase 1, is a new PET tracer with the potential ability to accumulate in proliferating tissues and malignant tumors^[106]. A higher accumulation of ¹⁸F-FLT was reported in gastric cancer than in normal gastric mucosa. ¹⁸F-FLT uptake is significantly correlated with gastric cancer differentiation and cellular density^[107]. ¹⁸F-FLT PET was more sensitive than ¹⁸F-FDG PET in imaging gastric cancer, especially in tumors frequently presenting with or without low ¹⁸F-FDG uptake, and may improve early evaluation of response to neoadjuvant treatment^[103]. However, F-FLT PET/CT imaging is not recommended for pre-treatment assessment of metastatic gastric cancer as it does not show significant advantages in evaluating liver and bone metastases, compared with ¹⁸F-FDG PET/CT imaging^[108].

Role of glucose metabolites in gastric cancer screening and diagnosis

Currently, gastroscopy still represents the gold standard for diagnosis of gastric cancer. Despite its uncomfortable and invasive features, gastroscopy is widely used in the surveillance, early screening and follow-up of gastric cancer. Over the past few years, several tumor serum biomarkers have been used as novel non-invasive tools for early diagnosis of gastric cancer. However, due to low specificity and sensitivity, current serum biomarkers such as CEA and carbohydrate antigens are not as effective as other screening methods.

Recently, serum and urine metabolomic studies of gastric cancer based on the use of highly sensitive detection techniques may present a novel opportunity to seek potential new biomarkers for early screening of asymptomatic gastric cancer and its follow-up. Metabolomics is defined as a quantitative description of all endogenous low-molecular-weight components (< 1 kDa) in a biological sample, such as tissue, urine

or plasma, and aims to diagnose various diseases by analyzing the data^[109]. The small molecule endogenous metabolites are mainly composed of the intermediate products produced by the four metabolic cycles, which include the glucose metabolism, lipid metabolism, amino acid metabolism and nucleic acid metabolism, in which glucose metabolism is the core (Figure 1)^[110]. The intermediate metabolites are important in biological systems and are promising candidates for understand disease phenotypes^[111]. Metabolomic studies, comparing the metabolite profiles of cancer cells vs normal cells, offer an opportunity to identify the changes in metabolic pathways, which prompt carcinogenesis. Compared with genomics, transcriptomics and proteomics, metabolomics provides terminal molecular data of the biological system which may be an effective way to elucidate the phenotypic changes associated with cancer. Recently, metabolomic studies have been successfully conducted in gastric cancer^[24-36] and other human cancers^[112-115].

Metabolomics approaches in gastric cancer may be applied largely in three ways. First, specific metabolites responsible for phenotypes associated with cancer-related mutations should be identified. Second, the common metabolites with altered levels in gastric cancer cells compared with normal cells, need to be found. Third, the response of cancer metabolism to environmental changes needs investigation^[28,29]. Advances in the highly sensitive metabolomic methods and data analysis techniques may facilitate such applications in a single study^[116]. Metabolomic profiles may offer a chance to identify the potential biomarkers for early diagnosis, prognosis, drug target identification and treatment response. Recently, several metabolites were suggested as diagnostic and prognostic biomarkers for gastric cancer^[117,118]. Gastric carcinogenesis is a complex phenomenon involving multiple epigenetic and genetic factors including several genetic, environmental and infectious agents causing a cumulative effect in the early stages. The model of gastric carcinogenesis is well known and includes the following sequential stages: chronic atrophic gastritis (CAG), intestinal metaplasia (IM), gastric dysplasia (DYS) and finally gastric cancer (GC). Plasma metabolomic studies, in which fifteen identified metabolites were quantitatively detected, showed unique metabolic profiles in the different stages of GC. The metabolic phenotype of chronic superficial gastritis (CSG) is significantly different from CAG, IM, DYS and GC, whose plots clustered closely. A similar metabolic pattern was shown in IM and GC^[119]. The discriminative metabolites characterizing the different stages of GC may be widely used in gastric cancer screening and early diagnosis combined with endoscopy. As previously mentioned, several serum metabolomic studies in GC suggested significant metabolic differences between cancer and control groups, suggesting potential biomarkers for the early diagnosis of GC.

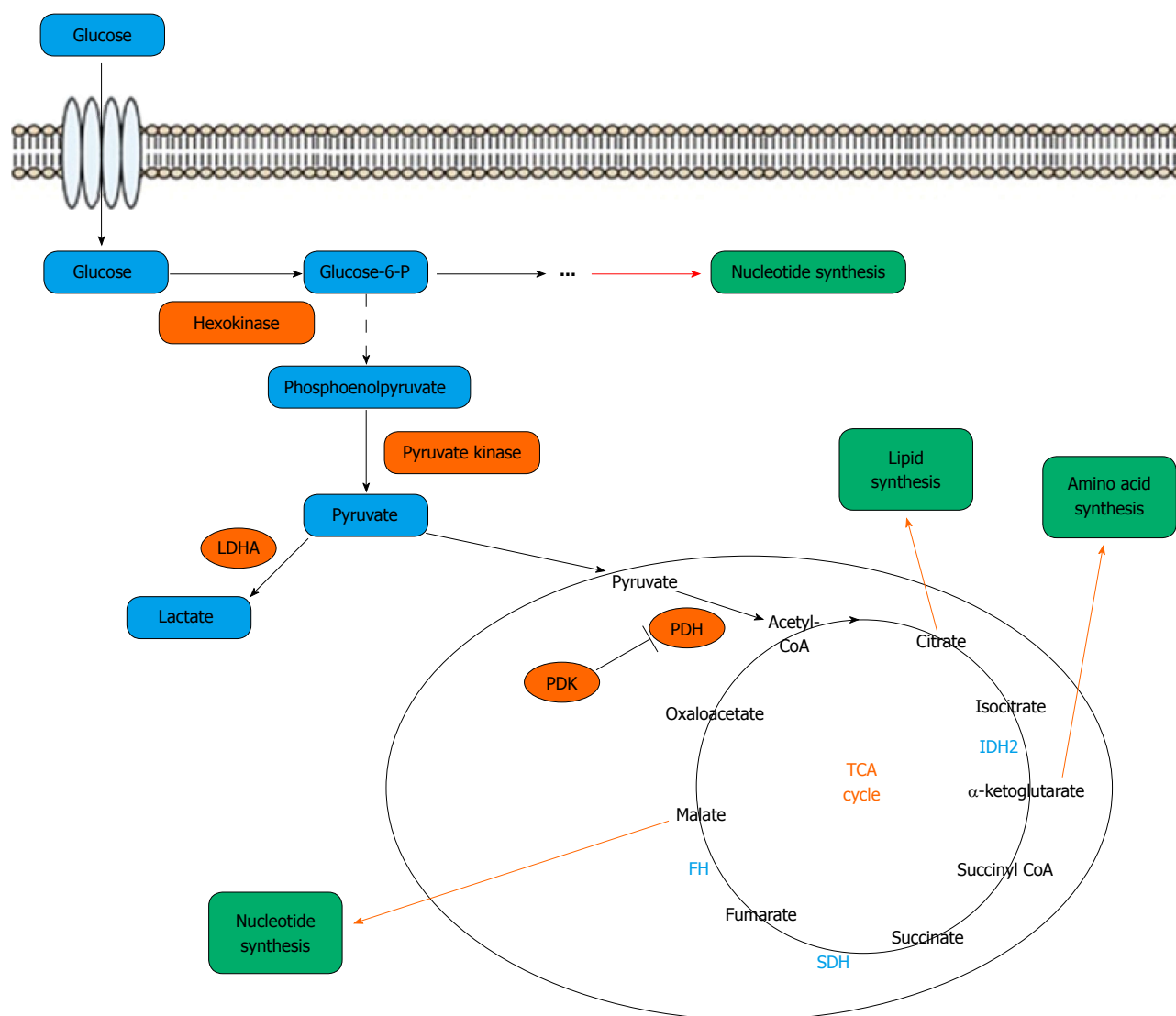


Figure 1 Glucose metabolism in gastric cancer. The figure incorporates the interplay between glucose and the other three metabolic pathways^[110].

Kim *et al.*^[33] studied urinary metabolomic biomarkers of gastric cancer in mouse models and found the presence of significant endogenous metabolic differences between tumor-bearing mice and controls. The study indicated that trimethylamine oxide (TMAO), 3-indoxylsulfate, hippurate, and citrate might serve as useful urinary biomarkers for the detection of gastric cancer in a mouse model. Jung *et al.*^[35] demonstrated that metabolomic changes in amino acid and lipid metabolism of urine samples resembled those in gastric cancer tissue, and were highly accurate in predicting gastric cancer with a much higher sensitivity than carbohydrate antigen 19-9 and CEA. Further, 4-hydroxyphenylacetate, alanine, phenylacetylglutamine, mannitol, glycolate, and arginine levels were significantly related to T stage of gastric cancer. Compared with serum and urine samples, tissue samples require invasive approaches such as endoscopy and surgery, which limits their application in the screening and early diagnosis of gastric cancer. However, identification of new biomarkers

simultaneously in tissue and serum or urine samples is helpful. Tissue metabolic markers in gastric cancer were identified by some studies^[23,24]. Integrated the recent studies on the profiling of glucose metabolites in gastric cancer, lactate and fumarate were recognized as the most commonly biomarkers for gastric cancer screening and diagnosis^[120].

Role of glucose metabolites in gastric cancer prognosis

Recent advances in metabolomics have offered new avenues to predict gastric cancer metastasis. Hur *et al.*^[28] analyzed the levels of Krebs cycle components in gastric cancer tissues and found that the levels of pyruvic acid, lactic acid and ketone bodies were associated with histopathology. In particular, the levels of ketone bodies were significantly higher in cancer tissues with differentiated tumors than in undifferentiated tumors. Wu *et al.*^[25] found that the levels of phenanthrenol and butanoic acid were significantly decreased in invasive cancers (T3/T4) compared with non-invasive cancers (T1/T2). Compared with

glucose metabolites, amino acid and lipid metabolites seem to be more potential in predicting gastric cancer prognosis. Using animal models of human gastric cancer, Chen *et al.*^[121] demonstrated that proline and serine metabolism play an important role in metastasis, and may be used in predicting gastric cancer metastasis and progression. Further, Hu *et al.*^[34] investigated urinary metabolite profiling to identify possible biomarkers in gastric cancer metastasis. They found that the levels of alanine, glycerol and L-proline were lower and the level of myo-inositol was higher in the metastasis group. These urinary metabolites may be a potential prognostic biomarker for gastric cancer metastasis.

Role of glucose metabolism in gastric cancer therapy

Predicting drug response: Currently, chemotherapy has become a first-line therapy for advanced gastric cancer. However, chemotherapy is not the gold standard due to its lower effectiveness compared with its role in colorectal and breast cancers. Chemoresistance is a major challenge. Recent studies suggested that metabolomics may play an important role in investigating cellular responses to chemotherapy of cancer^[122-126]. Morvan *et al.*^[124] investigated metabolic changes associated with tumor response to chloroethyl nitrosourea (CENU), an anticancer agent, in two tumor models *in vivo* and observed the activation of metabolic pathways of DNA repair and adaptation to treatment. Metabolomics of tumor response to anticancer agents may enable identification of metabolic pathways of drug efficacy and evaluate the effectiveness of treatment. Wang *et al.*^[125] assessed metabolomic prediction of chemosensitivity in a human xenograft model of gastric cancer and determined that a series of endogenous metabolites, including 1-acyl-lysophosphatidylcholines, polyunsaturated fatty acids and their derivatives, were predictive of chemosensitivity in gastric cancer. Sasada *et al.*^[126] conducted a similar metabolomic analysis to investigate the intracellular response of human gastric cancer cells to 5-fluorouracil (5-FU), and showed a dramatic alteration in the number of metabolites, especially proline, glutamate and proline dehydrogenase (PRODH), in non-5-FU-resistant cancer cells during short-term treatment with 5-FU. However, the proline and glutamate levels and PRODH mRNA expression were less affected in 5-FU-resistant cancer cells. In the future, metabolic biomarkers may play an important role in evaluating treatment response to anticancer drugs.

Targeting glucose metabolism in gastric cancer therapy

Enhanced glucose metabolism *via* aerobic glycolysis followed by lactic acid fermentation plays a predominant role in rapid energy (ATP) synthesis. However, generation of large number of metabolites contributes to an acidic micro-environment conducive to cancer proliferation. Therapeutically targeting cancer cell

metabolism such as glucose metabolism is more convenient and associated with fewer side effects compared with the other biologic systems since cellular metabolic pathways represent the terminus of systems biology and control the other systems genetically.

Ketogenic diets consist of high fat, with moderate-to-low protein content, and very low carbohydrates. It reduced tumor growth and improved survival in a mouse model of malignant gastric cancer^[127]. Furthermore, ketogenic diets have been suggested to increase the effects of radiochemotherapy in non-small cell lung cancer xenograft models^[128]. Recent studies revealed that ketogenic diets act as adjuvant cancer therapy *via* mechanisms that increased oxidative stress and inhibited glucose metabolism *via* lipid metabolism.

Metformin, a first-line anti-diabetic drug, inhibited proliferation and induced apoptosis in cancer cells. Metformin decreased mitochondrial respiration chain activity and ATP production and induced the activation of LKB1-AMPK, causing the inhibition of Raptor-mTOR complex^[129,130]. High glucose concentrations reduced the effectiveness of metformin on cancer cell proliferation and failure to maintain glucose homeostasis may promote aggressive breast cancer phenotype^[129].

Tanshinone II A, a diterpene quinone extracted from the plant Danshen, has been recently reported as an effective adjunctive reagent in the treatment of gastric cancer^[131]. Lin *et al.*^[132] confirmed that TIIA treatment inhibited cell growth and the proliferation of gastric cancer by suppressing glucose metabolism in cancer cells. Another study conducted by Bhattacharya *et al.*^[133] revealed that hypoglycemia and enhanced glycolysis increased resistance to chemotherapy in gastric cancer.

Another very promising strategy is to design gastric cancer-specific and even individualized inhibitors of all the steps of the glycolytic pathway *via* metabolomics studies. Granchi *et al.*^[134] reviewed recent advances of new bioactive molecules which disturb cancer glycolysis. Different kinds of small molecules that inhibit all the steps of the glycolytic pathway have been identified, such as hexokinase II (HK II), 2-deoxy-D-glucose and 3-bromopyruvate (3-BrPA), which were accepted as cancer therapeutic targets in several studies. Similar potential and promising therapy for gastric cancer was described by Ngo *et al.*^[135] (Figure 2). Hexokinase II is a key factor catalyzing the first step of glycolysis, which consists of the transfer of glucose to glucose-6-phosphate^[136]. HKII is accepted as a very potential and attractive anticancer target. 2-deoxy-D-glucose, a glucose analogue, binds and inhibits HK II, resulting in cellular ATP depletion, cell cycle suppression and cell death^[137]. 3-BrPA, an alkylating agent and glycolysis inhibitor and designated as an orphan drug by FDA for liver cancer, has been identified to hinder glucose metabolism by inhibiting HK II^[138]. In addition, 3-BrPA inactivated the

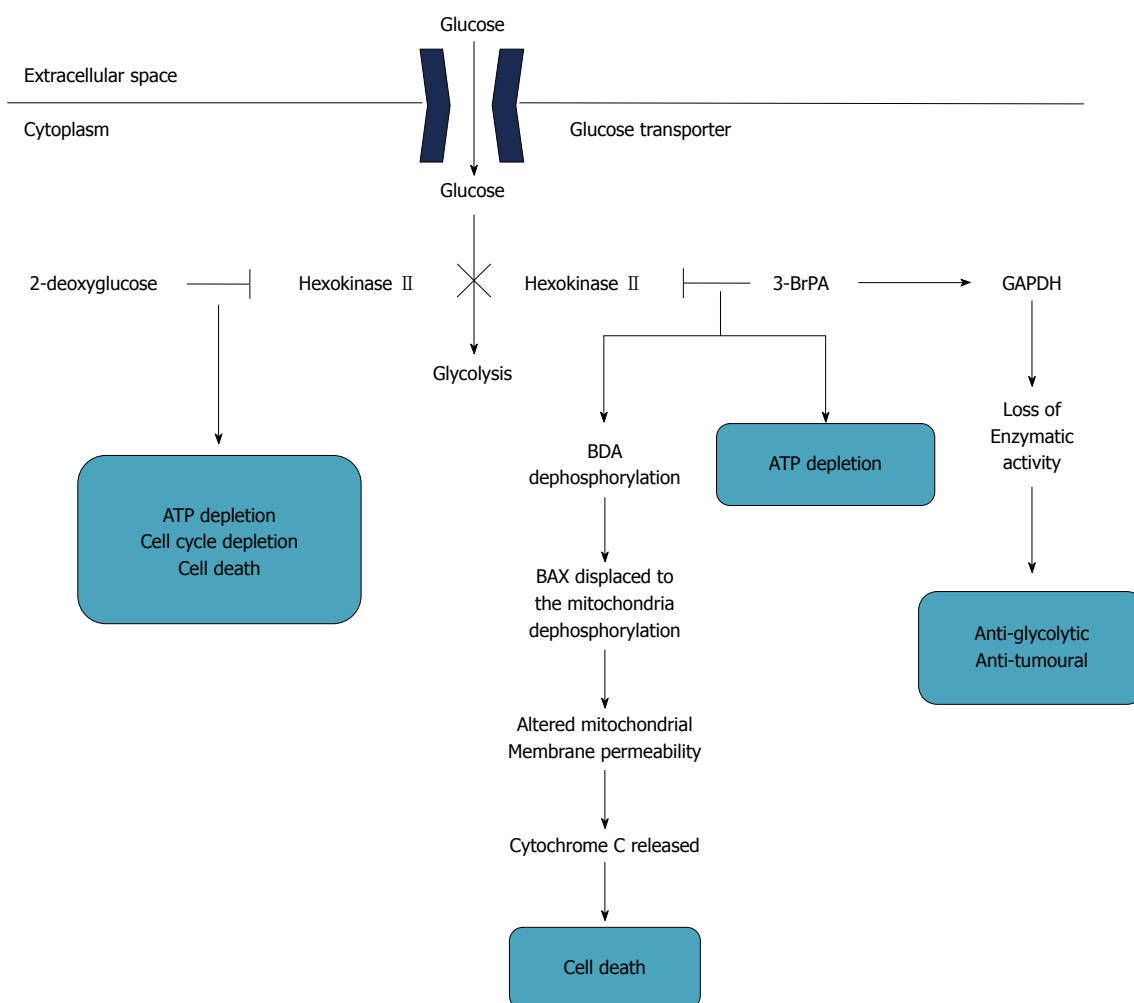


Figure 2 Targeting hexokinase II as a potential mechanism to inhibit the Warburg effect in cancer cells^[141].

glyceraldehydes-3-phosphate dehydrogenase (GAPDH) by GAPDH pyruvylation, leading to anti-glycolytic and antitumoral effects and mediating cancer cell death^[139].

Generally, most of the current glycolytic inhibitors showed only moderate efficacy when used as single agents, but in some cases demonstrated high potential when combined with other therapies. Currently, few therapeutic studies target gastric cancer metabolism. It is anticipated that further research will investigate the role of cancer-specific glycolytic inhibitors to develop effective therapeutic regimens for gastric cancer.

CONCLUSION

Altered glucose metabolism is a hallmark of gastric cancer, which provides new insights into gastric carcinogenesis and identification of biomarkers targeting specific metabolic aspects of gastric cancer. However, several hurdles remain before altered glucose metabolism is clinically used in the diagnosis and treatment of gastric cancer. The use of ¹⁸F-FDG PET is an exception. However, a major clinical application of metabolomics requires creation of spectral databases of metabolites of the normal population, similar to the

cancer genomic and proteomic databases. Further, the metabolites consist of a large group of small-molecule intermediates, which are too large for analysis using currently available technology. Another challenge is related to identification of cancer-specific biomarkers for gastric cancer, since metabolites may be considered as potential biomarkers for a range of cancers. It is anticipated that further research will focus on these aspects and promote the clinical application of glucose metabolism in gastric cancer in the not-too-distant future.

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Basic Study

***hsa-miR-29c* and *hsa-miR-135b* differential expression as potential biomarker of gastric carcinogenesis**

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Abstract

AIM: To investigate the expression profiles of *hsa-miR-29c* and *hsa-miR-135b* in gastric mucosal samples and their values as gastric carcinogenesis biomarkers.

METHODS: The expression levels of *hsa-miR-29c* and *hsa-miR-135b* in normal gastric mucosa, non-atrophic chronic gastritis, intestinal metaplasia and intestinal-type gastric adenocarcinoma were analysed using quantitative real-time PCR. The difference between *hsa-miR-29c* and *hsa-miR-135b* expression profiles in the grouped samples was evaluated by ANOVA and Student's *t*-test tests. The results were adjusted for multiple testing by using Bonferroni's correction. *P* values ≤ 0.05 were considered statistically significant. To evaluate *hsa-miR-29c* and *hsa-miR-135b* expressions as potential biomarkers of gastric carcinogenesis, we performed a receiver operating characteristic curve analysis and the derived area under the curve, and a Categorical Principal Components Analysis. *In silico* identification of the genetic targets of *hsa-miR-29c* and *hsa-miR-135b* was performed using different prediction tools, in order to identify possible genes involved in gastric carcinogenesis.

RESULTS: The expression levels of *hsa-miR-29c* were higher in normal gastric mucosal samples, and decreased progressively in non-atrophic chronic gastritis samples, intestinal metaplasia samples and intestinal-type gastric adenocarcinoma samples. The expression of *hsa-miR-29c* in the gastric lesions showed that non-atrophic gastritis have an intermediate profile to gastric normal mucosa and intestinal-type gastric adenocarcinoma, and that intestinal metaplasia samples presented an expression pattern similar to that in intestinal-type gastric adenocarcinoma. This microRNA (miRNA) has a good discriminatory accuracy between normal gastric samples and (1) intestinal-type gastric adenocarcinoma; and (2) intestinal metaplasia, and regulates the *DMNT3A* oncogene. *hsa-miR-135b* is up-regulated in non-atrophic chronic gastritis and intestinal metaplasia samples and down-regulated in normal gastric mucosa and intestinal-type gastric adenocarcinoma samples. Non-atrophic chronic gastritis and intestinal metaplasia are significantly different from normal gastric mucosa samples. *hsa-miR-135b* expression presented a greater discriminatory accuracy between normal samples and gastric lesions. This miRNA was associated with *Helicobacter pylori* presence in non-atrophic chronic gastritis samples and regulates the *APC* and *KLF4* tumour suppressor genes.

CONCLUSION: Our results provide evidence of epigenetic alterations in non-atrophic chronic gastritis and intestinal metaplasia and suggest that *hsa-miR-29c* and *hsa-miR-135b* are promising biomarkers of gastric carcinogenesis.

Key words: Gastric cancer; Gastric lesions; MicroRNA; Biomarker; Carcinogenesis

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Core tip: The miRNAs *hsa-miR-29c* and *hsa-miR-135b* were reported as potential biomarkers of intestinal-type gastric adenocarcinoma. We evaluated and compared the expression profile of these miRNAs in gastric mucosal samples, including normal gastric mucosa, non-atrophic chronic gastritis, intestinal metaplasia and intestinal-type gastric adenocarcinoma. Our results provided evidence of epigenetic alterations in non-atrophic chronic gastritis and intestinal metaplasia and suggest that *hsa-miR-29c* and *hsa-miR-135b* are promising biomarkers of gastric carcinogenesis.

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INTRODUCTION

Since the middle of the last century, the histological classification of adenocarcinomas has been largely based on the criteria proposed by Lauren^[1]. According to this classification, there are three gastric adenocarcinoma types: intestinal, diffuse and undifferentiated, which is also classified as indeterminate^[1]. Intestinal-type and diffuse-type gastric adenocarcinomas have their own characteristics and specific risk factors^[2].

In 1988, Pelayo Correa proposed a paradigm for intestinal-type gastric adenocarcinoma carcinogenesis, which became known as the Correa cascade. According to this cascade, a subset of patients who develop intestinal-type gastric adenocarcinomas undergo a multi-stage and complex process of carcinogenesis, initiated by (1) chronic superficial gastritis, also called non-atrophic chronic gastritis; followed by (2) chronic atrophic gastritis; then (3) intestinal metaplasia; and finally (4) dysplasia^[3,4].

Helicobacter pylori (*H. pylori*) infection is the major risk factor among all the main risk factors involved in chronic gastritis, intestinal metaplasia, dysplasia and intestinal-type gastric adenocarcinoma. For this reason, in 1994, this bacterium was classified as a type 1 carcinogen by the World Health Organization^[5].

Despite the fact that the different inflammatory stages of the Correa cascade are pathologically well defined, the molecular signatures of these stages have not been well explored and the mechanisms that lead to carcinogenic progression are still unknown^[6].

The pre-cancerous or pre-malignant lesions are defined as those that precede invasive cancers which many of the molecular changes and phenotypic

characteristics of invasive cancer are present but not fully expressed^[7]. Thus, it is assumed that the changes found in intestinal-type gastric adenocarcinomas may also be present in different stages of gastric lesions, such as chronic gastritis, intestinal metaplasia and dysplasia.

Several studies have shown that gastric cancer is a complex disease involving changes in oncogenes, tumour suppressor genes, DNA repair regulatory genes, cell cycle and cell adhesion, as well as numerous epigenetic changes^[8]. A class of small non-coding RNAs, called microRNAs (miRNAs), have emerged as key agents in these epigenetic changes^[9].

MiRNAs are short (approximately 22 nucleotides in length), endogenous, noncoding RNAs that regulate the expression of target mRNAs at a post-transcriptional level^[10]. Based on the results obtained in studies by Ribeiro-dos-Santos *et al.*^[11], Moreira *et al.*^[12], Gomes *et al.*^[13] and Darnet *et al.*^[14], *hsa-miR-29c* and *hsa-miR-135b* were reported as potential biomarkers of intestinal-type gastric adenocarcinoma. However, more studies are needed to confirm and validate *hsa-miR-29c* and *hsa-miR-135b* as potential biomarkers.

The objective of this study was to investigate the expression profiles of *hsa-miR-29c* and *hsa-miR-135b* in gastric mucosal samples, including normal gastric mucosa, non-atrophic chronic gastritis, intestinal metaplasia and intestinal-type gastric adenocarcinoma, and their values as gastric carcinogenesis biomarkers. Additionally, *in silico* prediction was performed to identify potential driver genes involved in the carcinogenic mechanism^[15] regulated by these miRNAs.

MATERIALS AND METHODS

Patient tissues

This study comprised randomly selected frozen tissue samples of normal gastric mucosa ($n = 20$), FFPE samples of non-atrophic chronic gastritis ($n = 20$) and of intestinal metaplasia ($n = 10$) from patients undergoing endoscopic gastric biopsy samples, and gastric intestinal adenocarcinoma frozen tissue samples obtained from patients undergoing gastrectomies ($n = 14$). All cases investigated in this study were reviewed and confirmed by a pathologist.

Histological processing was performed using glass slide-mounted 3 μ m-thick rotary microtome slices (Leica 2125RT). These preparations were deparaffinised, stained with haematoxylin-eosin (HE) and analysed by light microscopy. After histological processing, manual microdissection was performed to increase the accuracy of histopathological characterisation.

Non-atrophic chronic gastritis samples were defined by the presence of lymphocytes and plasmocytes in the lamina propria. The presence or absence of neutrophils permeating the glandular and the surface epithelia, and the presence or absence of lymphoid follicles were also evaluated^[16].

Samples of intestinal metaplasia were histopathologically diagnosed by the replacement of the surface and glandular gastric columnar epithelial cells by metaplastic cells of intestinal morphology, such as absorptive and goblet cells.

Fresh tissue samples were immediately stored in *RNAlater Solution* (Ambion) at -80°C until total RNA extraction. Only samples with a pure tumour area occupying at least 80% of the slide were used. Pathological TNM staging was evaluated according to the 2010 criteria of The American Joint Committee on Cancer.

The histological sections of gastric mucosal biopsy were stained with HE and cresyl fast violet to perform the *H. pylori* detection.

Samples were obtained from the Hospital Universitário João de Barros Barreto - Federal University of Pará (Belém, Pará, Brazil) and from the Hospital São Camilo e São Luís (Macapá, Amapá, Brazil). Informed consent was obtained from all individual participants included and the study protocol was approved by the Local Ethics Committee (Protocol number: 657 666) in accordance with the Helsinki Declaration of 1964.

Total RNA isolation and quantification

The total RNA was extracted using the High Pure Kit miRNA Isolation Kit (Roche Diagnostics) according to manufacturer's protocol, and stored at -80°C to avoid degradation. The total RNA concentrations were determined by the Qubit® 2.0 Fluorometer (Life Technologies) using the Qubit RNA HS Assay kit (Life Technologies). The samples were diluted to the final concentration of 4 ng/ μL .

Quantitative real-time polymerase chain reaction

Assays for measuring the miRNAs expression were performed using TaqMan MicroRNA Assays (Applied Biosystems) according to the manufacturer's instructions. Initially, 10 ng of total RNA was subjected to reverse transcription polymerase chain reaction using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems) according to manufacturer's protocol. The thermocycling conditions were: 30 min at 16°C , followed by 30 min at 42°C , 5 min at 85°C and 5 min at 4°C .

The quantitative real-time polymerase chain reaction (qRT-PCR) was performed using TaqMan Universal PCR Master Mix Kit (Applied Biosystems) according to the manufacturer's protocol and the equipment 7500 Real-Time PCR System (Applied Biosystems). The reactions were performed in triplicate and incubated in optical 96-well reaction plates. The thermocycling conditions were: 95°C for 10 min, and 40 cycles of 15 s at 95°C , followed by 1 min at 60°C .

After finalization of the qRT-PCR experiments, the average values of the cycle threshold (Ct) of the reactions in triplicate were determined. The comparative Ct method was adopted, and Z30 was used as an

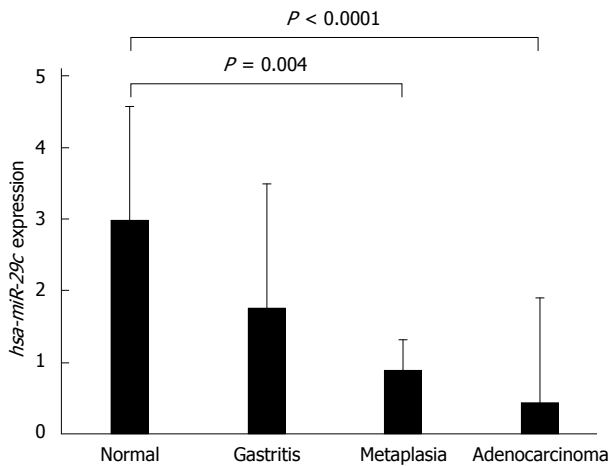


Figure 1 Expression values of *hsa-miR-29c* in samples of normal gastric mucosa, non-atrophic chronic gastritis, intestinal metaplasia and intestinal-type gastric adenocarcinoma, respectively (values in log scale). *P* values were obtained by ANOVA test (adjusted by using Bonferroni's correction).

endogenous control. The relative amount of miRNA expression was normalized by the average values of Ct_{30} and calculated by the equation $2^{-\Delta Ct}$, where $\Delta Ct = Ct_{miRNA} - Ct_{30}$.

In silico prediction of *hsa-miR-29c* and *hsa-miR-135b* target genes

In order to identify genes that may be involved in gastric carcinogenesis process, we compared the driver genes ranked by Vogelstein *et al.*^[15] with the target genes of *hsa-miR-29c* and *hsa-miR-135b*.

In silico identification of the target genes was performed using miRecords (<http://mirecords.biolead.org>) (which integrates 11 prediction tools), TargetCompare (<http://54.187.40.156:8080/targetcompare/>), miRTarBase (<http://mirtarbase.mbc.ntu.edu.tw>), MicroCosm/miRBase (<http://ebi.ac.uk>), miRDB (<http://mirdb.org>), miRo (<http://ferrolab.dmi.unict.it>) and miRNAmap (<http://mirnamap.mbc.ntu.edu.tw>). We considered target genes the ones that were observed in no less than 10 tools.

We also used the miRTarBase database to check which miRNA target genes have already been validated experimentally. The mRNA sequences of target genes were obtained from NCBI.

Statistical analysis

The pattern of distribution of the data was determined by the Shapiro-Wilk test. The difference between *hsa-miR-29c* and *hsa-miR-135b* expression profiles in the grouped samples was evaluated by ANOVA and Student's *t*-test tests. The results were adjusted for multiple testing by using Bonferroni's correction. *P* values ≤ 0.05 were considered statistically significant.

To evaluate *hsa-miR-29c* and *hsa-miR-135b* as potential biomarkers of gastric carcinogenesis, we performed a receiver operating characteristic (ROC)

curve analysis and the derived area under the curve (AUC), and a Categorical Principal Components Analysis (CATPCA). Statistical tests and graphics were performed using IBM SPSS Statistics software (version 20), GraphPad Prism (GraphPad Software), MATLAB® 8.3 (Release 2014a) and RStudio (version 0.98.1103).

RESULTS

Expression levels of *hsa-miR-29c* and *hsa-miR-135b*

To determine and compare the expression levels of *hsa-miR-29c* and *hsa-miR-135b* in gastric mucosal samples, we performed qRT-PCR.

We used the ANOVA test to compare the miRNAs expression levels between normal gastric mucosa, non-atrophic chronic gastritis, intestinal metaplasia and intestinal-type gastric adenocarcinoma samples. The results were adjusted for multiple testing by using Bonferroni's correction.

The expression levels of *hsa-miR-29c* were higher in normal gastric mucosa samples, and decreased progressively in non-atrophic chronic gastritis, intestinal metaplasia and intestinal-type gastric adenocarcinoma samples (Figure 1). The decrease in the expression of *hsa-miR-29c* as the Correa cascade stages progress is molecular evidence for the Correa cascade pathogenesis.

ANOVA test showed that non-atrophic chronic gastritis samples have an intermediate *hsa-miR-29c* profile to gastric normal mucosa and intestinal-type gastric adenocarcinoma. Furthermore, intestinal metaplasia *hsa-miR-29c* expression is significantly different to its in normal gastric mucosa samples (*P* = 0.004) but is similar to its in intestinal-type gastric adenocarcinoma. There is no difference between non-atrophic chronic gastritis and intestinal metaplasia expression profiles (Figure 1).

The expression levels of *hsa-miR-135b* showed higher values in the non-atrophic chronic gastritis and the intestinal metaplasia samples and lower values in the normal gastric mucosa and intestinal-type gastric adenocarcinoma. ANOVA test showed non-atrophic chronic gastritis (*P* < 0.0001) and the intestinal metaplasia (*P* = 0.003) are different to normal gastric mucosa, but similar to each other. Non-atrophic chronic gastritis showed a significant difference in expression of *hsa-miR-135b* in comparison to intestinal-type gastric adenocarcinoma (*P* = 0.001) (Figure 2).

Expression levels of *hsa-miR-29c* and *hsa-miR-135b* and its relationship to *H. pylori* infection

To determine whether the presence of *H. pylori* affects the expression of *hsa-miR-29c* and *hsa-miR-135b*, a Student's *t*-test was used to compare the *H. pylori*-positive and *H. pylori*-negative non-atrophic chronic gastritis samples. No significant difference was found in the expression of *hsa-miR-29c* (*P* = 0.0939) between those groups, however, for *hsa-miR-135b*, there was a significant difference (*P* = 0.011). Samples of *H.*

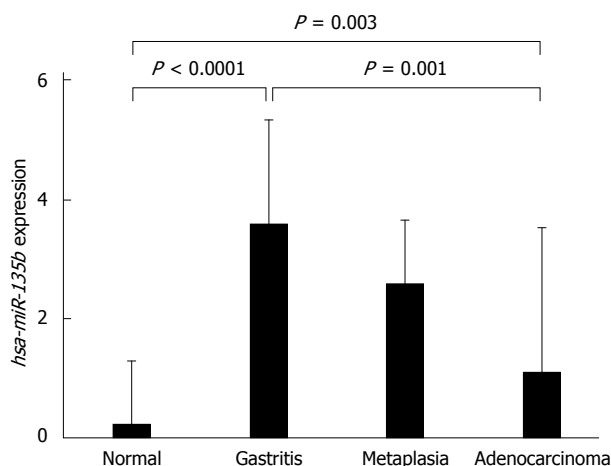


Figure 2 Expression values of *hsa-miR-135b* in samples of normal gastric mucosa, non-atrophic chronic gastritis, intestinal metaplasia and intestinal-type gastric adenocarcinoma, respectively (values in log scale). *P* values were obtained by ANOVA test (adjusted by using Bonferroni's correction).

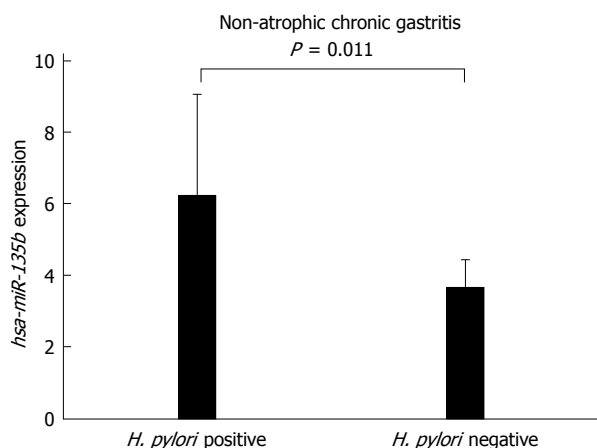


Figure 3 Comparison of the expression values of *hsa-miR-135b* between samples of *Helicobacter pylori*-positive non-atrophic chronic gastritis and *Helicobacter pylori*-negative non-atrophic chronic gastritis ($P = 0.011$). *H. pylori*: *Helicobacter pylori*.

pylori-positive non-atrophic chronic gastritis have a higher expression of *hsa-miR-135b* than the *H. pylori*-negative non-atrophic chronic gastritis samples (Figure 3), indicating that this miRNA may be involved in the immune response modulation in association with *H. pylori* infection.

Evaluation of *hsa-miR-29c* and *hsa-miR-135b* as potential biomarkers

To evaluate *hsa-miR-29c* and *hsa-miR-135b* expression as potential biomarker of gastric carcinogenesis, ROC curve analysis and the discriminatory accuracy by AUC values were performed. As shown in Figure 4, *hsa-miR-29c* expression presented a greater discriminatory accuracy between normal gastric samples and (1) intestinal-type gastric adenocarcinoma (Figure 4A); and (2) intestinal metaplasia (Figure 4C). Otherwise,

hsa-miR-135b expression presented a greater discriminatory accuracy between normal samples and gastric lesions (Figure 4G-I).

To provide a global view of *hsa-miR-29c* and *hsa-miR-135b* expression in all samples groups studied simultaneously, CATPCA analysis was performed. The CATPCA analysis of *hsa-miR-29c* expression resulted in two dimensions (first component: Cronbach's alpha = 0.778 and eigenvalue = 2.401; second component: Cronbach's alpha = 0.422 and eigenvalue = 1.463). According to the angles between the vectors (Figure 5), the *hsa-miR-29c* expression was able to distinguish each group of samples. It was not possible to construct a three-dimensional graphic due to the negative value of the third component's Cronbach's alpha.

The CATPCA analysis for *hsa-miR-135b* expression resulted in three dimensions (first component: Cronbach's alpha = 0.938 and eigenvalue = 8.051; second component: Cronbach's alpha = 0.829 and eigenvalue = 4.424; third component: Cronbach's alpha = 0.81 and eigenvalue = 4.101). Figure 6 shows the same CATPCA in a three-dimensional space in two different angles (Figure 6). According to the angles between the vectors, *hsa-miR-135b* expression was able to distinguish the gastric normal mucosa samples to *H. pylori*-positive non-atrophic chronic gastritis and intestinal metaplasia samples.

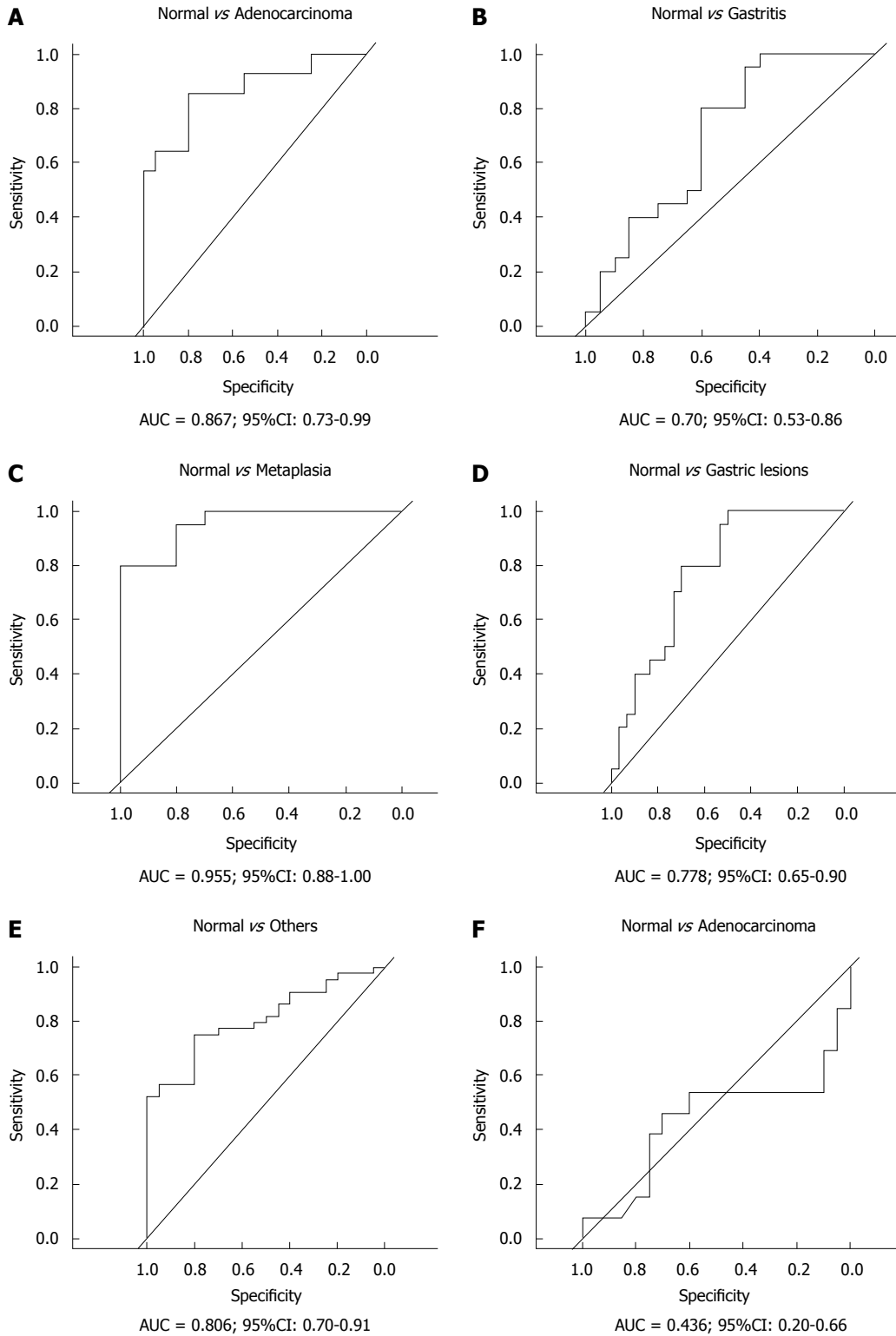
In silico prediction of *hsa-miR-29c* and *hsa-miR-135b* target genes

To predict the target genes of *hsa-miR-29c* and *hsa-miR-135b* and identify possible genes involved in gastric carcinogenesis, we used 17 different tools and compared the results with the driver genes list ranked by Vogelstein *et al.*^[15]. The *DNMT3A* driver gene is a validated target of *hsa-miR-29c*, and the *APC* and *KLF4* driver genes are validated targets of *hsa-miR-135b* (Table 1).

DISCUSSION

This study compared the expression levels of two miRNA candidates for gastric cancer biomarkers (*hsa-miR-29c* and *hsa-miR-135b*) between normal gastric mucosa, gastric lesions (non-atrophic chronic gastritis and intestinal metaplasia) and intestinal-type gastric adenocarcinoma.

The results showed a progressive down-regulation of *hsa-miR-29c* in normal gastric mucosa, non-atrophic chronic gastritis, intestinal metaplasia and intestinal-type gastric adenocarcinoma, providing evidence for the pathogenesis of the Correa cascade. The expression of this miRNA in the gastric lesions showed that non-atrophic gastritis have a intermediate profile to gastric normal mucosa and intestinal-type gastric adenocarcinoma samples, and that intestinal metaplasia samples presented an expression pattern similar to that in intestinal-type gastric



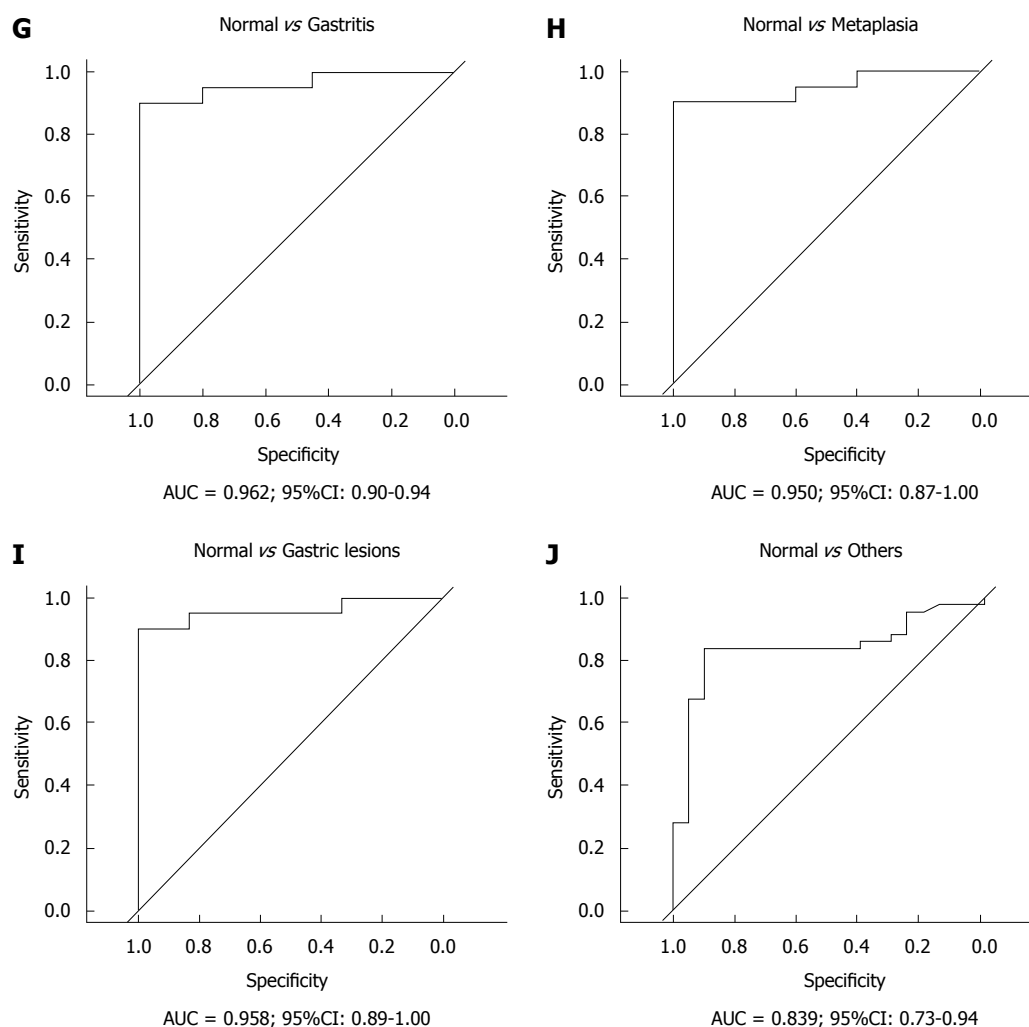


Figure 4 Receiver operating characteristic curve analysis of *hsa-miR-29c* (A-E) and *hsa-miR-135b* (F-J) expression. Gastric lesions: Non-atrophic chronic gastritis and intestinal metaplasia; Others: Non-atrophic chronic gastritis, intestinal metaplasia and intestinal-type gastric adenocarcinoma; AUC: Area under the curve.

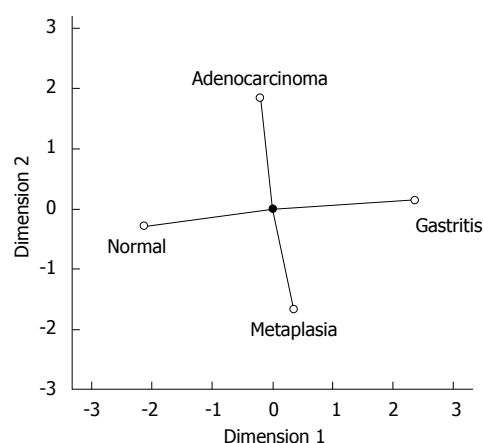


Figure 5 Categorical principal components analysis of *hsa-miR-29c* expression in two dimensions. Vectors making 180-degree indicate they are closely and negatively related. Vectors making a 90-degree angle indicate they are not related.

adenocarcinoma.

Furthermore, we observed a significantly difference between *hsa-miR-29c* profiles in normal gastric

mucosa and intestinal-type gastric adenocarcinoma. Different studies analysed the expression of *hsa-miR-29c* in gastric cancer and found results consistent with those of this investigation^[17-23].

The down-regulation of *hsa-miR-29c* has been reported in several human malignancies, including nasopharyngeal carcinoma^[24], bladder carcinoma cells^[25], lung cancer^[26], esophageal cancer^[27,28], chronic lymphocytic leukaemia^[29,30] and melanoma^[31].

Considering the expression profile of *hsa-miR-29c* in gastric cancer, it is suggested that *hsa-miR-29c* acts as a TS-miR. Therefore, down-regulation of this miRNA can lead to overexpression of oncogenes, such as *DNMT3A*.

The *DNMT3A* gene encodes the DNA methyltransferase 3A, an enzyme responsible for the dynamics of DNA methylation during embryogenesis and pathogenesis. The de-regulation of DNA methylation patterns can shut-down or cripple the normal transcriptional activity and is considered an early event in tumour development^[32]. This gene was validated to be a target of *hsa-miR-29c* in three different papers^[32-34].

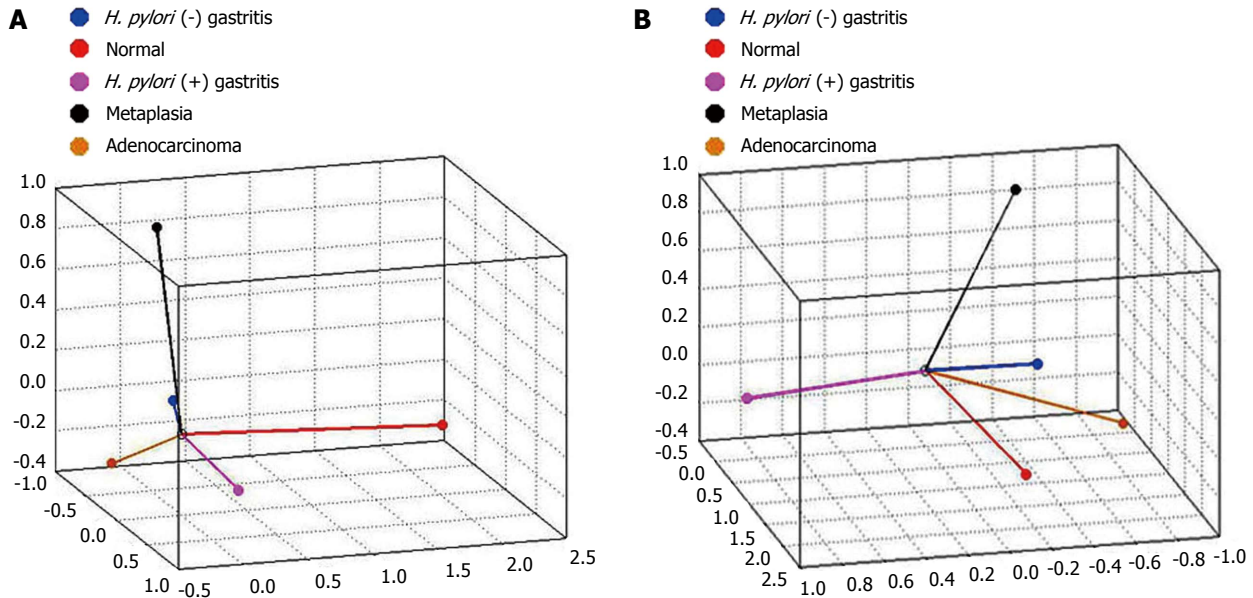


Figure 6 Categorical principal components analysis of *hsa-miR-135b* expression in three dimensions in two different angles (A and B). Vectors making 180-degree indicate they are closely and negatively related. Vectors making a 90-degree angle indicate they are not related. *H. pylori*: *Helicobacter pylori*.

Table 1 Validated target genes of *hsa-miR-29c* and *hsa-miR-135b* according to the miRTarBase database

| MicroRNA | Target genes | Ref. Seq. RNA | Validation methods | | | | | No. of papers |
|---------------------|---------------|---------------|--------------------|--------------|------|------------|-------|---------------|
| | | | Reporter assay | Western blot | qPCR | Microarray | Other | |
| <i>hsa-miR-29c</i> | <i>DNMT3A</i> | NM_022552 | × | × | × | × | × | 3 |
| | | NM_153759 | | | | | | |
| | | NM_175629 | | | | | | |
| | | NM_175630 | | | | | | |
| <i>hsa-miR-135b</i> | <i>APC</i> | NM_000038 | × | | × | | × | 1 |
| | | NM_001127510 | | | | | | |
| | | NM_001127511 | | | | | | |
| | <i>KLF4</i> | NM_004235 | × | | × | × | × | 1 |

DNMT3A: DNA methyltransferase 3 alpha; APC: adenomatous polyposis coli; KLF4: Kruppel-like factor 4 (gut); Ref.Seq.RNA: Reference Sequence of messenger RNA; qPCR: Quantitative real-time polymerase chain reaction.

According to these papers, in normal conditions, highly expressed *hsa-miR-29c* may control *DNMT3A* through a conserved function. Therefore, expression of this miRNA in tumour cells can lead to reduced global DNA methylation, restoring expression of tumour suppressor genes and inhibiting tumourigenicity both *in vivo* and *in vitro*^[32-34].

Indeed, overexpression of *DNMT3A* was reported not only in gastric cancer itself but also in gastric lesions^[35-37]. Hyper-methylation of the *hMLH1*, *P16*, *DAP-kinase*, *THBS1* and *TIMP-3* genes was detected in chronic gastritis and intestinal metaplasia samples^[38].

It is possible that *hsa-miR-29c* inhibits tumourigenicity both *in vivo* and *in vitro*^[32] by restoring expression of tumour suppression genes involved in the control of cell proliferation. Indeed, Matsuo *et al.*^[18] demonstrated that *hsa-miR-29c* is involved in regulating the S phase of the cell cycle in gastric cancer. Furthermore, Wang *et al.*^[23] find that *hsa-miR-29c* act as metastasis suppressor in gastric

cancer. These findings suggested that this miRNA not only functioned as TS-miR in gastric cancer but also might serve as effective predictors for gastric cancer prevention^[23].

Up-regulated expression of *hsa-miR-135b* was observed in gastric lesions compared to normal gastric mucosa and intestinal-type gastric adenocarcinoma samples. Non-atrophic chronic gastritis and intestinal metaplasia are significantly different from normal gastric mucosa samples.

To date, the expression of *hsa-miR-135b* has been analysed in gastric cancer in a few studies^[17,21]. This miRNA has been most extensively studied in other types of human cancer, such as colon^[39], breast^[40], cutaneous squamous cell carcinoma^[41] and lung cancer^[42,43]. In all of these cases, its overexpression points to the hypothesis that this miRNA acts as oncomiR in the process of carcinogenesis.

In silico analysis showed that *hsa-miR-135b* has two validated target genes, *KLF4*^[44] and *APC*^[45], which

are both tumour suppressor genes.

The *KLF4* gene encodes a zinc-finger transcription factor, which is involved in mediating pro-inflammatory responses and regulating cell proliferation and differentiation^[46,47]. Down-regulation of this gene is reported in gastric cancer, indicating its participation in the regulation of homeostasis and maintenance of the gastric mucosa. In addition, restoration of *KLF4* expression was able to inhibit tumour growth *in vivo* and *in vitro* by inducing apoptosis in gastric cancer cells. Thus, altering *KLF4* expression plays a critical role in gastric cancer development and progression^[48].

The *APC* gene encodes a protein that binds to the transcription factor β -catenin and results in degradation of β -catenin. The loss of function of this gene causes the nuclear accumulation of APC-free β -catenin, which stimulates the Wnt signalling pathway and leads to de-regulated cell growth and adhesion^[45]. Mutations in *APC* have been identified in patients with gastric adenocarcinoma, especially in those with the intestinal type^[49-52]. These results suggest that the loss of *APC* expression plays an important role during gastric carcinogenesis.

Several studies have suggested that miRNAs represent a bridge between chronic gastritis and gastric cancer development^[53-56]. This study showed that *hsa-miR-29c* expression has a good discriminatory accuracy between normal gastric samples and (1) intestinal-type gastric adenocarcinoma; and (2) intestinal metaplasia; *hsa-miR-135b* expression presented a greater discriminatory accuracy between normal samples and gastric lesions.

In conclusion, our results suggest that *hsa-miR-29c* and *hsa-miR-135b* are promising biomarkers of gastric carcinogenesis and provide evidence of epigenetic alterations in non-atrophic chronic gastritis and intestinal metaplasia, indicating that better understanding of these gastric lesions is required for the prevention of gastric cancer.

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COMMENTS

Background

The molecular signatures of the gastric pre-cancerous lesions, such as non-atrophic chronic gastritis and intestinal metaplasia, have not been well explored and the mechanisms that lead to carcinogenic progression are still unknown. microRNAs (miRNAs) were reported as potential biomarkers of intestinal-type gastric adenocarcinoma. *hsa-miR-29c* and *hsa-miR-135b* are promising biomarkers of gastric carcinogenesis and provide evidence of epigenetic alterations in non-atrophic chronic gastritis and intestinal metaplasia.

Research frontiers

A subset of patients who develop intestinal-type gastric adenocarcinomas undergo a multi-stage and complex process of carcinogenesis, initiated

by chronic gastritis and intestinal metaplasia. *Hsa-miR-29c* and *hsa-miR-135b* expressions are altered in non-atrophic chronic gastritis and intestinal metaplasia, indicating that better understanding of these gastric lesions is required for the prevention of gastric cancer.

Innovations and breakthroughs

Previous studies have shown that *hsa-miR-29c* and *hsa-miR-135b* are potential biomarkers of intestinal-type gastric adenocarcinoma. This study investigated the expression profiles of *hsa-miR-29c* and *hsa-miR-135b* in gastric mucosal samples, including normal gastric mucosa, non-atrophic chronic gastritis, intestinal metaplasia and intestinal-type gastric adenocarcinoma. The expression of *hsa-miR-29c* in the gastric lesions showed that non-atrophic gastritis have a intermediate profile to gastric normal mucosa and intestinal-type gastric adenocarcinoma, and that intestinal metaplasia samples presented an expression pattern similar to that in intestinal-type gastric adenocarcinoma. This miRNA regulates the *DMNT3A* oncogene. *Hsa-miR-135b* is up-regulated in non-atrophic chronic gastritis and intestinal metaplasia samples and down-regulated in normal gastric mucosa and intestinal-type gastric adenocarcinoma samples. Non-atrophic chronic gastritis and intestinal metaplasia are significantly different from normal gastric mucosa samples. *Hsa-miR-135b* expression presented a greater discriminatory accuracy between normal samples and gastric lesions. This miRNA was associated with *Helicobacter pylori* presence in non-atrophic chronic gastritis samples and regulates the *APC* and *KLF4* tumour suppressor genes.

Applications

This study showed that *hsa-miR-29c* expression has a good discriminatory accuracy between normal gastric samples and (1) intestinal-type gastric adenocarcinoma; and (2) intestinal metaplasia; *hsa-miR-135b* expression presented a greater discriminatory accuracy between normal samples and gastric lesions. These results suggest that *hsa-miR-29c* and *hsa-miR-135b* are promising biomarkers of gastric carcinogenesis, indicating that better understanding of the gastric lesions is required for the prevention of gastric cancer.

Terminology

According to the Correa cascade, a subset of patients who develop intestinal-type gastric adenocarcinomas undergo a multi-stage and complex process of carcinogenesis, initiated by (1) non-atrophic chronic gastritis; followed by (2) chronic atrophic gastritis; then (3) intestinal metaplasia; and finally (4) dysplasia. *hsa-miR-29c* and *hsa-miR-135b* are miRNAs, a class of noncoding RNAs, that regulate the expression of target mRNAs at a post-transcriptional level. These miRNAs are related to the gastric carcinogenesis and may be potential biomarkers.

Peer-review

The authors used gastric tissues derived from normal, gastritis, metaplasia, and carcinoma to investigate expression of 2 miRNAs and evaluate their potential to be biomarkers to distinguish these 4 groups. The concept is clear and the flow of data presentation is straightforward.

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Basic Study

Oncogenic potential of IDH1R132C mutant in cholangiocarcinoma development in mice

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Abstract

AIM: To investigate whether IDH1R132C mutant in combination with loss of p53 and activated Notch signaling promotes intrahepatic cholangiocarcinoma (ICC) development.

METHODS: We applied hydrodynamic injection and sleeping beauty mediated somatic integration to induce loss of p53 (*via* shP53), activation of Notch [*via* intracellular domain of Notch1 (NICD)] and/or overexpression of IDH1R132C mutant together with the sleeping beauty transposase into the mouse liver. Specifically, we co-expressed shP53 and NICD (shP53/NICD, *n* = 4), shP53 and IDH1R132C (shP53/IDH1R132C, *n* = 3), NICD and IDH1R132C (NICD/IDH1R132C, *n* = 4), as well as NICD, shP53 and IDH1R132C (NICD/shP53/IDH1R132C, *n* = 9) in mice. Mice were monitored for liver tumor development and euthanized at various time points. Liver histology was analyzed by hematoxylin and eosin staining. Molecular features of NICD/shP53/IDH1R132C ICC tumor cells were characterized by Myc tag, Flag tag, Ki-67, p-Erk and p-AKT immunohistochemical staining. Desmoplastic

reaction in tumor tissues was studied by Picro-Sirius red staining.

RESULTS: We found that co-expression of shP53/NICD, shP53/IDH1R132C or NICD/IDH1R132C did not lead to liver tumor formation. In striking contrast, co-expression of NICD/shP53/IDH1R132C resulted in ICC development in mice ($P < 0.01$). The tumors could be identified as early as 12 wk post hydrodynamic injection. Tumors rapidly progressed, and by 18 wk post hydrodynamic injection, multiple cystic lesions could be identified on the liver surface. NICD/shP53/IDH1R132C liver tumors shared multiple histological features of human ICCs, including hyperplasia of irregular glands. Importantly, all tumor cells were positive for the biliary epithelial cell marker cytokeratin 19. Extensive collagen fibers could be visualized in tumor tissues using Sirius red staining, duplicating the desmoplastic reaction observed in human ICC. Tumors were highly proliferative and expressed ectopically injected genes. Together these studies supported that NICD/shP53/IDH1R132C liver tumors were indeed ICCs. Finally, no p-AKT or p-ERK positive staining was observed, suggesting that NICD/shP53/IDH1R132C driven ICC development was independent of AKT/mTOR and Ras/MAPK signaling cascades.

CONCLUSION: We have generated a simple, non-germline murine ICC model with activated Notch, loss of p53 and IDH1R132C mutant. The study supported the oncogenic potential of IDH1R132C.

Key words: IDH1 mutant; Notch pathway; Intrahepatic cholangiocarcinoma; Mouse liver cancer; p53

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Core tip: We established a novel murine intrahepatic cholangiocarcinoma (ICC) model *via* hydrodynamic transfection of activated form of Notch1 (NICD), shP53 and IDH1R132C into the mouse liver. This study is the first to demonstrate that IDH1R132C mutant can cooperate with other oncogenes or tumor suppressor genes to promote ICC development *in vivo*. In addition, it provides the ICC research community with an innovative and convenient approach to generate IDH1R132C mutant ICC in mice. Finally, ICC induced by NICD/shP53/IDH1R132C provides a useful tool to study IDH mutant in ICC pathogenesis and a novel preclinical murine model for testing drugs against the deadly malignancy.

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INTRODUCTION

Intrahepatic cholangiocarcinoma (ICC) is a deadly malignancy of the biliary epithelium arising within the liver. It is the second most common primary hepatic cancer, representing about 10% to 20% of all primary hepatic carcinomas^[1,2]. While ICC remains a relatively rare malignancy worldwide, its incidence rate has been rising rapidly over the past several decades^[3]. For the lack of apparent clinical symptoms, signs and deviant lab test results, ICC is generally diagnosed at the advanced stage. Treatment options for ICC are very limited. Indeed, there is no curative treatment except surgical resection at the early stage of ICC. The combination of gemcitabine and cisplatin is the first-line treatment for inoperable ICC patients^[3-5]. However, standard chemotherapies only offer very limited benefit. Clearly, effective molecular targeted therapies are urgently needed for the treatment of ICC.

Molecular genetics underlying ICC pathogenesis remains poorly understood^[6,7]. A rising number of genetics data points to a heterogeneous collection of underlying mutations in multiple oncogenes and tumor suppressor genes in human ICC, such as KRAS^[8], BRAF^[9], p53^[10-12], SMAD4^[11] and p16^[13].

Recently, mutations in metabolic genes, which can reprogram tumor metabolism to stimulate cell growth and proliferation, have been identified in various human tumors^[14]. These mutations are considered to be novel targets for cancer therapies. Isocitrate dehydrogenase (IDH) is an enzyme that participates in NADP⁺ or NAD⁺ dependent tricarboxylic acid (TCA) cycle. It localizes to the cytoplasm, mitochondrion, and peroxisome in cells, and catalyzes the oxidative decarboxylation of isocitrate to produce α -ketoglutarate (α -KG) and CO₂. There are three isoforms in human: IDH1, IDH2 and IDH3. Mutations of IDH1 and IDH2 have been identified in multiple tumor types. For example, IDH1 mutants are found in brain tumors (including gliomas^[15] and glioblastomas^[16]), acute myeloid leukemia^[17,18], thyroid carcinomas^[19,20], cartilaginous tumors^[21,22] and ICCs^[23,24]. Previous studies have demonstrated that mutant IDH1 is oncogenic *via* regulating TCA cycle and increasing tumor cells' dependence on oxidative mitochondrial metabolism^[25]. IDH1 mutation alters its enzymatic activity, leading to the production of (D)-2-hydroxyglutarate (2-HG) rather than α -KG^[26,27]. 2-HG is structurally similar to α -KG, and acts as a α -KG antagonist to competitively inhibit multiple α -KG-dependent dioxygenases, including lysine histone demethylases and the ten-eleven translocation (TET) family of DNA hydroxylases^[28,29]. The oncogenic potential of IDH mutants in ICC was recently validated *in vivo* and the study demonstrated that IDH mutants function to block hepatocyte differentiation while promoting ICC pathogenesis^[30]. However, the study utilized transgenic mice with IDH2R140Q or IDH2R172K mutant in combination with K-RasG12D

to induce ICC formation in mice. In human ICCs, IDH2 mutant is relatively rare. On the other hand, IDH1R132C is the most common IDH mutant found in human ICC. According to COSMIC database, among all ICCs with IDH1 mutations, 47 of all the 76 identified mutations (about 61.8%) are substitution of arginine by cysteine at position 132, *i.e.*, IDH1R132C. However, the *in vivo* oncogenic potential of IDH1R132C has not been investigated.

The abnormal activation of Notch signaling pathway plays critical roles in tumor development^[31], including in ICC^[32]. The contact of Notch ligands with their receptors induces proteolytic cleavage, and releases the Notch intracellular domain (NICD) resulting in the activation of the Notch pathway^[33]. Previous studies demonstrate that the Notch pathway can control liver development by regulating biliary differentiation^[34], and activated Notch 1 (NICD) synergizes with activated AKT signaling to promote ICC development^[35]. As a canonical tumor suppressor gene, silencing of p53 has been implicated in ICC development^[36,37]. The genetic interaction between Notch pathway and the tumor suppressor gene p53 in ICC development has not been studied.

In this study, we investigated the oncogenic potential of IDH1R132C in ICC development. We applied hydrodynamic transfection to overexpress IDH1R132C together with NICD1 or shP53 into mouse liver^[38]. We found that co-expression of NICD/shP53, IDH1R132C/shP53 or NICD/IDH1R132C into the mouse liver did not lead to ICC formation in mice. In contrast, all NICD/shP53/IDH1R132C injected mice developed ICC starting at 12 wk post hydrodynamic transfection. Our results provided evidence, for the first time, that IDH1R132C mutant can promote ICC development in combination with activated Notch signaling and loss of p53. ICC induced by NICD/shP53/IDH1R132C therefore provides a useful tool to study IDH mutant in ICC pathogenesis and a novel preclinical murine model for testing drugs against ICC.

MATERIALS AND METHODS

Ethics statement

Hydrodynamic transfection induced mouse ICC used in this study was generated as previously described^[38]. Mice were housed, fed, and monitored in accordance with protocols approved by the committee for animal research at the University of California, San Francisco (IACUC approval number: AN108577). Mice were monitored closely for liver tumor development. Mice with noticeable swelling abdominal mass or with a body condition score of 2 or less were euthanized by carbon dioxide inhalation followed by cervical dislocation according to the IACUC protocol.

Constructs and reagents

All the constructs, including Myc tagged pT3-EF5 α -

NICD, shRNAmir-based silencing of p53 (pT2-shP53) and pCMV/sleeping beauty transposase (SB) used for mouse injection were previously described^[35,38-40]. Flag tagged human IDH1 cDNA clone was kindly provided by Dr. Yue Xiong (University of Northern Carolina), and IDH1R132C mutant was generated using the QuickChange Site-Directed Mutagenesis kit (Stratagene, Santa Clara, CA). IDH1R132C was subsequently cloned into pT3-EF5 α vector by the Gateway PCR cloning strategy (Invitrogen, Carlsbad, CA). Plasmids were purified using the Endotoxin-free Maxi-prep kit (Sigma, St. Louis, MO) before injecting into mice.

Hydrodynamic tail vein injection

Wild type FVB/N mice were obtained from Charles River (Wilmington, MA). Hydrodynamic injections were performed as described previously^[35,38,41]. Briefly, ten micrograms of the plasmids encoding pT3-EF5 α -NICD (with Myc tag) and/or pT2-shP53 and/or pT3-EF5 α -IDH1R132C (with Flag tag) along with sleeping beauty transposase (pCMV/SB) at a ratio of 25:1 were diluted in 2 mL saline (0.9% NaCl) for each mouse. Saline solution was filtered through a 0.22 μ m filter and injected into the lateral tail vein of 6- to 8-wk-old FVB/N mice in 5-7 s.

Histology and immunohistochemical staining

Liver samples were fixed overnight in zinc formalin (Anatech Ltd.), embedded in paraffin, cut into 5- μ m-thick sections, and placed on glass slides. The rabbit polyclonal anti-Myc (Invitrogen; dilution 1:1000), Flag tag (Cell Signaling Technology; dilution 1:200), anti-CK19 (Abcam; dilution 1:100), anti-Ki67 (Thermo Scientific; dilution 1:150), anti-p-AKT (Cell Signaling Technology; dilution 1:100), and anti-p-ERK1/2 (Cell Signaling Technology; dilution 1:100) antibodies were used. Briefly, slides were deparaffinized in xylene, rehydrated through a graded alcohol series and rinsed in PBS. Endogenous peroxidase was inactivated using 3% hydrogen peroxide in methanol. After boiled in 0.01 M citrate buffer (pH 6.0) for 10 min in a microwave oven, slides were incubated with primary antibodies overnight at 4 °C and subsequently with goat anti-rabbit biotin conjugated secondary antibody (1:500 dilution in PBS) for 30 min at room temperature. Then, signal was visualized using Vectastain ABC Elite kit (Vector Laboratories Inc, Burlingame, CA) and developed with 3,3'-diaminobenzidine (DAB). Sections were counterstained with hematoxylin (Sigma). Negative controls were performed with the same procedure, and PBS was incubated as a substitute for the primary antibodies.

Picro-Sirius red staining

Liver samples were fixed as described previously. Slides were deparaffinized in xylene, rehydrated through a graded alcohol series and rinsed in PBS, and incubated with Picro-Sirius red solution for 60 min. Slides were

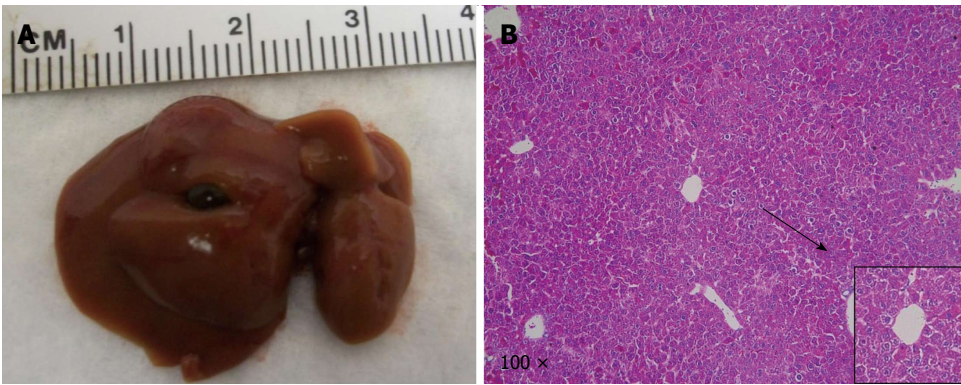


Figure 1 Hydrodynamic co-transfection of NICD and IDH1R132C does not lead to liver tumor formation in mice. A: Macroscopic appearance of the liver from an NICD/IDH1R132C injected mouse harvested at 22 wk post injection; B: Hematoxylin-eosin (HE) staining image of representative NICD/IDH1R132C liver tissue. Inset: Expanded view of the HE image.

| Table 1 Tumor development in mice coinjected with NICD/IDH1R132C, NICD/shP53, shP53/IDH1R132C or NICD/shP53/IDH1R132C | | | | | | | |
|---|----------|-----|----------|-----|--------------------|--------------|--------------------|
| | Code | Sex | Age (wk) | WPI | Tumor ¹ | Tumor number | Tumor size (mm) |
| NICD/IDH1R132C | ND-F4.1 | F | 28 | 21 | N | 0 | 0 |
| | ND-F4.2 | F | 28 | 22 | N | 0 | 0 |
| | ND-F4.3 | F | 28 | 22 | N | 0 | 0 |
| | ND-F4.4 | F | 28 | 22 | N | 0 | 0 |
| NICD/shP53 | NP-F4.1 | F | 31 | 24 | N | 0 | 0 |
| | NP-F4.2 | F | 31 | 24 | N | 0 | 0 |
| | NP-F4.3 | F | 31 | 24 | N | 0 | 0 |
| | NP-F4.4 | F | 31 | 24 | N | 0 | 0 |
| shP53/IDH1R132C | PD-F3.1 | F | 27 | 20 | N | 0 | 0 |
| | PD-F3.2 | F | 27 | 20 | N | 0 | 0 |
| | PD-F3.3 | F | 27 | 20 | N | 0 | 0 |
| | NDP-F4.1 | F | 16 | 9 | N | 0 | 0 |
| NICD/shP53/IDH1R132C | NDP-F4.2 | F | 19 | 12 | Y | 1 | 1.5 |
| | NDP-F4.3 | F | 19 | 12 | Y | 2 | 1, 2 |
| | NDP-F4.4 | F | 20 | 13 | Y | 2 | 1, 1 |
| | NDP-F4.5 | F | 20 | 13 | Y | 4 | 1, 1, 2, 3 |
| | NDP-F4.6 | F | 22 | 15 | Y | 3 | 1.5, 2, 2 |
| | NDP-F4.7 | F | 22 | 15 | Y | 5 | 1, 1.5, 2, 2, 3 |
| | NDP-F4.8 | F | 25 | 18 | Y | 6 | 1, 2, 2.5, 4, 4, 6 |
| | NDP-F4.9 | F | 25 | 18 | Y | 4 | 1, 1, 2, 4 |
| | | | | | | | |

¹Tumor. N: No tumor nodules observed in liver; Y: Liver tumor in liver. WPI: Weeks post injection.

then rinsed in PBS and quickly dehydrated in xylene.

Statistical analysis

Students' *t*-test was used to compare tumor incident rates in mouse groups.

RESULTS

Hydrodynamic co-transfection of IDH1R132C and NICD does not lead to liver tumor formation in mice

To study the oncogenic potential of IDH1R132C

mutant, we generated pT3-EF5α-IDH1R132C plasmid which can be delivered into and stably expressed in mouse hepatocytes *via* sleeping beauty mediated somatic integration. It has been widely recognized that tumor development is a complex process and requires the activation of multiple signaling pathways. In our study, the IDH1 mutant, as a metabolic gene, is unlikely to be sufficient to promote any tumor formation. Deregulation of Notch pathway and p53 is known to be involved in human ICC pathogenesis. We attempted to develop mouse ICC models in which Notch, p53 and IDH1 are deregulated, either alone or in combination.

In our previous studies, we showed that NICD alone is only able to promote ICC development over long latency^[35]. We therefore investigated whether co-expression of IDH1R132C accelerated NICD induced ICC development in mice. Towards this goal, we co-expressed NICD/IDH1R132C into wild type FVB/N mice (*n* = 4) by hydrodynamic transfection. All mice appeared to be healthy and were harvested at 22 wk post injection (Table 1). We found that the livers appeared to be normal in all mice; and none of the mice had visible nodules on the liver surface (Figure 1A). The result was corroborated by histological examination (Figure 1B). Together, the data suggest that IDH1R132C is unable to cooperate with NICD to promote liver tumor development *in vivo*.

Hydrodynamic transfection of IDH1R132C mutant cooperates with NICD and shP53 to promote ICC development in mice

A previous study showed that IDH mutants are associated with loss of p53 activity in human ICCs^[23]. We therefore investigated whether loss of p53 expression is able to synergize with NICD and IDH1R132C to induce ICC formation in mice.

At the first step, we investigated whether loss of p53 (*via* shP53) accelerated NICD1 induced ICC development. We co-expressed NICD/shP53 into the mice (*n* = 4) by hydrodynamic transfection. All mice appeared to be healthy and were harvested at 24

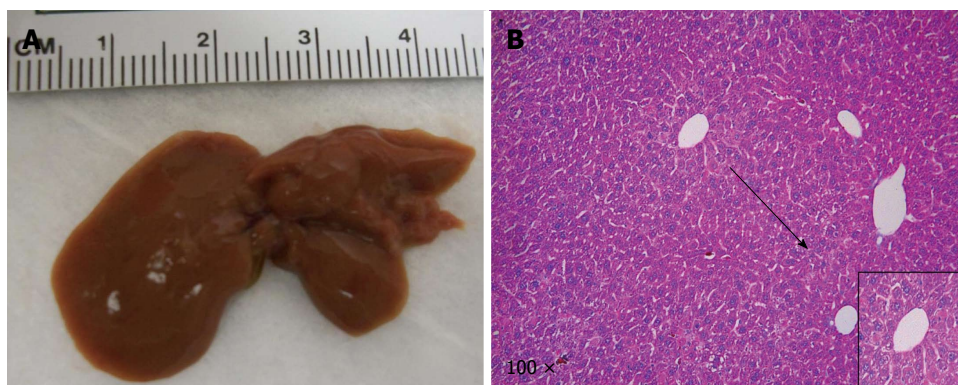


Figure 2 Hydrodynamic co-transfection of NICD and shP53 cannot promote liver tumor development in mice. A: Macroscopic appearance of the liver from a NICD/shP53 injected mouse harvested at 24 wk post injection; B: Hematoxylin-eosin (HE) stained image of representative NICD/shP53 liver tissue. Inset: Expanded view of the HE image.

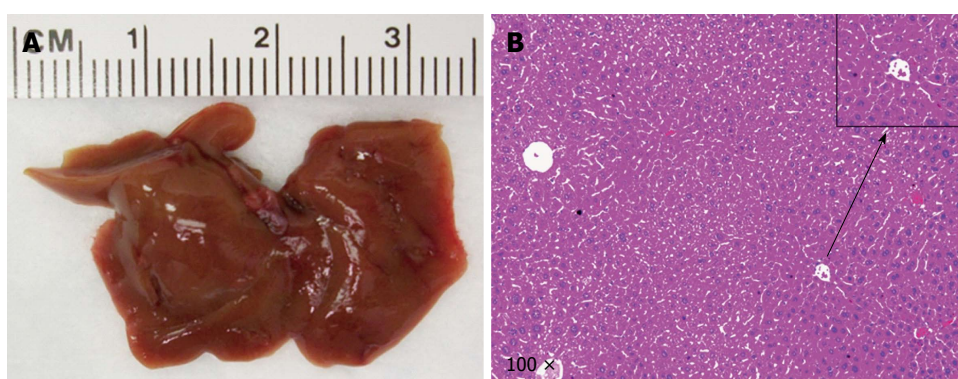


Figure 3 Hydrodynamic co-transfection of shP53 and IDH1R132C cannot lead to liver tumor in mice. A: Macroscopic appearance of the liver from a NICD/shP53 injected mouse harvested at 20 wk post injection; B: Hematoxylin-eosin (HE) stained image of representative shP53/IDH1R132C liver tissue. Inset: Expanded view of the HE image.

wk post injection. We found that none of the mice showed any sign of liver tumor formation (Figure 2), suggesting that loss of p53 is unable to cooperate with NICD to induce ICC formation in mice.

As a second step, we examined whether loss of p53 and overexpression of IDH1R132C mutant could promote ICC development in mice. We co-expressed IDH1R132C/shP53 into the mice ($n = 3$) by hydrodynamic transfection. Again, all mice appeared to be healthy with no lesions on the liver (Figure 3). The result indicates that loss of p53 and overexpression of IDH1R132C cannot promote ICC development *in vivo*.

Next, we tested the hypothesis that IDH1R132C mutant cooperates with NICD and shP53 to promote ICC development in mice. In this study, a mixture of NICD/shP53/IDHR132C plasmids was hydrodynamically transfected to mice ($n = 9$). Mice were harvested at five time points [9 wk ($n = 1$), 12 wk ($n = 2$), 13 wk ($n = 2$), 15 wk ($n = 2$) and 18 wk ($n = 2$)] in order to better understand the tumor initiation and progress processes.

Macroscopically, livers of these mice appeared normal at 9 wk after injection. However, small and cyst-like lesions were present on the liver surface of both mice at 12 wk post-injection (Table 1 and Figure

4A-a). By 18 wk post injection, multiple cystic lesions could be identified on the liver surface (Table 1 and Figure 4A-b).

At the microscopic level, small tumors of ductular phenotype could be identified on the mouse liver at 12 wk post injection (Figure 4B-a). The tumors grew markedly by 18 wk post injection and exhibited either a ductular or cystic phenotype (Figure 4B-b). The tumors were indeed induced by the ectopically expressed genes, as tumor cells strongly and uniformly expressed Myc-tagged NICD and Flag-tagged IDHR132C (Figure 5B and C). The liver tumors shared multiple histological features of human ICCs, such as hyperplasia of irregular glands (Figure 5A). Importantly, immunohistochemical staining for the biliary marker cytokeratin 19 (CK19)^[42,43] demonstrated that tumor cells were all CK19 positive (Figure 5D). Many cells in NICD/shP53/IDHR132C induced ICC were positive for the proliferation marker Ki67 (Figure 5E), which is in accordance with what has been shown in human high-grade ICC^[44]. Furthermore, extensive collagen fibers could be visualized in tumor tissues using Sirius red staining (Figure 5H), duplicating the desmoplastic reaction observed in human ICC. Altogether, these results confirm that the tumors

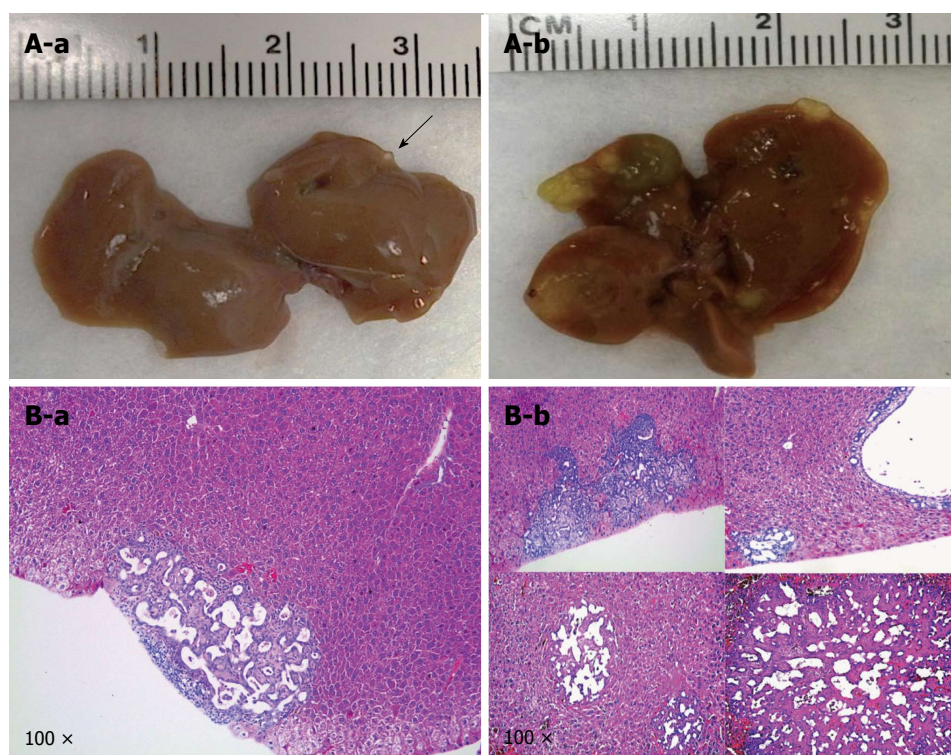


Figure 4 NICD/shP53/IDH1R132C induced ICC formation in mice. A: Gross images of NICD/shP53/IDH1R132C injected mice harvested at 12 wk post injection (a) and 18 wk post injection (b). The arrow indicates a cystic lesion on the liver surface; B: Hematoxylin-eosin stained images of the corresponding liver sections showing liver tumors from NICD/shP53/IDH1R132C injected mice.

exhibited exclusively biliary differentiation (Figure 5), supporting the classification as murine ICC.

Activation of AKT/mTOR and Ras/MAPK signaling has been implicated in ICC development^[35,40,41], we therefore investigated whether these pathways are activated in NICD/shP53/IDH1R132C ICC tumor cells. We found that neither p-AKT nor p-ERK1/2 was expressed in the ICC tumor cells (Figure 5F and G), suggesting that ICC development in this model was independent of activated AKT/mTOR and Ras/MAPK cascades.

In summary, our results indicate that NICD/shP53/IDH1R132C can drive ICC development *in vivo* ($P < 0.01$ when compared with other mouse cohorts). The results support the oncogenic potential of IDH1R132C mutant in ICC pathogenesis. ICC induced by NICD/shP53/IDH1R132C therefore provides a useful tool to further characterize the functional contribution of IDH1R132C mutant in ICC development. These mice also can be utilized as a novel and useful preclinical murine model for testing drugs against ICC.

DISCUSSION

Our present study was designed to evaluate the effect of mutant IDH1 in the development of liver tumors. Mutant IDH1 induces accumulation of 2-HG, which increases DNA methylation and decreases expression of tumor suppress genes, resulting in cellular proliferation in the tissue^[23]. Thus, mutant

IDH1 is generally considered a candidate oncogene in previous liver cancer studies. It is interesting to note that in human ICC samples, IDH1 mutant is associated with better prognosis^[23], suggesting that most likely, mutant IDH1 *per se* is not oncogenic. Rather, it functions to modify the tumor progression initiated by other oncogenes or loss of tumor suppressor genes. In our current studies, we show that hydrodynamic transfection of NICD/shP53, shP53/IDH1R132C or NICD/IDH1R132C cannot promote liver tumor formation in mice. In striking contrast, overexpression of all the three factors, NICD, shP53 and IDH1R132C, is sufficient for ICC development in mice. The results support that mutant IDH1 is capable of stimulating ICC development in the combination of other genetic events, and in this case, activation of Notch pathway and loss of p53 tumor suppressor.

Recently, transgenic mice overexpressing IDH2 mutants (IDH2R140Q or IDH2R172K) were generated^[30]. It was found that overexpression of IDH2 mutants did not lead to tumor development or any abnormal liver phenotype in the absence of liver injury. Importantly, the study demonstrated that co-expression of IDH2R192K and KRasG12D oncogene in the mouse liver was able to promote ICC development in mice. The tumor development required long latency with liver tumors detected between 33 and 58 wk of age. Mechanistically, the study suggested that it is likely that IDH2R192K and KRasG12D cooperated to initiate the activation and expansion of hepatic

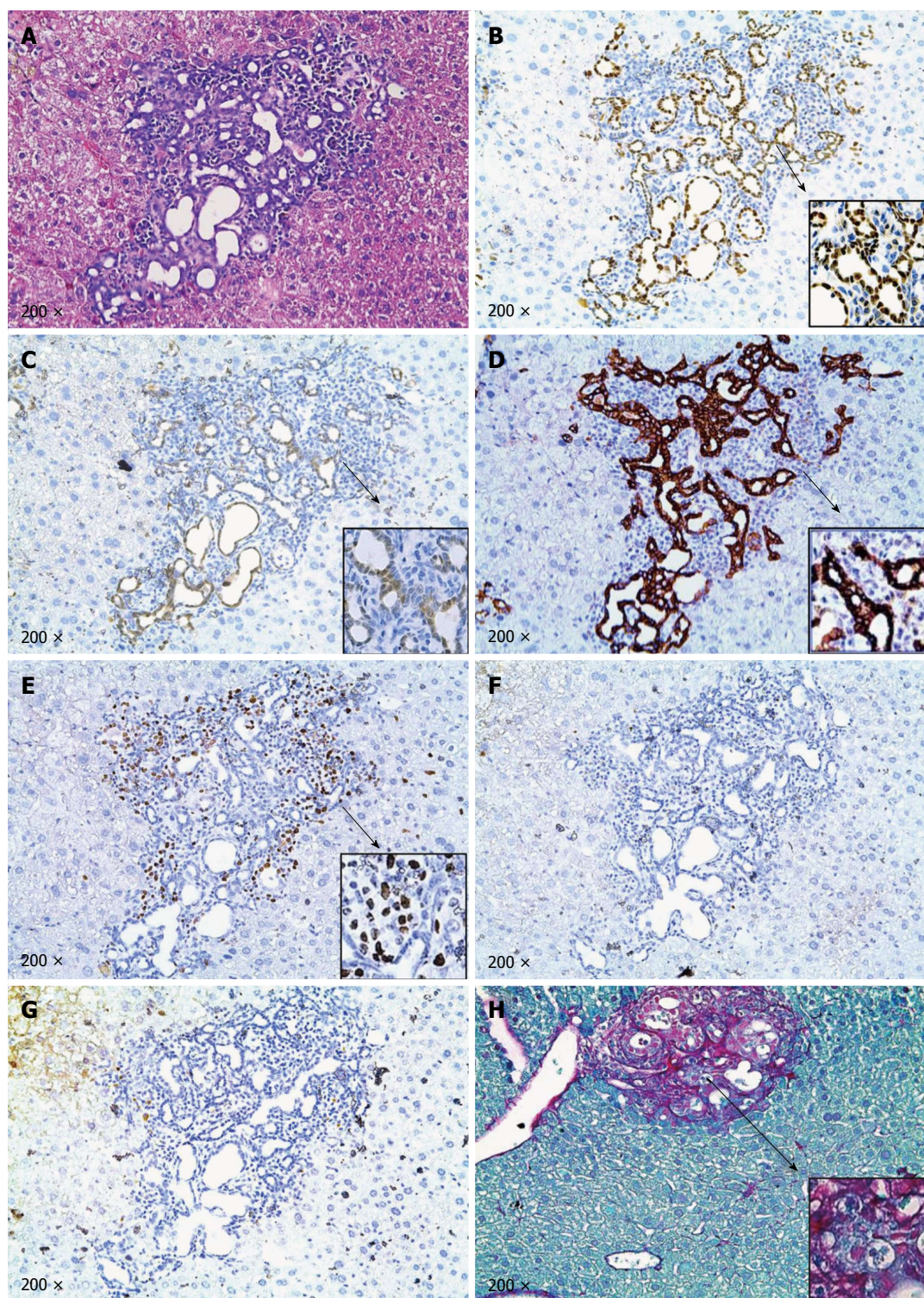


Figure 5 Molecular characterization of NICD/shP53/IDH1R132C induced ICCs. A: Representative hematoxylin-eosin (HE) stained image of NICD/shP53/IDH1R132C ICC tumors; B: Myc tag; C: Flag tag; D: CK19; E: Ki67; F: p-AKT and G: p-ERK1/2 immunostaining in NICD/shP53/IDH1R132C ICC tumor samples; H: Picro-Sirius red staining image of a liver section showing profound desmoplastic reaction in a NICD/shP53/IDH1R132C ICC tumor sample. Inset: Expanded view of the immunostaining images.

progenitor cells, eventually leading to ICC formation. However, IDH2 mutants are relatively rare in human ICCs, and it is not known whether IDH1 mutant can cooperate with other oncogenes to stimulate ICC development. Our study, therefore, is the first *in vivo* study to demonstrate that IDH1 mutant can

indeed cooperate with other oncogenic stimuli, such as activated Notch and loss of p53, to promote ICC development in mice. Intriguingly, we did not observe any sign of progenitor or oval cell-like cell expansion in non-tumor liver tissues adjacent to the ICC lesions in NICD/shP53/IDH1R132C injected mice. It remains

unknown whether NICD/shP53/IDH1R132C promotes ICC pathogenesis *via* progenitor cell expansion. As IDH1 is a metabolic gene, it would be also of great interest to further characterize the metabolic events in NICD/shP53/IDH1R132C ICC tumor cells. For example, it is important to analyze whether 2-HG accumulates in NICD/shP53/IDH1R132C tumor cells, and whether tumor cells show increased glycolysis.

Traditionally, genetically engineered mouse models, including knockout or transgenic mice, are required to demonstrate the oncogenic or tumor suppressor potential of the target genes and to illustrate how these genes contribute to tumor initiation and progression. Recently, however, hydrodynamic transfection, which combines hydrodynamic transfection and sleeping beauty mediated somatic integration, was developed and this technology has been applied to study HCC and ICC^[38]. The most important features of hydrodynamic transfection reside in its flexibility and cost effectiveness. For example, using the traditional transgenic model, Saha and colleagues need to first generate *LSL-IDH2R172K* transgenic mice. These mice need to be maintained, and crossed to *Alb-Cre* and *LSL-KRasG12D* in order to generate the triple transgenic mice, *i.e.*, *Alb-Cre;LSL-KRasG12D;LSL-IDH2R172K* to study whether IDH2R172K synergized with KRasG12D to induce ICC development in mice. Clearly, these studies are labor intensive, expansive and require a large number of mice. For other investigators to duplicate the study, one has to import the mice into his or her own institute. In contrast, hydrodynamic transfection is highly flexible, and one only needs wild type mice and plasmids required for injection in order to determine the genetic and biochemical crosstalk among multiple oncogenic pathways and their potential to promote liver tumor development *in vivo*. Our study therefore provides a convenient and cost-effective approach to generate IDH mutant related ICC murine models.

In our current study, we co-expressed IDH1R132C with NICD and shP53 in the mouse liver. It would be highly interesting to expand the study in order to further elucidate the functional contribution of IDH1 mutant in ICC pathogenesis. Other signaling pathways which are important for ICC tumorigenesis include activation of Yap^[45], hypermethylation of Pten^[46], and mutant Smad4^[47], etc. Using hydrodynamic transfection, one can combine IDH1R132C with these genetic events to determine whether the combination can lead to ICC formation. In addition, it would be important to co-express other IDH1 mutants, such as IDH1R132L (about 13.2% of IDH1 mutations in human ICCs) and IDH1R132G (about 18.4% of IDH1 mutations in human ICCs) with NICD1 and shP53, and determine whether these IDH1 mutants have similar functions *in vivo*.

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COMMENTS

Background

Intrahepatic cholangiocarcinoma (ICC) is a deadly disease lacking effective treatment options. IDH1 mutation is identified in about 10% of all human ICC cases, and IDH1R132C represents the most frequent IDH1 mutation in ICC. However, the oncogenic potential of IDH1R132C in ICC pathogenesis remains unknown, especially *in vivo*.

Research frontiers

In this study, the authors applied hydrodynamic transfection to study the oncogenic potential of IDH1R132C mutant in driving ICC development *in vivo*. Hydrodynamic transfection is an innovative cost effective and reliable method for generating preclinical murine models of liver cancer, including hepatocellular carcinoma and cholangiocarcinoma. Furthermore, IDH1 mutants are identified as critical genetic events in ICC pathogenesis, and it is important to clarify the oncogenic potential of IDH1 mutants using *in vivo* approaches.

Innovations and breakthroughs

In this manuscript, the authors described the establishment of a novel murine ICC model *via* hydrodynamic transfection of activated form of Notch1 (NICD), silencing of p53 (shP53) and IDH1R132C (NICD/shP53/IDH1R132C) into the mouse liver. This study is the first to demonstrate that IDH1R132C mutant can cooperate with other oncogenes or tumor suppressor genes to promote ICC development *in vivo*, supporting the oncogenic potential of IDH1R132C mutant during ICC pathogenesis.

Applications

ICC induced by NICD/shP53/IDH1R132C provides a useful tool to further characterize the functional contribution of IDH1R132C mutant in ICC development. These mice also can be utilized as a novel and useful preclinical murine model for testing drugs against ICC.

Terminology

Hydrodynamic transfection: it is a novel approach for stable gene expression in mouse hepatocytes by hydrodynamic injection in combination with sleeping beauty mediated somatic integration. Specifically, to achieve the goal of long-term gene expression in hepatocytes, two plasmids are needed: one encoding the SB transposase, and the other encoding the gene of interest under a mammalian promoter and flanked by inverted repeats. The two plasmids are then mixed together, diluted into saline, and injected into lateral vein of mouse tail *via* hydrodynamic injection. Hydrodynamic transfection is now widely used to generate novel murine models of liver cancer.

Peer-review

Good article and interesting topic. This study provides a useful tool to further characterize the functional contribution of IDH1R132C mutant in ICC development.

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Basic Study

Interleukin-22 contributes to liver regeneration in mice with concanavalin A-induced hepatitis after hepatectomy

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Abstract

AIM: To investigate the therapeutic effects and mechanisms of interleukin (IL)-22 in liver regeneration in mice with concanavalin A (ConA)-induced liver injury following 70% hepatectomy.

METHODS: Mice were injected intravenously with ConA at 10 µg/g body weight 4 d before 70% hepatectomy to create a hepatitis model, and recombinant IL-22 was injected at 0.125 µg/g body weight 30 min prior to 70% hepatectomy to create a therapy model. Control animals received an intravenous injection of an identical volume of normal saline.

RESULTS: IL-22 treatment prior to 70% hepatectomy performed under general anesthesia resulted in reductions in the biochemical and histological evidence of liver injury, earlier proliferating cell nuclear antigen expression and accelerated recovery of liver mass. IL-22 pretreatment also significantly induced signal transducer and activator of transcription factor 3 (STAT3) activation and increased the expression of a variety of mitogenic proteins, such as Cyclin D1. Furthermore, alpha fetal protein mRNA expression was significantly elevated after IL-22 treatment.

CONCLUSION: In this study, we demonstrated that IL-22 is a survival factor for hepatocytes and prevents and repairs liver injury by enhancing pro-growth pathways *via* STAT3 activation. Treatment with IL-22 protein may represent a novel therapeutic strategy for preventing liver injury in patients with liver disease who have undergone hepatectomy.

Key words: Interleukin-22; Concanavalin A; Partial hepatectomy; Liver regeneration

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Core tip: Interleukin (IL)-22 appears to play a protective role in inflammation and has also been demonstrated to have proliferative effects in a hepatocyte cell line, however, it has rarely been reported that the protective and proliferative effects exist simultaneously. In this article, we investigated the therapeutic effects and mechanisms of IL-22 in liver regeneration in mice with concanavalin A-induced liver injury following 70% hepatectomy. IL-22 played protective and survival roles against liver injury in this model.

Zhang YM, Liu ZR, Cui ZL, Yang C, Yang L, Li Y, Shen ZY. Interleukin-22 contributes to liver regeneration in mice with concanavalin A-induced hepatitis after hepatectomy. *World J Gastroenterol* 2016; 22(6): 2081-2091 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i6/2081.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i6.2081>

INTRODUCTION

Interleukin-22 (IL-22) is an inducible cytokine of the IL-10 superfamily that was identified by the Belgian Renauld team as early as 2000 and was previously known as the IL-10-related factor from T cells^[1]. IL-22 is produced by activated T cells and natural killer (NK) cells and acts *via* a heterodimeric receptor complex consisting of IL-22 receptor α (IL-22R α) and IL-10 receptor β (IL-10R β).

IL-22 has been demonstrated to exhibit a variety of effects. IL-22 appears to play an important role in inflammation and has also been noted to exert proliferative effects in a hepatocyte cell line *in vitro*^[2-5]. In 2004, the Bin Gao team demonstrated that IL-22 expression is significantly induced in T cell-mediated hepatitis and that IL-22 blockade markedly enhances liver injury in this model, while administration of recombinant IL-22 prevents concanavalin A (ConA)-induced liver injury^[5]. These findings suggest that IL-22 acts as a protective cytokine that attenuates liver injury in T cell-mediated hepatitis. Furthermore, *in vitro* studies also revealed that IL-22 has no obvious toxicity in liver cell lines or primary liver cells and has proliferative and survival effects on

these cells. Additionally, a recent study supported a potential therapeutic role for IL-22 as a protective factor in hepatic resection. The authors of this study observed significant increases in hepatic IL-22 receptor expression and serum IL-22 levels after 70% hepatectomy and a significant decrease in liver regeneration after IL-22 blockade^[6]. However, the precise mechanism of IL-22-mediated liver protection remains unclear.

Currently, all of the evidence supporting the role of IL-22 in liver protection is from liver injury models, and the evidence for the liver proliferative effects is almost entirely from the simple 2/3 liver resection model without other injury. However, clinical patients with hepatectomy nearly always have liver disease and thus significantly decreased liver regeneration abilities. In this article, we sought to investigate the therapeutic effects and mechanisms of IL-22-mediated liver regeneration in mice with ConA-induced liver injury following 70% hepatectomy.

MATERIALS AND METHODS

Materials

Recombinant IL-22 protein was purchased from Pepro Tech Inc (New Jersey, United States). Anti-STAT3, Cyclin D1 and proliferating cell nuclear antigen (PCNA) antibodies were obtained from Cell Signaling Technology Inc (CST, United States). Female C57/BL6 mice were purchased from HFK Bioscience Co., Ltd. (Beijing, China).

70% hepatectomy model

Female C57/BL6 mice (6-8 wk of age, 20-25 g) were maintained under specific pathogen-free conditions with free access to water and food before each experiment. The animals were anesthetized with chloral hydrate injections. After a midline incision was created under microscopic guidance, and the middle and left hepatic lobes of the liver were fully freed, 7-0 vascular sutures were used to ligate the branches of the hepatic artery and portal vein of the median and left lateral lobes of the liver. Next, the bile duct was ligated with 7-0 vascular sutures, and the gallbladder was removed. Finally, the median and left lateral lobes of the liver were resected after a 4-0 silk suture ligation was secured around the base of each lobe.

ConA-induced liver injury model

ConA was injected intravenously at 10 μ g/g body weight 4 d before the operation, and the 70% hepatectomy model animals received intravenous injections of identical volumes of normal saline.

ConA-induced liver injury and 70% hepatectomy model

ConA was injected intravenously at 10 μ g/g body weight, and 70% hepatectomies were performed 4 d later.

IL-22 treatment model

Four days after the intravenous injections of ConA at 10 µg/g body weight, recombinant murine IL-22 was injected intravenously at 0.125 µg/g body weight 30 min prior to 70% hepatectomy. Control animals received intravenous injections of identical volumes of normal saline.

Liver weight/body weight ratio

At 32 h, 40 h, 48 h, 1 wk, and 2 wk, the mice were humanely killed under general anesthesia once moribund. The liver and body weights of all mice in each group were measured, and the liver weight/body weight ratio was then calculated to observe the liver regeneration conditions.

Examination of liver injury

To assess the damage to the hepatic parenchyma, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured using a serum analyzer (Cobas-Mira Plus, Roche, Mannheim, Germany). The liver specimens were fixed in 10% buffered formalin and embedded in paraffin, and the paraffin embedded liver tissue sections were then stained with hematoxylin and eosin (HE) for histological examinations.

Immunohistochemistry

Tissue specimens were fixed in neutral buffered formalin and then embedded in paraffin. The 4-µm paraffin sections were deparaffinized in xylene and rehydrated in a graded series of alcohol. Endogenous peroxidase was inhibited with 0.3% H₂O₂ in methanol. The sections were heated in a microwave oven (in 10 mmol/L citrate buffer, pH 6.0) for 20 min for epitope retrieval, followed by incubation with the primary antibody against PCNA (1:4000; Abcam). The slides were then incubated with a biotinylated bridging antibody (dilution: 1/200, DAKO) for 60 min. The sections were counterstained with Mayer's hematoxylin. PCNA antigen expression levels were evaluated by counting the positively stained cells in the portal triads of five high-power fields (HPFs) per slide, and the results are expressed as the average number of positive cells/HPF.

Quantitative real-time PCR analysis

RNA was extracted from snap-frozen liver tissue samples using the TRIzol reagent. Five micrograms of RNA was reverse-transcribed into cDNA using oligo-dT primers with a Superscript III First-Strand Synthesis System (Invitrogen). Quantitative real-time PCR was performed with iCycler IQ system (Bio-Rad, Hercules, CA). The primer sequences for alpha fetal protein (AFP) gene were 5'-CAA AGC ATT GCA CGA AAA TGA G-3' (forward) and 5'-AAC AAA CTG GGT AAA GGT GAT GGT-3' (reverse). β -actin was measured

as a housekeeping gene. The cycling conditions comprised a 5 min polymerase activation at 95 °C, 40 cycles of 95 °C for 5 s and 60 °C for 30 s and a single fluorescence measurement. Melting curve analysis based on increasing the temperature from 60 to 95 °C at a rate of 0.5 °C/s with continuous fluorescence measurement revealed a single, narrow peak for the suspected fusion temperature.

Western blot analysis

The mice were euthanized at baseline and at 32 h, 40 h, 48 h, 1 wk and 2 wk after hepatectomy, and liver samples were obtained for Western blot analyses. The proteins were extracted from the liver tissues and quantified using a protein assay (Bio-Rad Laboratories, CA). The protein samples (30 µg) were fractionated by SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblotting was conducted using antibodies against STAT3 and Cyclin D1 (Cell Signaling Technology Inc., United States). The results were visualized via an enhanced chemiluminescent detection system (Pierce ECL Substrate Western blot detection system, Thermo Scientific, IL) and exposure to autoradiography film (Kodak XAR film).

Statistical analysis

All parametric data are presented as the mean \pm SD. The data were analyzed for significance using Student's *t*-tests. One-way analyses of variance with Fisher's protected least significant difference (PLSD) tests were used to compare the means. The log-rank test was applied to compare survival curves. Differences were considered statistically significant at $P < 0.05$.

RESULTS**Effects of IL-22 on the liver weight/body weight ratio after partial hepatectomy**

At 32 h, 40 h, 48 h, 1 wk, or 2 wk, the mice were humanely killed under general anesthesia once moribund. The liver and body weights were measured, and the liver weight/body weight ratios were then calculated. As illustrated in Figure 1, increases in the liver weight/body weight ratios were observed in the PHX, ConA + PHX and ConA + PHX + IL-22 groups, and all groups returned to normal liver weights by 2 w. Compared with the ConA + PHX group, the ratio of the ConA + PHX + IL-22 group increased more rapidly, and significant differences between these two groups were observed at 40 h, 48 h, 1 wk and 2 wk. Similarly, compared to the ConA + PHX group, the ratios of the PHX group increased more rapidly, and the differences reached significance at 48 h or 1 wk. However, the increase in the PHX group was less than that in the ConA + PHX group at 32 h. These data correlated with the cellular swelling in the liver at 32 h in the ConA + PHX group.

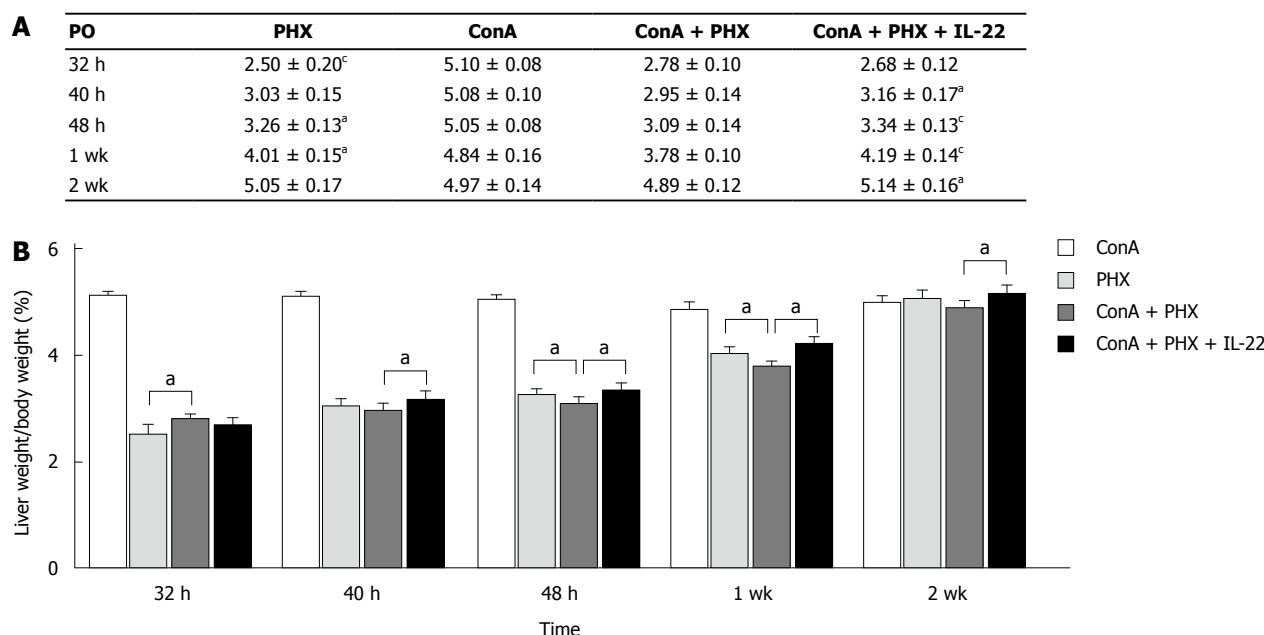


Figure 1 Liver weight/body weight ratios following partial hepatectomy. A and B: Increases in the liver weight/body weight ratio were observed in the PHX, concanavalin A (ConA) + PHX and ConA + PHX + interleukin (IL)-22 groups, and all groups returned to normal liver weights by 2 wk. Compared with the ConA + PHX group, the ConA + PHX + IL-22 group exhibited greater increases that reached significance at 40 h, 48 h, 1 wk and 2 wk (^a $P < 0.05$); these differences were particularly notable at 48 h and 1 wk (^b $P < 0.01$). The increases in the PHX group were significantly different from those in the ConA + PHX group at 48 h and 1 wk (^c $P < 0.05$); however, the increase in the PHX group at 32 h was less than that in the ConA + PHX group (^d $P < 0.01$). These data were correlated with the cellular swelling in the liver at 32 h in the ConA + PHX group.

Effects of IL-22 on liver damage following partial hepatectomy

At 32 h, 40 h, 48 h, 1 wk and 2 wk after 70% hepatectomy, serum samples were collected, and the ALT and AST levels were measured *via* biochemical analyses. As illustrated in Figure 2, compared with the ConA + PHX group, the ALT and AST serum levels in the ConA + PHX + IL-22 group were reduced, and these differences were significant at all of the time points. Furthermore, with the recombinant IL-22 pretreatment, the decreases in the ALT and AST levels of the ConA + PHX + IL-22 group were significantly greater than those of the ConA and PHX groups at 40 h, 48 h and 1 wk.

We also investigated the histologic features of the liver by HE staining. As illustrated in Figure 3, the HE staining of the ConA + PHX group demonstrated severe sinusoidal narrowing that was noted as early as 32 h after hepatectomy. By 48 h, swelling, nuclear condensation and laminar necrosis of the hepatocytes were observed in addition to the near-total loss of the hepatic sinusoids. By 2 wk, the hepatic sinusoids and the complete structure of the hepatic lobule were not observed in the hepatocytes. In contrast, the HE staining of the ConA + PHX + IL-22 group revealed much less evidence of injury at 48 h; the cytoplasm was preserved, and some less severe swelling was present. At 2 wk, normal nuclear morphologies, hepatic sinusoids, complete structures of the hepatic lobules and significant regeneration in the hepatocytes were observed.

Effects of IL-22 on hepatocyte proliferation after partial hepatectomy

Hepatocyte proliferation was determined by the expression of PCNA, which is a nuclear antigen that is associated with hepatocyte proliferation. As illustrated in Figure 4, the PCNA labeling indices in the four groups began to increase postoperatively and peaked at 48 h. Compared with the ConA + PHX group, the PCNA labeling indices were significantly increased in the ConA, PHX and ConA + PHX + IL-22 groups at all of the time points, particularly at 32, 40, and 48 h. Furthermore, the PCNA levels in the ConA + PHX + IL-22 group increased to greater extents than those of the ConA and PHX groups at 32 h, 40 h and 48 h.

Effects of IL-22 on AFP mRNA expression after partial hepatectomy

The mice underwent 70% hepatectomy or sham laparotomy, and quantitative analysis of the liver AFP mRNA expression was performed by real-time RT-PCR. At 32 h, 40 h, 48 h, 1 wk, and 2 wk, the hepatic AFP mRNA expression levels in the ConA + PHX group were 0.55 ± 0.06 , 0.93 ± 0.08 , 1.72 ± 0.11 , 0.66 ± 0.05 and 0.43 ± 0.04 , respectively. In the IL-22 pretreatment group, the corresponding values were 0.74 ± 0.08 , 1.81 ± 0.14 , 2.80 ± 0.26 , 0.86 ± 0.05 and 0.51 ± 0.03 . Figure 5 illustrates that the AFP mRNA expression began to increase at 32 h and had significantly increased by 48 h after hepatectomy in all four groups. Additionally, the AFP mRNA levels in the ConA + PHX + IL-22 group were significantly increased

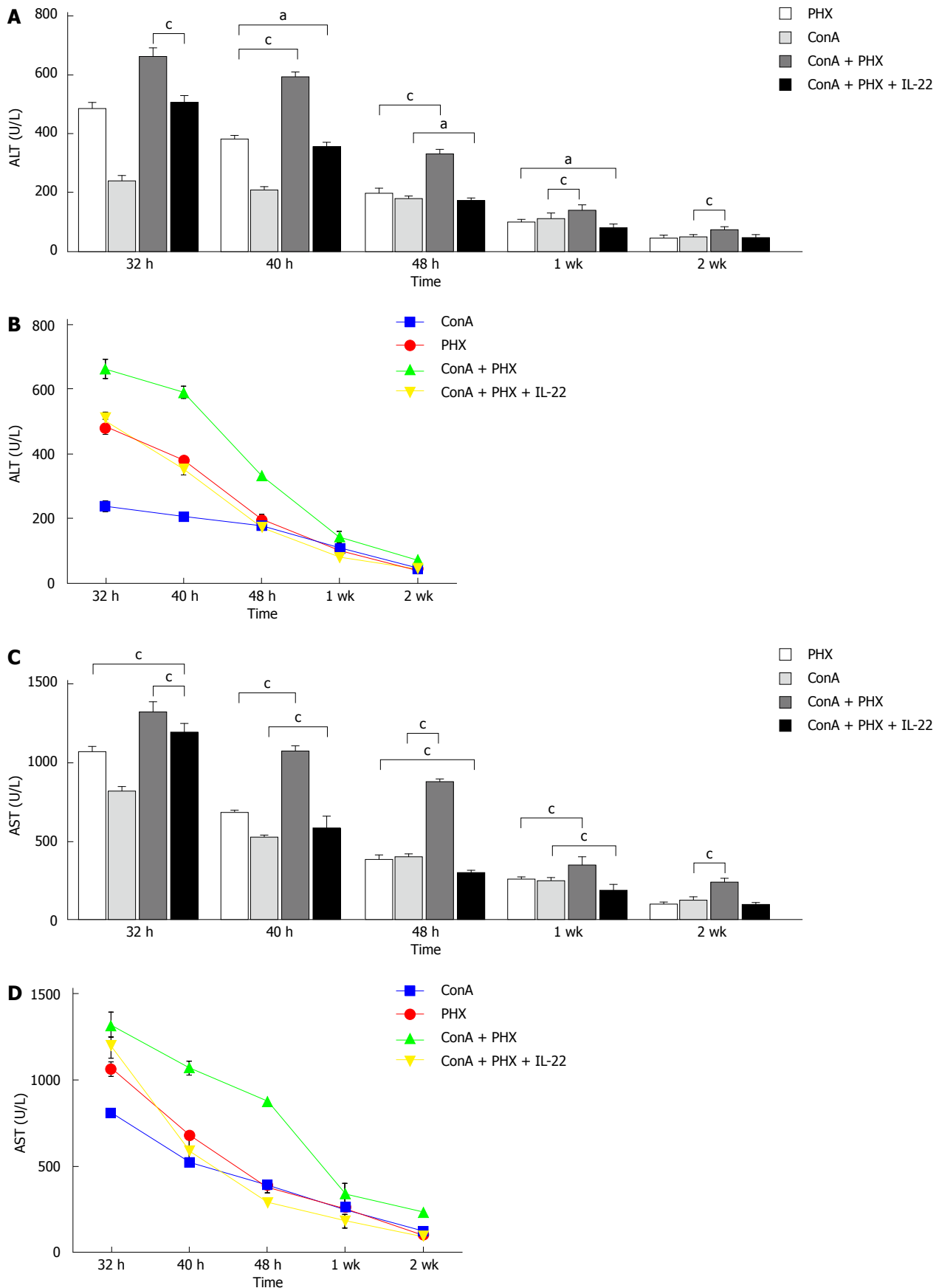


Figure 2 Serum alanine aminotransferase and aspartate aminotransferase levels after partial hepatectomy. A-D: Decreases in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were observed after 32 h, particularly from 40 h to 1 wk. Compared to the concanavalin A (ConA) + PHX group, the serum ALT and AST levels of the ConA + PHX + interleukin (IL)-22 group decreased, and the difference were significant at all time points ($^cP < 0.01$). Furthermore, with the recombinant IL-22 pretreatment, the ALT and AST levels in the ConA + PHX + IL-22 group decreased to significantly greater extents than the levels in the ConA and PHX groups at 40 h, 48 h and 1 wk ($^aP < 0.05$).

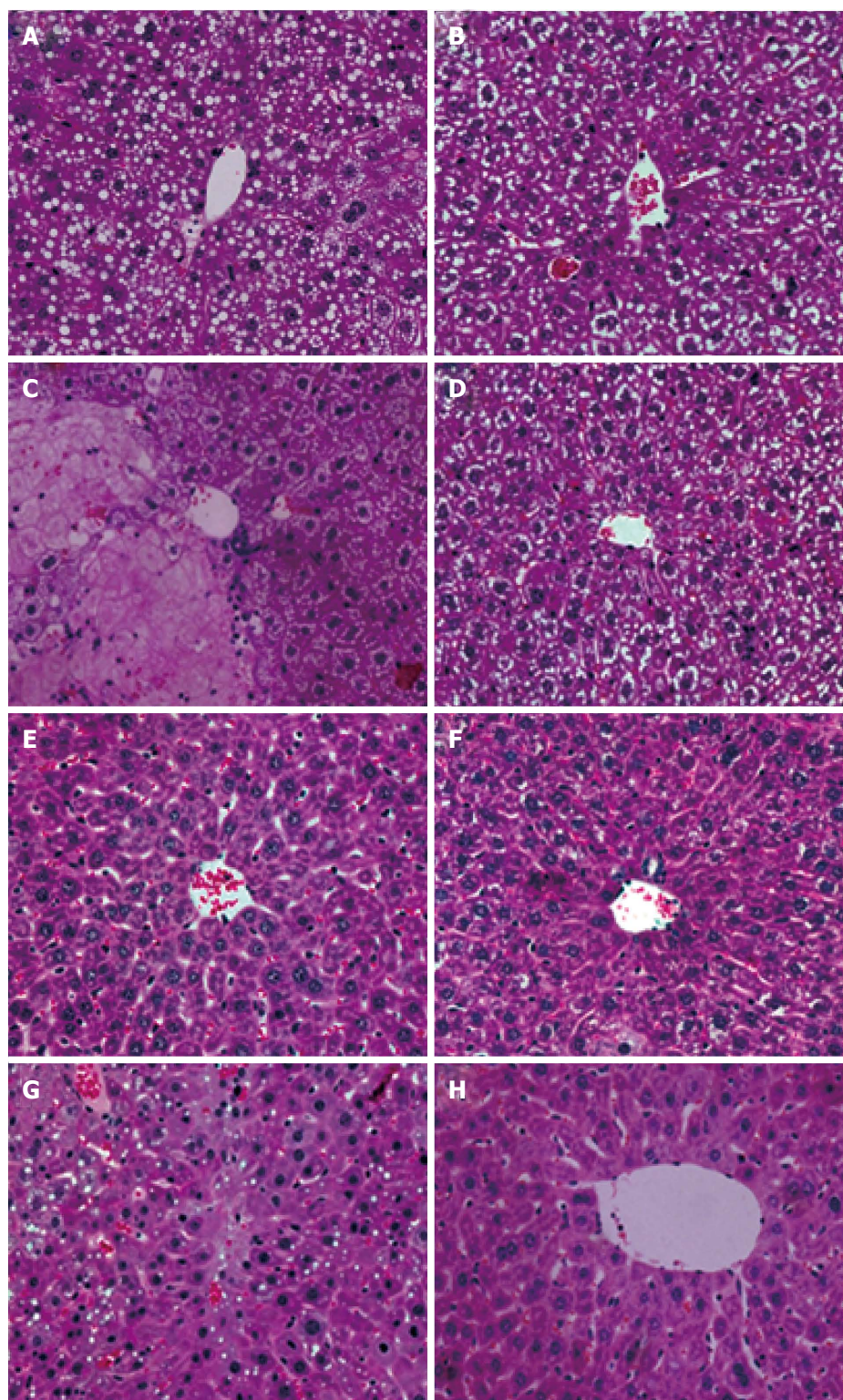


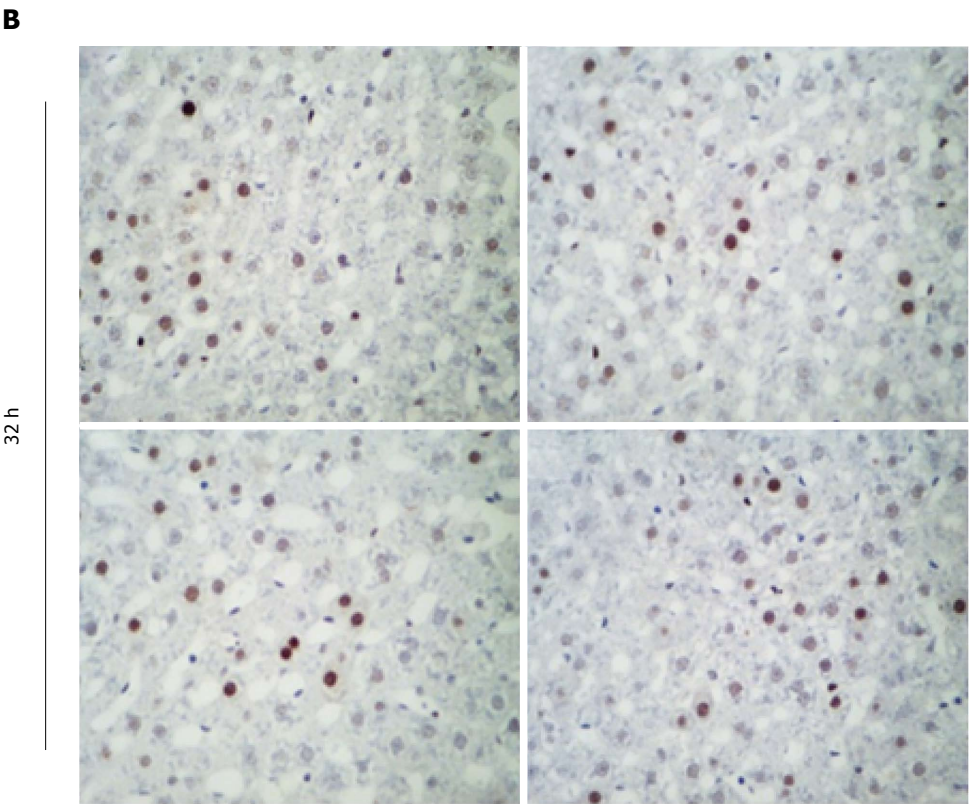
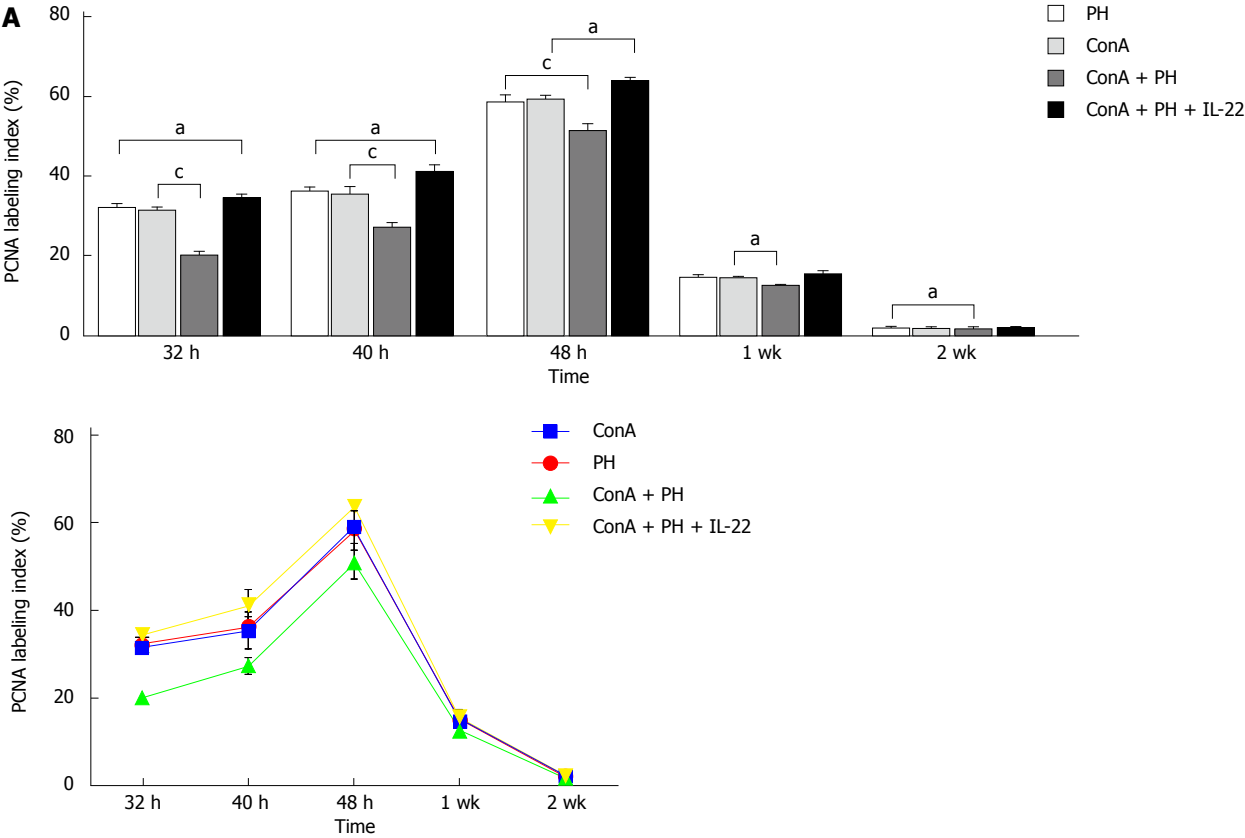
Figure 3 Representative hematoxylin and eosin staining of the remnant livers after partial hepatectomy. A-D: The PHX, concanavalin A (ConA), ConA + PHX and ConA + PHX + interleukin (IL)-22 groups, respectively, at 48 h after partial hepatectomy; E-H: The PHX, ConA, ConA + PHX and ConA + PHX + IL-22 groups, respectively, at 2 wk after partial hepatectomy.

at 32 h, 40 h, 48 h and 1 wk after partial hepatectomy compared with the ConA + PHX group.

Effects of IL-22 on STAT3 and Cyclin D1 activation after partial hepatectomy

The activation of the STAT3 and Cyclin D1 was

measured using Western blot analysis to assess the effects of IL-22 after partial hepatectomy. As illustrated in Figure 6, no significant activation of STAT3 or Cyclin D1 was observed at 32 h after partial hepatectomy in the ConA + PHX group, and the activation increased gradually at 48 h and 2 wk. Although STAT3 and Cyclin



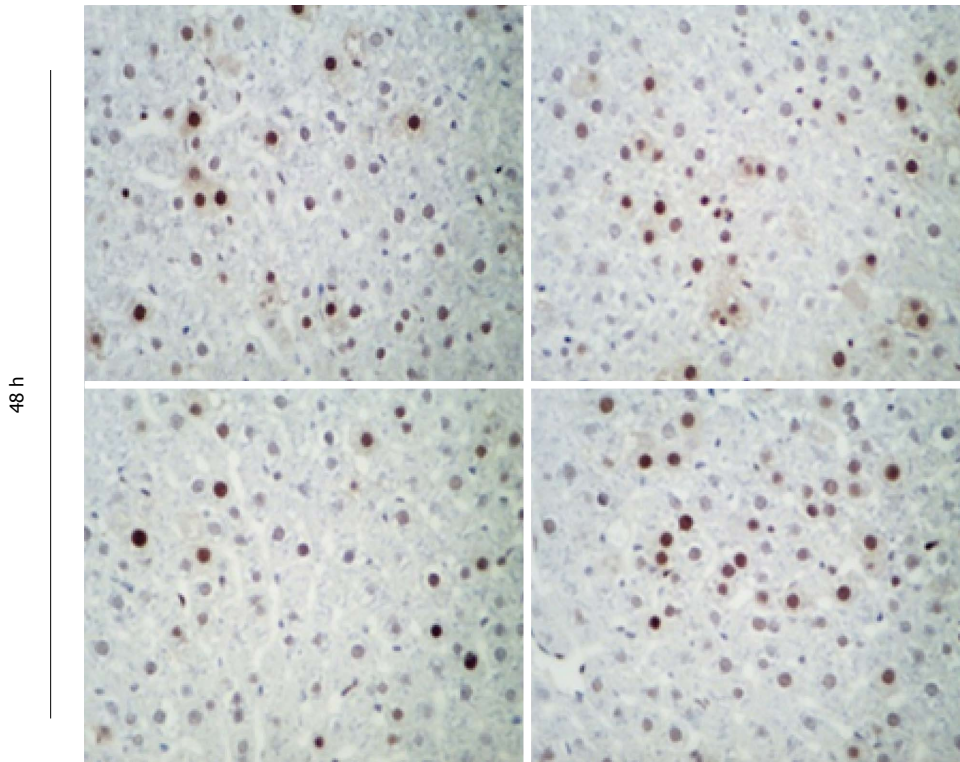


Figure 4 Proliferating cell nuclear antigen labeling indices in the remnant livers after partial hepatectomy. A: Compared with the concanavalin A (ConA) + PHX group, the proliferating cell nuclear antigen (PCNA) labeling indices were significantly increased in the ConA, PHX and ConA + PHX + interleukin (IL)-22 groups at all of the time points ($^aP < 0.05$), particularly at 32, 40, and 48 h ($^cP < 0.01$). Furthermore, the increases in the PCNA levels in the ConA + PHX + IL-22 group were significantly greater than those in the ConA and PHX groups at 32 h, 40 h and 48 h ($^bP < 0.05$); B: PCNA labeling of the cells at 32 h and 48 h after hepatectomy in the PHX, ConA, ConA + PHX and ConA + PHX + IL-22 groups, respectively.

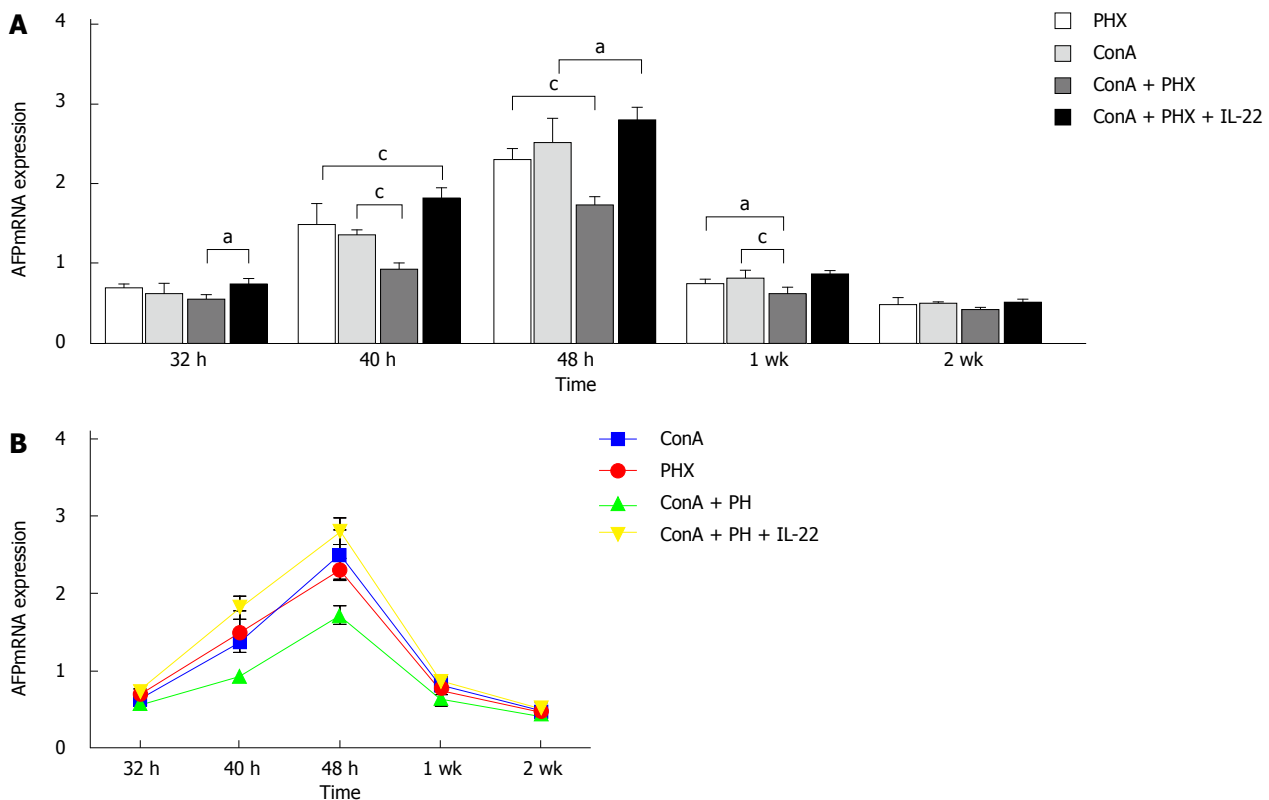


Figure 5 Hepatic alpha fetal protein mRNA expression in the remnant liver after partial hepatectomy. A, B: Alpha fetal protein (AFP) mRNA expression began to increase at 32 h and significantly increased to peak at 48 h after hepatectomy. With the interleukin (IL)-22 pretreatment, the AFP mRNA levels significantly increased at 32 h after partial hepatectomy compared with the concanavalin A (ConA) + PHX group ($^aP < 0.05$), and marked increases were observed at 40 h, 48 h and 1 wk ($^bP < 0.01$). However, the difference was not significant at 2 wk ($P > 0.05$).

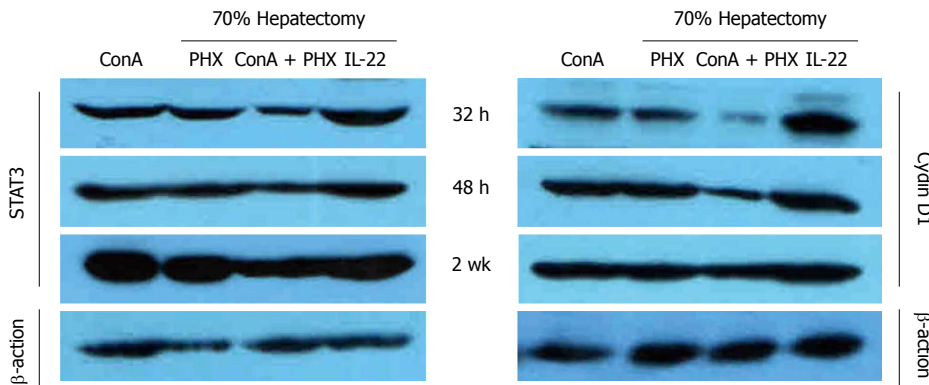


Figure 6 Western blot detection of the activation of STAT3 and Cyclin D1 in the remnant livers after partial hepatectomy. With the administration of interleukin (IL)-22, STAT3 and Cyclin D1 were activated much earlier, and the most significant increases in the four groups were observed at 32 h, 48 h, and 2 wk; whereas the least significant increases were observed in the concanavalin A (ConA) + PHX group at all of the time points.

D1 activation was apparent in the remnant livers of the other three groups, STAT3 and Cyclin D1 were activated much earlier in the ConA + PHX + IL-22 group than the PHX and ConA groups. Furthermore, among the four groups, the STAT3 and Cyclin D1 levels exhibited the greatest increases in the ConA + PHX + IL-22 group and the smallest increases in the ConA + PHX group across all of the time points.

DISCUSSION

IL-22 has previously been shown to have a variety of effects. IL-22 appears to play a protective role in inflammation^[7-10] and has also been demonstrated to have proliferative effects in a hepatocyte cell line^[11]. Hepatectomy in T cell-mediated hepatitis induced by ConA models clinical hepatectomy with liver disease well. We demonstrated that IL-22 acts as a protective cytokine that attenuates liver injury in this model. The present study revealed that pre-treatment with IL-22 prior to hepatectomy significantly decreases the serum ALT and AST levels and increases the serum ALB level following 70% hepatectomy. Our findings suggest that IL-22 plays a protective role against liver injury in ConA-induced hepatitis following 70% hepatectomy and that IL-22 is a survival factor for hepatocytes. With the administration of exogenous IL-22, the liver weight/body weight ratio increased significantly and returned to the normal level by 2 wk. Additionally, the nuclear morphologies, hepatic sinusoids, complete structures of the hepatic lobules returned to normal, and significant regeneration was observed in the hepatocytes. In contrast, the ConA + PHX group that was not administered exogenous IL-22 exhibited swelling, nuclear condensation and laminar necrosis of the hepatocytes and a near-total loss of the hepatic sinusoids.

In the liver, IL-22 plays an important role in the acute-phase response and possibly also plays a role in the promotion of liver regeneration^[2,6,12,13]. IL-22 acts *via* a heterodimeric receptor complex that consists of IL-22R α and IL-10R β ^[2,5,14,15]. Examinations

of the downstream signaling events following IL-22 administration in the context of partial hepatectomy demonstrated an increase in STAT3 activation. A substantial volume of published evidence supports the notion of STAT3-mediated cell survival and proliferation^[16-21]. Our present investigation revealed that the injection of IL-22 rapidly induced STAT3 activation in the liver and that STAT3 induced the expression of genes that are important for cell cycle progression (*e.g.*, Cyclin D1) and concurrently significantly increased PCNA staining to eventually promote cell survival and proliferation. These findings suggest that IL-22 was also partially responsible for hepatic STAT3 activation in this model. Thus, the activation of STAT3 by IL-22 was likely responsible for the protective role of IL-22 in the hepatocytes.

AFP is a specific marker of liver cancer tumors and is closely related to individual development, tissue regeneration, apoptosis and tumorigenesis^[22-25]. The main role of AFP in liver regeneration is the regulation of hepatocyte growth. It has also been demonstrated that in the context of the synergy of various growth factors, AFP mediates cell growth regulation *via* an interaction with a special cell membrane receptor that results in the uptake of arachidonic acid and AFP into the cell. The process provides the necessary substrate and signal transduction for the M phase of mitosis^[26,27]. Our present study found that following pretreatment with recombinant IL-22, AFP mRNA began to be expressed from 32 h, and this expression increased significantly by 48 h after hepatectomy. This increase in expression was probably due to the loss of the negative regulation of the transcription inhibitory factor. This expression trend reflects the promotion of cell proliferation in the liver by AFP mRNA. These findings suggest that IL-22 can decrease the expression of the transcription inhibitory factor to induce the expression of AFP mRNA and provide the necessary material basis for mitosis and thus eventually promote cell survival and proliferation.

In summary, the model of hepatectomy in T cell-mediated hepatitis induced by ConA simulates clinical

hepatectomy with liver disease accurately, and our findings suggest that IL-22 played protective and survival roles against liver injury in this model. Thus, IL-22 treatment should be considered to be a novel therapeutic option for liver injury and regeneration.

COMMENTS

Background

Interleukin (IL)-22 appears to play a protective role in inflammation and has also been demonstrated to exert proliferative effects in a hepatocyte cell line; however, it has rarely been reported that the protective and proliferative effects exist simultaneously. In this article, the authors sought to investigate the therapeutic effects and mechanisms of IL-22 in liver regeneration in mice with concanavalin A (ConA)-mediated liver injury following 70% hepatectomy.

Research frontiers

IL-22 has been demonstrated to play a protective role in inflammation and proliferative effects in a hepatocyte cell line, however, it has rarely been reported that the protective and proliferative effects exist simultaneously.

Innovations and breakthroughs

In this article, the authors investigated the therapeutic effects and mechanisms of IL-22 in liver regeneration in mice with ConA-mediated liver injury following 70% hepatectomy. IL-22 was demonstrated to play a protective role and have proliferative effects together.

Applications

IL-22 treatment should be considered to be a novel therapeutic option for liver injury and regeneration.

Peer-review

In this study, the authors demonstrated that IL-22 is a valuable factor for hepatocyte proliferation and can protect the liver from injury induced by ConA. The work is well-done and provides the interesting results.

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Basic Study

Human urokinase-type plasminogen activator gene-modified bone marrow-derived mesenchymal stem cells attenuate liver fibrosis in rats by down-regulating the Wnt signaling pathway

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Abstract

AIM: To evaluate the therapeutic effects of bone marrow-derived mesenchymal stem cells (BMSCs) with

human urokinase-type plasminogen activator (uPA) on liver fibrosis, and to investigate the mechanism of gene therapy.

METHODS: BMSCs transfected with adenovirus-mediated human urokinase plasminogen activator (Ad-uPA) were transplanted into rats with CCl₄-induced liver fibrosis. All rats were sacrificed after 8 wk, and their serum and liver tissue were collected for biochemical, histopathologic, and molecular analyzes. The degree of liver fibrosis was assessed by hematoxylin and eosin or Masson's staining. Western blot and quantitative reverse transcription-polymerase chain reaction were used to determine protein and mRNA expression levels.

RESULTS: Serum levels of alanine aminotransferase, aminotransferase, total bilirubin, hyaluronic acid, laminin, and procollagen type III were markedly decreased, whereas the levels of serum albumin were increased by *uPA* gene modified BMSCs treatment. Histopathology revealed that chronic CCl₄-treatment resulted in significant fibrosis while *uPA* gene modified BMSCs treatment significantly reversed fibrosis. By quantitatively analysing the fibrosis area of liver tissue using Masson staining in different groups of animals, we found that model animals with CCl₄-induced liver fibrosis had the largest fibrotic area (16.69% ± 1.30%), while fibrotic area was significantly decreased by BMSCs treatment (12.38% ± 2.27%) and was further reduced by *uPA*-BMSCs treatment (8.31% ± 1.21%). Both protein and mRNA expression of β -catenin, Wnt4 and Wnt5a was down-regulated in liver tissues following *uPA* gene modified BMSCs treatment when compared with the model animals.

CONCLUSION: Transplantation of *uPA* gene modified BMSCs suppressed liver fibrosis and ameliorated liver function and may be a new approach to treating liver fibrosis. Furthermore, treatment with *uPA* gene modified BMSCs also resulted in a decrease in expression of molecules of the Wnt signaling pathway.

Key words: Bone marrow-derived mesenchymal stem cells; Liver fibrosis; Urokinase plasminogen activator; Wnt signaling pathway

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Core tip: It has been confirmed that urokinase plasminogen activator (uPA) has a protective effect in liver fibrosis. Bone marrow-derived mesenchymal stem cells (BMSCs) have been discovered to provide effective therapy for liver fibrosis. Therefore, the present study was designed to investigate the therapeutic effects of *uPA* gene modified BMSCs in a rat model of CCl₄-induced liver fibrosis, and the impact on the Wnt signaling pathway which is involved in the pathogenesis of liver fibrosis. *uPA* gene modified BMSCs can suppress liver fibrosis and ameliorate liver function. Furthermore, it also resulted in down-regulation of molecules of the

Wnt signaling pathway and may be a new approach to treating liver fibrosis.

Ma ZG, Lv XD, Zhan LL, Chen L, Zou QY, Xiang JQ, Qin JL, Zhang WW, Zeng ZJ, Jin H, Jiang HX, Lv XP. Human urokinase-type plasminogen activator gene-modified bone marrow-derived mesenchymal stem cells attenuate liver fibrosis in rats by down-regulating the Wnt signaling pathway. *World J Gastroenterol* 2016; 22(6): 2092-2103 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i6/2092.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i6.2092>

INTRODUCTION

Liver fibrosis results from a sustained wound-healing response of the liver to a chronic injury, including viral infection, alcohol abuse, drug toxicity and autoimmune hepatopathy. It is characterized by excessive remodeling of extracellular matrix (ECM) and deposition of collagen^[1]. The activation of quiescent hepatic stellate cells (HSCs) to a myofibroblast-like phenotype is considered the key event in the pathogenesis of liver fibrosis^[2]. Currently, aside from liver transplantation, no other effective strategy exists to reverse or prevent fibrosis. Therefore, other approaches to treating fibrosis need to be investigated.

Bone marrow-derived mesenchymal stem cells (BMSCs) are non-hematopoietic cells with multi-lineage potential^[3,4]. They can be isolated from bone marrow and various other sources. BMSCs were recently discovered to provide effective therapy for liver fibrosis because of their multipotent differentiation, high self-renewal ability, and low immunogenicity^[5,6]. Several studies have demonstrated that BMSCs could reduce liver fibrosis or improve the liver function in rats with carbon tetrachloride (CCl₄)-induced liver fibrosis^[7,8]. However, the therapeutic effects were limited and needed to be improved^[9]. BMSCs have a particular pattern of cell proliferation and differentiation, enabling easy introduction and expression of the foreign gene and making it a potentially targeted cell for genetic treatment. Therefore, gene modified BMSCs may be a novel therapy to treat liver fibrosis.

For the continuing progress in the field of gene therapy for liver fibrosis, intensive efforts have been put in to design gene therapy strategies. Furthermore, the focus is on blocking any of the fibrogenic pathways and regulating the fibrinolytic homeostasis in a liver fibrosis animal model^[1]. The plasminogen activation system is involved in proteolysis, cell migration, tissue remodeling, and cell adhesion. Amongst them the urokinase plasminogen activator (uPA) system has been recently implicated in the inhibition of liver fibrosis^[10,11]. The uPA system consists of the serine protease uPA, its inhibitors, and receptor^[1]. uPA is a particular serine protease that converts inactive plasminogen into active plasmin, which degrades ECM

directly and catalyzes the activation of latent matrix metalloproteinases (MMPs)^[12]. Studies of knockout mice with an inactive uPA system have showed that uPA was involved in the pathological liver process after injury and the knockout mice had abnormally high liver fibrogenesis^[13]. The role of uPA has also been investigated in rats using CCl₄. In the CCl₄-induced acute liver injury model, lack of uPA led to the accumulation of fibrin and fibronectin within injured areas, insufficient removal of necrotic cells, and delayed repair. Similarly, plasminogen deficiency also caused excessive matrix accumulation and prominent activation of HSCs after liver injury^[14]. Moreover, Pohl *et al.*^[15] found that there was decreased expression of uPA in cultured-activated HSC, which resulted in low uPA activity and failed to resolve the fibrotic scarring.

Given the evidence above, we believed that uPA is a protective factor in liver fibrosis, therefore, increasing *uPA* expression in fibrotic liver tissues may reverse fibrosis and regenerate functional hepatocytes. Increasing studies re searching for the effect of *uPA* gene therapy have been carried out. However, the specific molecular mechanism remains unclear.

The Wnt signaling pathway consists of a highly conserved family of secreted glycoproteins that play an essential role in diverse arrays of biologic processes such as organogenesis, tissue homeostasis, and pathogenesis of many human diseases^[16-18]. On the basis of previous studies, the Wnt signaling pathway has been divided into canonical (β -catenin-dependent) and non-canonical (β -catenin-independent) signaling pathways^[19,20]. In the canonical Wnt signaling pathway, β -catenin is a chief downstream effector that mediates the Wnt signaling from the cell membrane to the cytoplasm^[21]. The non-canonical pathway is characterized by β -catenin independence, including the non-canonical Wnt/Ca²⁺ pathway and planar cell polarity (PCP) pathway^[22].

Lately, accumulating studies show that the Wnt signaling pathway is involved in the pathogenesis of liver fibrosis^[16,23,24]. It was reported that the Wnt signaling pathway obviously participates in HSCs activation, leading to liver fibrosis. Cheng *et al.*^[16] found that expression of both canonical (β -catenin) and non-canonical (Wnt4 and Wnt5a) Wnt genes was increased approximately 3-12 fold in culture-activated HSCs compared with quiescent HSCs. Kordes *et al.*^[25] also showed that the canonical Wnt signaling was active in freshly isolated HSCs from rats. A subsequent analysis of Kyoto encyclopedia of genes and genomes (KEGG) pathway also revealed that Wnt5a was involved in the activation of HSCs and played a role in liver fibrogenesis^[26]. Therefore, the Wnt signaling pathway contributes to HSCs activation, leading to excessive ECM deposition. There seems to be some sort of association between the *uPA* gene and Wnt signaling pathway. To date, no studies have been carried out to investigate the association between *uPA* gene and Wnt

signaling pathway in liver fibrosis.

Hence, in the present study, we introduced the human *uPA* gene into BMSCs by using an adenoviral vector and investigated its effect on liver fibrosis. The aims of our study were to evaluate the antifibrogenic effect of *uPA* gene modified BMSCs on liver fibrosis and to investigate the impact of *uPA* gene modified BMSCs treatment on Wnt signaling pathway.

MATERIALS AND METHODS

Animals and ethics statement

Male Sprague-Dawley (SD) rats were included in our study (Experimental Animal Center of Guangxi Medical University, China). All the rats were provided with standard feed and water ad libitum and individually housed at a constant temperature (18 °C-20 °C) and humidity (60%-70%) with a 12 h light/dark cycle. All animal experiments were approved by the Institutional Animal Care and Use Committee of Guangxi Medical University and the animal protocol was designed to minimize the pain and discomfort of the animals.

Cell culture and subculture

BMSCs derived from the SD rat were purchased from ScienCell Research Laboratories (San Diego, California, United States). Briefly, the cells were seeded at a density of 3×10^3 cells/cm² in T-25 culture flasks and incubated in Dulbecco's modified Eagle's medium-low glucose (DMEM; Gibco, Grand Island, United States) supplemented with 13% fetal bovine serum (FBS; Gibco), 50 U/mL penicillin and 50 mg/mL streptomycin at 37 °C in 50 mL/L carbon dioxide (CO₂). The culture medium was replaced after the first 24 h and every three days after that. After primary cultivation, the adherent cells reached almost 80% confluence. The plastic adherent cells were lifted up with 0.25% trypsin, suspended in fresh medium and transferred to a new flask for expansion. BMSCs at passage 4 (P4) were used for subsequent transduction and transplantation experiments.

Adenovirus infection

Replication-deficient E1 and E3 adenoviral vectors coding for non-secreted human uPA (Ad-uPA) cDNA were purchased from Biowit Biotechnologies (Shenzhen, China). The Ad-uPA expressed both green fluorescence protein (GFP) and human uPA. Adenoviral vector without the therapeutic gene (Ad-GFP) was obtained as the control adenovirus. For adenoviral transduction, the P4 BMSCs (1×10^6 cells/well) were seeded into 6-well plates for 24 h. BMSCs were then infected with Ad-uPA at different levels of multiplicity of infection (MOI) (20, 40, 60, 80, and 100). BMSCs were transfected with Ad-GFP under the same condition. After 48-72 h, light and fluorescent microscopy was performed to observe transfection efficiency and cell viability according to GFP expression

and cell morphology. The optimal MOI was chosen for both highest GFP expression and viability and used throughout the study.

Detection of human uPA expression in transfected BMSCs by Western blot

After the third day of *in vitro* adenovirus infection, transfected (Ad-uPA or Ad-GFP) and untransfected BMSCs were harvested and solubilized in protein lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitors. Protein concentration was determined with the BCA (bicinchoninic acid) protein assay kit (Beyotime, Jiangsu, China), and protein lysates were aliquoted and stored at -80 °C until use. Equal amounts of protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Beyotime, Jiangsu, China) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Boston, United States). The membrane was blocked with 50 g/L skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) at room temperature for 2-3 h. After a brief rinse, the membrane was incubated overnight at 4 °C in TBST with a rabbit anti-human uPA antibody (1:6000; Abcam, Cambridge, United States) and horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies (1:1000; Cell Signaling, Boston, United States) for 1-2 h at room temperature. Then the membrane was washed in TBST and protein was detected by enhanced chemical luminescence (ECL) (Beyotime Institute of Biotechnology, Haimen, China).

Experimental liver fibrosis model and cell transplantation

To induce liver fibrosis, 6-8-wk-old male SD rats ($m = 200-250$ g, $n = 30$) were injected with 400 mL/L CCl₄ subcutaneously (CCl₄:olive oil, 2:3) at a dose of 3 mL/kg every three days for 8 wk^[27]. Mock-treated rats were injected with olive oil alone as a normal control group ($n = 10$). At the end of the 4th week, the model rats were randomly divided into three groups: (1) uPA-BMSCs group (injected *via* the tail vein with 2×10^6 Ad-uPA-transfected BMSCs, $n = 10$); (2) BMSCs group (injected *via* the tail vein with 2×10^6 untransfected BMSCs, $n = 10$); and (3) model group (injected *via* the tail vein with an equal volume of normal saline, $n = 10$). Meanwhile, rats in the control group were given the same dose of normal saline. All rats were sacrificed at the end of the 8th week, and biological samples (liver tissues and blood samples) were obtained for molecular and histological analyses. Liver tissue was excised for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and western blot analysis. The remaining tissue was fixed and processed for histological analysis.

Biochemical assays

Immediately after blood sample collection, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL) and albumin (ALB) levels in serum were measured with an automated analyser (LX20; Beckman Coulter, Fullerton, CA, United States) at the First Affiliated Hospital of Guangxi Medical University. The levels of serum hyaluronic acid (HA), laminin (LN), and procollagen type III (PCIII) were detected by enzyme-linked immunosorbent assay (ELISA).

Histopathological analysis

Liver tissues were fixed in 40 g/L formaldehyde, embedded in paraffin and sectioned at a thickness of 5 μ m. Hematoxylin and eosin (HE) staining and Masson's trichrome staining were used for histological structure analysis and fibrosis area analysis, respectively. Five random views of Masson trichrome-stained sections from each sample ($n = 10$ /group) were captured by a light microscope (Olympus, Tokyo, Japan). The fibrotic area was checked with the Image J 1.44s software (National Institutes of Health, Bethesda, MD, United States)^[28]. The percentage of the fibrotic area was calculated by comparing the collagen stained area to the total area.

Quantitative detection of mRNA expression by qRT-PCR

qRT-PCR was used to assess the mRNA expression of molecules involved in Wnt signaling (β -catenin, Wnt4, and Wnt5a). Total RNA was extracted from liver tissues of each rats using chloroform and Trizol solution (TaKaRa Bio Inc, Shiga, Japan). First-strand cDNA synthesis was performed with 1 μ g of total RNA. Moreover, cDNA samples were thereafter amplified in the ABI Prism 7500 Sequence Detection system (Applied Biosystems, Massachusetts, United States) for 40 cycles (95 °C for 3 s, 60 °C for 34 s) with specific oligonucleotide primers (TaKaRa Bio Inc., Shiga, Japan). Each sample was analyzed in triplicate, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used for normalization. The relative quantification of target genes was determined using the $\Delta\Delta$ CT method^[29]. Primers used in qRT-PCR analyzes are listed in Table 1.

Western blot analysis of uPA, β -catenin, Wnt4, and Wnt5a protein expression

Total protein from liver tissue samples was extracted by the standard procedure^[30]. The BCA assay was used to estimate protein concentration. The following primary antibodies were used: rabbit anti-rat uPA antibody (1:6000 dilution; Abcam, Cambridge, United States), mouse anti-rat β -catenin antibody (1:1000 dilution; Origene Technologies, Maryland, United States), goat anti-rat Wnt4 antibody (1:5000 dilution; Origene

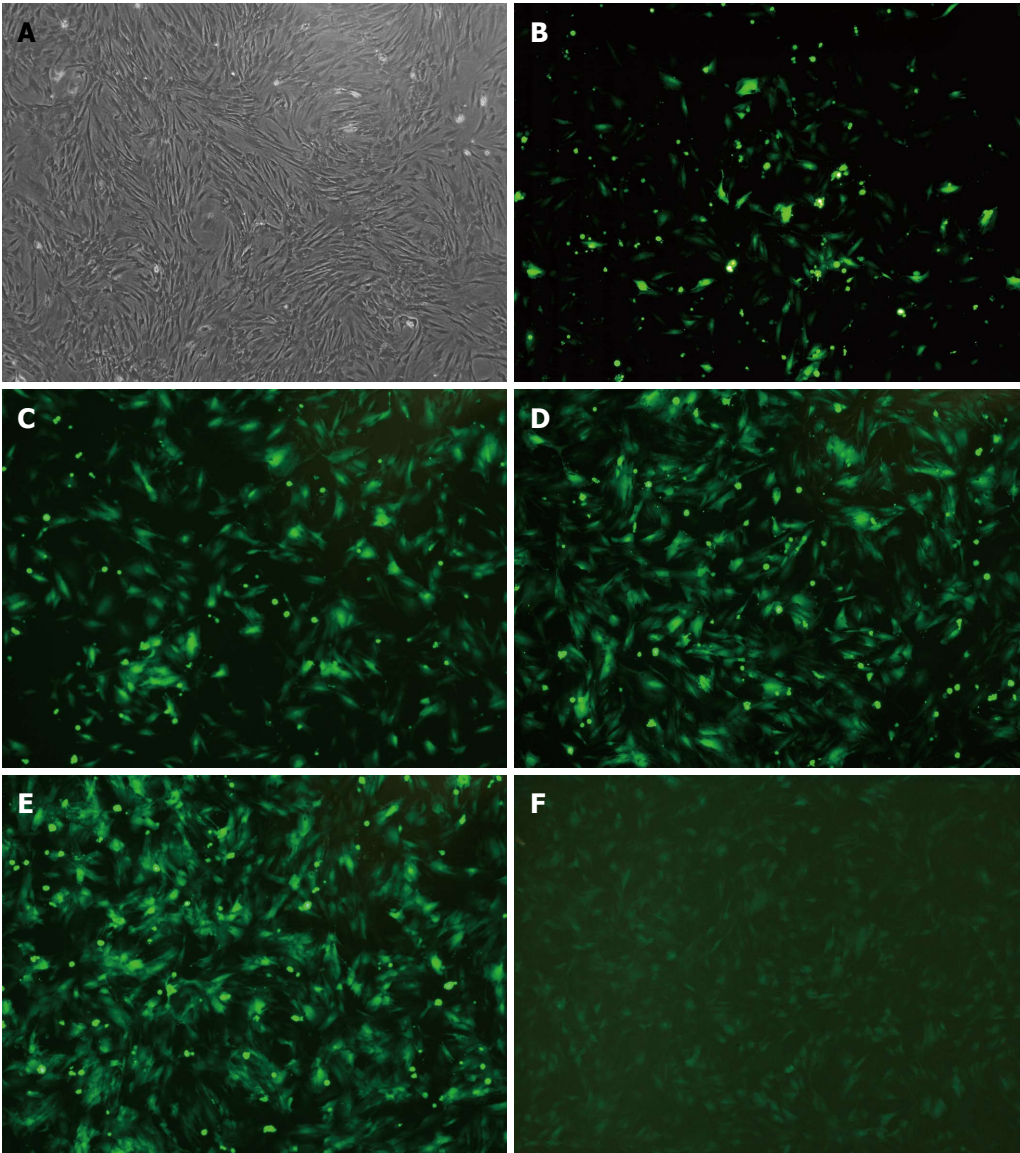


Figure 1 Morphological characterization of bone marrow-derived mesenchymal stem cells. A: The morphological appearance of bone marrow-derived mesenchymal stem cells (BMSCs) at passage 4 of cell culture (magnification 40 ×); B-F: Expression of green fluorescence protein 72 h after BMSCs transfection by Ad-uPA at different levels of multiplicity of infection (MOI) (MOI = 20, 40, 60, 80, and 100; magnification 40 ×).

| Table 1 Primer sequences for polymerase chain reaction | | |
|--|-----------|------------------------|
| Gene name | Direction | Sequence (5'-3') |
| <i>β-catenin</i> | Forward | ACGGCAATCAGGAAAGCAA |
| | Reverse | ACAGACAGCACCTTCAGCACTC |
| <i>Wnt4</i> | Forward | GCCATCTCTTCAGCAGGTGTG |
| | Reverse | CATAGGCGATGTTGTCCGAGC |
| <i>Wnt5a</i> | Forward | ACTTGCAACAATGAAGCAGGTC |
| | Reverse | CATAGGCGATGTTGTCCGAGC |
| <i>uPA</i> | Forward | CGAAGACTTCAGCGACGAAAC |
| | Reverse | CGAAGACTTCAGCGACGAAAC |
| <i>GAPDH</i> | Forward | TATGACTCTACCCACG |
| | Reverse | ATACTCAGCACCAGCATCACC |

Technologies), and rabbit anti-rat Wnt5a antibody (1:1000 dilution; Origene Technologies). Western blot was done using the method described above.

Statistical analysis

All data are expressed as the mean ± SD. Comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls *post hoc* test. *P*-values less than 0.05 were considered statistically significant. All the statistical analyses were performed with the statistical software package SPSS version 16.0 (SPSS Inc., Chicago, United States).

RESULTS

Morphological characterization of BMSCs

Rat BMSCs were successfully isolated and cultured in whole bone marrow adherent culture system. As shown in Figure 1A, after being subcultured four times, the cells remained in good status and the shape of

Table 2 Comparison of functional liver levels and liver fibrosis parameters in serum

| Group | n | ALT (U/L) | AST (U/L) | TBIL (μ mol/L) | ALB (g/L) | HA (μ g/L) | LN (μ g/L) | PCIII (μ g/L) |
|-----------|----|----------------------------------|-----------------------------------|---------------------------------|---------------------------------|-----------------------------------|-----------------------------------|---------------------------------|
| uPA-BMSCs | 10 | 74.95 \pm 12.66 ^{b,c} | 173.68 \pm 21.10 ^{b,c} | 18.66 \pm 2.52 ^{b,c} | 30.63 \pm 4.25 ^{b,c} | 105.71 \pm 15.21 ^{b,c} | 143.82 \pm 18.99 ^{b,c} | 27.14 \pm 5.93 ^{b,c} |
| BMSCs | 10 | 96.34 \pm 14.97 ^b | 228.65 \pm 25.66 ^b | 23.95 \pm 5.09 ^b | 26.65 \pm 3.79 ^b | 127.24 \pm 17.79 ^b | 173.15 \pm 22.32 ^b | 53.38 \pm 6.31 ^b |
| Model | 10 | 126.76 \pm 19.29 ^a | 287.67 \pm 26.17 ^a | 32.13 \pm 7.11 ^a | 22.60 \pm 4.35 ^a | 187.66 \pm 22.88 ^a | 224.45 \pm 23.40 ^a | 73.89 \pm 10.76 ^a |
| Control | 10 | 31.45 \pm 7.75 | 67.51 \pm 11.86 | 13.39 \pm 3.23 | 35.95 \pm 5.02 | 47.15 \pm 6.58 | 97.23 \pm 17.59 | 16.13 \pm 3.13 |

^a*P* < 0.05 vs control group; ^b*P* < 0.05 vs model group; ^c*P* < 0.05 vs BMSCs group. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TBIL: Total bilirubin; ALB: Albumin; HA: Hyaluronic acid; LN: Laminin; PCIII: Procollagen type III.

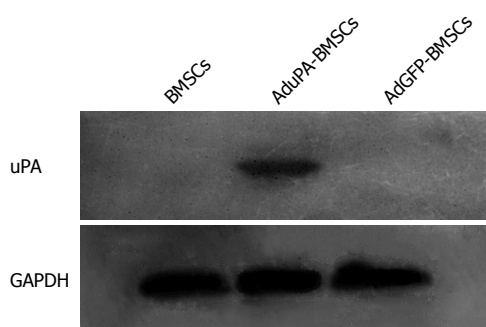


Figure 2 Western blot detection of urokinase plasminogen activator protein expression in bone marrow-derived mesenchymal stem cells after Ad-urokinase plasminogen activator transfection. Lane 1, uninfected bone marrow-derived mesenchymal stem cells (BMSCs); Lane 2, BMSCs infected with recombinant adenovirus containing human urokinase plasminogen activator (uPA) cDNA; Lane 3, BMSCs infected with control adenovirus. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

BMSCs changed from round shape initially to long spindle shape after that. Meanwhile, it was found that the P4 BMSCs formed colonies and distributed radially in the flask.

Detection of Ad-uPA transfection efficiency

To test the transfection efficiency, BMSCs were infected with adenoviral vectors (Ad-uPA or Ad-GFP) at MOI of 20, 40, 60, 80 and 100. As shown in Figure 1B-F, 72 h later, BMSCs were found to be in good condition when transfected at an MOI of 80 under the fluorescence microscope, with highest cell viability and transfection efficiency. The BMSCs were in better status when transfected at MOI of 20, 40 and 60. However, it featured poor transfection efficiency. There was strong green fluorescence at an MOI of 100, yet under the microscope, morphology retraction was found in some of the adherent cells as well as a few floating cells. The result suggested that 80 was the optimal MOI to be used for adenoviral infection.

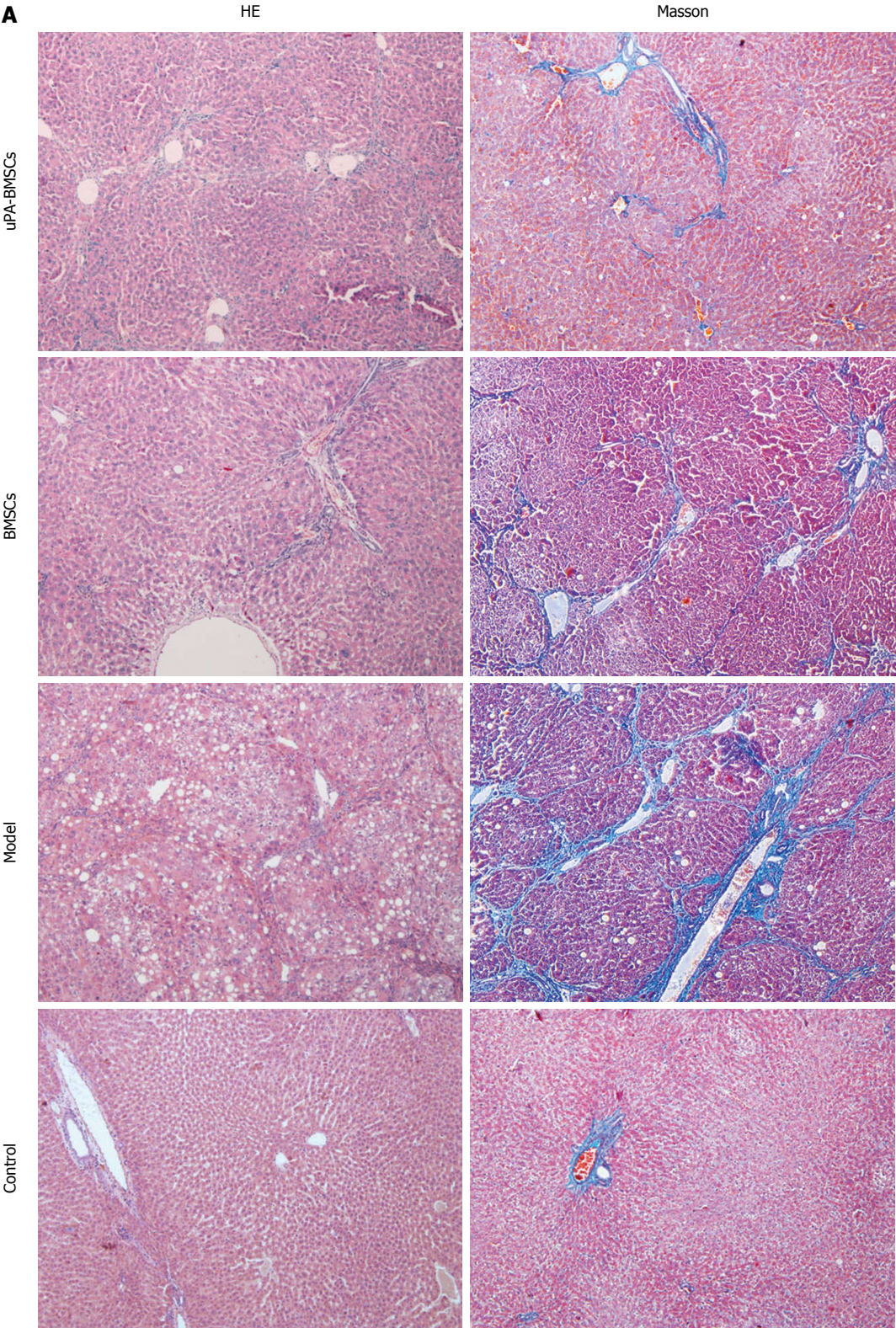
To further determine the expression of uPA in Ad-uPA-transfected BMSCs, Western blot analysis was performed. As showed in Figure 2, there was no positive band in Ad-GFP-transfected BMSCs and untransfected BMSCs. However, a specific band was only found in Ad-uPA-transfected BMSCs. These results indicated that uPA adenovirus had been successfully transfected into BMSCs, leading to significant expression of uPA *in vitro*.

Effect of uPA-BMSCs on concentrations of biochemical markers

As shown in Table 2, rats in the model group displayed chronic liver injury, with higher serum ALT, AST, and TBIL levels and lower ALB levels than in the control group (*P* < 0.01 for all). However, the levels of ALT, AST, and TBIL were significantly decreased, whereas the levels of ALB were increased in the uPA-BMSCs group compared with the model group (*P* < 0.05 for all). Serum ALT, AST, and TBIL levels in the BMSCs group were also decreased but higher than those in the uPA-BMSCs group. Moreover, as compared to the control group, higher levels of serum HA, LN, and PCIII were detected in the model group. Moreover, the BMSCs treatment significantly reduced all the above parameters compared with the model group (*P* < 0.05 for all) with an enhanced effect in the uPA-BMSCs group.

Effect of uPA-BMSCs on histopathologic characteristics

Histological examination using HE and Masson's staining were performed to show the extent of liver damage (Figure 3A). For HE staining, liver tissue samples from the normal control group showed normal lobular architecture with radiating hepatic cords pointing to central veins, whereas the model group exhibited fatty degeneration, ballooning changes of hepatocytes and necrosis. In contrast, uPA-BMSCs treatment remarkably ameliorated the adipose degeneration of hepatocytes and reduced the immigration of inflammatory cells compared with the model group or BMSCs group. For Masson's staining, the control group showed the normal architecture while the model group presented extensive liver bridging fibrosis and substantial collagen deposition. However, bridging fibrosis and collagen were distinctly decreased by the uPA-BMSCs or BMSCs treatment compared with the model group (Figure 3A). Also, quantitative analyzes of fibrosis area were consistent with the histological changes. The model group had the largest fibrotic area. Fibrotic area was significantly decreased by BMSCs treatment and was further reduced by uPA-BMSCs treatment (Figure 3B, control group, 1.03% \pm 0.66% vs model group, 16.69% \pm 1.30% vs BMSCs group, 12.38% \pm 2.27% vs uPA-BMSCs group, 8.31% \pm 1.21%; control group vs model group, *P* < 0.05; model group vs BMSCs group, *P* < 0.05; model group vs uPA-BMSCs group, *P* < 0.05; BMSCs group vs uPA-



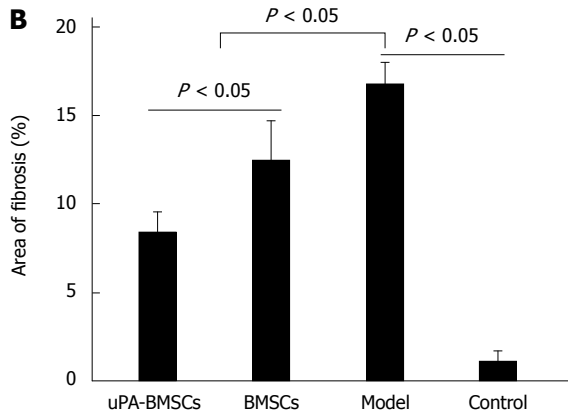


Figure 3 Histopathological change of liver tissue in different groups. A: HE and Masson staining was used to detect structural changes in liver tissues; B: Quantitative analyses of liver fibrosis were performed using Masson stained sections. Five random views from each sample in each group were analyzed. BMSCs: Bone marrow-derived mesenchymal stem cells; uPA: Urokinase plasminogen activator.

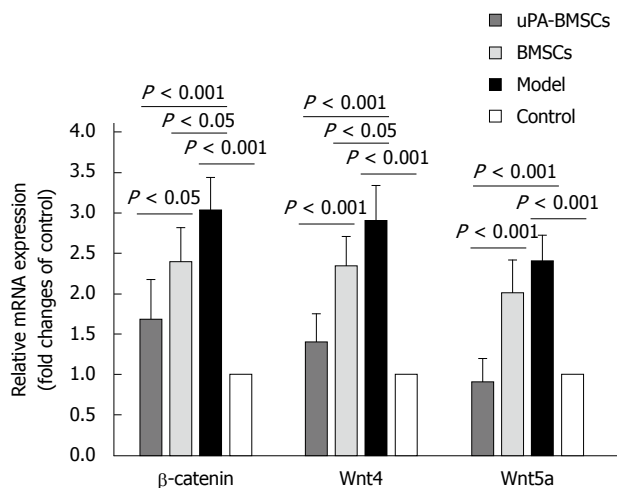


Figure 4 Effect of urokinase plasminogen activator gene modified-bone marrow-derived mesenchymal stem cells transplantation on the mRNA expression of molecules of the Wnt signaling pathway. The bar graph shows mean relative mRNA expression levels of β-catenin, Wnt4 and Wnt5a in liver tissues. Each sample was repeated three times from each cluster. Data are normalized to GAPDH mRNA expression levels. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; BMSCs: Bone marrow-derived mesenchymal stem cells; uPA: Urokinase plasminogen activator.

BMSCs group, $P < 0.05$).

Effect of uPA-BMSCs on the mRNA expression of β-catenin, Wnt4, and Wnt5a

We examined β-catenin, Wnt4, and Wnt5a mRNA expression levels in liver tissues by qRT-PCR (Figure 4). As revealed in Figure 4, low mRNA expression levels of β-catenin, Wnt4, and Wnt5a were detected in the control group, while they were significantly increased in the model group ($P < 0.05$ for all). Interestingly, uPA-BMSCs treatment could further reduce the expression levels of all these indexes compared with the model group or BMSCs group ($P < 0.05$ for all). According to these results, we hypothesized that uPA-BMSCs treatment could attenuate liver fibrosis *via* mechanisms that may be associated with decreased

activation of the Wnt signaling pathway.

Effect of uPA-BMSCs on the protein expression of uPA, β-catenin, Wnt4, and Wnt5a

It has been widely accepted that the Wnt signaling pathway participates in liver fibrosis. To further explore the association between the antifibrogenic effect of uPA-BMSCs transplantation and the Wnt signaling pathway in liver fibrosis, we examined the protein expression levels of uPA, β-catenin, Wnt4, and Wnt5a in liver tissues by Western blot (Figure 5A). The densitometric quantification (Figure 5B) of these bands showed that protein expression of uPA was apparently increased in the uPA-BMSCs group, suggesting the successful expression of uPA *in vivo*. Also, the protein expression levels of β-catenin, Wnt4, and Wnt5a were significantly increased in the model group compared with the control group ($P < 0.05$ for all). However, the uPA-BMSCs group had markedly lower protein expression levels of β-catenin, Wnt4, and Wnt5a than the model group or BMSCs group ($P < 0.05$ for all). Moreover, the BMSCs group displayed a slight increase in the expression levels of uPA with a slight decrease of β-catenin, Wnt4, and Wnt5a compared with the model group ($P < 0.05$ for all).

DISCUSSION

Liver fibrosis is a reversible consequence of chronic damage to the liver mainly in conjunction with the excessive deposition of ECM proteins in the liver^[1]. Progression of liver fibrosis eventually leads to cirrhosis, which can be associated with hepatocellular carcinoma and liver failure. Early intervention or treatment of liver fibrosis greatly reduces the risk of cirrhosis. However, there is no effective drugs to attenuate liver fibrosis presently. Therefore, new therapy strategies are being intensely investigated. In this study, we made a new attempt to deliver uPA gene modified BMSCs into rats with CCl₄-induced liver

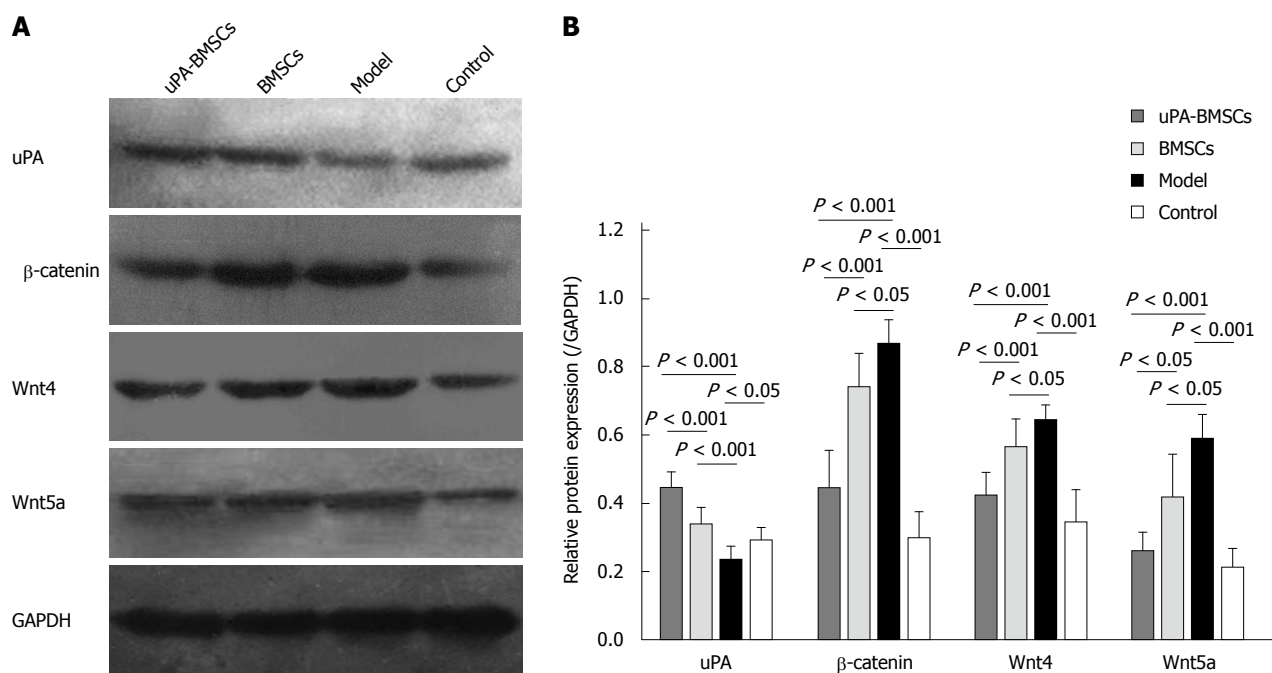


Figure 5 Effect of urokinase plasminogen activator gene modified-bone marrow-derived mesenchymal stem cells transplantation on the protein expression of molecules of the Wnt signaling pathway. A: Representative Western blot analysis of uPA, β-catenin, Wnt4, and Wnt5a protein expression levels in four groups; B: The bar graph represents mean relative protein expression levels of urokinase plasminogen activator, β-catenin, Wnt4, and Wnt5a. Data are normalized to GAPDH protein expression levels. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; BMSCs: Bone marrow-derived mesenchymal stem cells; uPA: Urokinase plasminogen activator.

fibrosis.

Numerous previous studies have showed that BMSCs can improve tissue repair, reduce inflammation^[31,32], and differentiate into hepatic cells *in vitro* and *in vivo*^[33,34]. As a result, BMSCs have attracted much attention over the past decade as a novel therapeutic paradigm for chronic liver diseases, such as liver failure^[35]. Both Sakaida *et al.*^[36] and Fang *et al.*^[37] showed that transplantation of BMSCs reduced CCl₄-induced liver damage and collagen deposition in mice. A therapeutic effect of transplanting BMSCs was also documented in CCl₄-injured rats^[7]. In our study, we found that the serum levels of liver function indexes were improved in the BMSCs treatment group as compared with the CCl₄-induced liver fibrosis rat model group. Meanwhile, BMSCs treatment significantly attenuated bridging fibrosis. These results were consistent with the previous studies. However, some reports indicated that the therapeutic effect of simple BMSCs therapy has been limited^[38-40]. Although BMSCs may enhance the regeneration of hepatocytes, it is hard to break down the reconstructed fibrous scar, even with increased secretion of anti-fibrotic factors. Therefore, the genetic modification of BMSCs might be a more promising option for improving their therapeutic potential.

In the present study, we investigated the effect of uPA gene modified-BMSCs on CCl₄-induced liver fibrosis in rats and the mechanism by which uPA-BMSCs ameliorate fibrosis. These data show that administration of uPA gene modified-BMSCs resulted

in a further improvement of liver fibrosis than BMSCs alone. Extensive evidence supports the fact that the plasminogen activation system participates in the matrix remodeling process, and alternative expression of the plasminogen activation system was found in fibrotic organs. Until now, the anti-fibrotic activity of uPA was confirmed in animal models of liver fibrosis^[41,42]. So we believe that a combination of BMSCs transplantation and uPA gene therapy may provide a novel tool for the treatment of liver fibrosis. In the present study, we chose the classical method by injecting with CCl₄ subcutaneously for 8 wk to develop the liver fibrosis rat model. Administration of CCl₄ to rodents is widely used to study the therapeutic method for hepatic injury. The reason for selecting CCl₄ was that CCl₄-induced fibrosis in rats shares similar pathological changes to human liver fibrosis that is characterized by centrilobular necrosis followed by hepatic fibrosis. According to our results, the uPA-BMSCs group had lower serum levels of ALT, AST, and TBIL, and higher ALB levels than the model or BMSCs group. Moreover, serum levels of HA, LN, and PCIII, which reflect ECM deposition, were significantly lower in the uPA-BMSCs group than in the model group or BMSCs group. Histological examination also indicated that uPA-BMSCs treatment remarkably reduced the deposition of collagen fibers compared with the model group or BMSCs group. These findings were also observed by Sun *et al.*^[43] who suggested that transplantation of uPA gene modified cell could suppress hepatic fibrosis and ameliorate liver function.

As mentioned above, a therapeutic effect of *uPA* gene modified BMSCs in liver fibrosis has been established more thoroughly than single BMSCs transplantation, and it may be a more favorable therapeutic option than BMSCs alone. As a result, the present study deduced the reason that increased expression of *uPA* gene not only improved degradation of ECM components directly or indirectly by activating the MMPs, but also possibly enhanced the transplantation of BMSCs by participating in cell proliferation, adhesion, migration, and angiogenesis in a plasmin-independent manner^[44]. However, the precise molecular mechanism remained to be defined and further investigated.

HSCs have been regarded as the main cells for synthesizing and secreting ECM components. Inhibition of the activation of HSCs has become an important treatment strategy for liver fibrosis. It has been proven that the Wnt signaling pathway is related to HSCs activation and fibrogenesis^[45,46]. It was found that inhibition of Wnt/ β -catenin signaling resulted in the down-regulation of HSC activation and attenuated CCl₄-induced liver fibrosis eventually^[24,25]. Hence, we assumed that the anti-fibrotic activity of *uPA* may be related to the down-regulation of the Wnt signaling pathway. In the present study, we investigated the effect of *uPA*-BMSCs on the expression of molecules of the Wnt signaling pathway by qRT-PCR and western blot. Results showed that the Wnt signaling pathway was abnormally activated in the model group, and *uPA*-BMSCs treatment can down-regulate the expression of molecules of the Wnt signaling pathway. Both significantly lower mRNA levels and protein levels of β -catenin, Wnt4 and Wnt5a in liver tissues were observed in the *uPA*-BMSCs treatment group compared with the model group. To some degree, we also concluded that *uPA*-BMSCs attenuated the development of liver fibrosis possibly partly by down-regulating the Wnt signaling pathway. To the best of our knowledge, the present study is the first attempt to report the potential association between *uPA* and the Wnt signaling pathway.

In conclusion, the present study displayed that *uPA* gene modified BMSCs significantly improved liver function and attenuated CCl₄-induced liver fibrosis, thus providing a new and efficient approach for the treatment of liver fibrosis by enhancing *uPA* expression to improve ECM degradation. Furthermore, it also resulted in decreased mRNA and protein expression of molecules involved in Wnt signaling, suggesting that it is antifibrotic partly due to the down-regulation of the Wnt signaling pathway. As the molecular mechanism involved in liver fibrosis was complicated, it needed to be further explored. While all of these conclusions are consistent with our data, the mechanisms by which *uPA*-BMSCs transplantation inhibits liver fibrosis remain to be defined. Thus, further studies should be carried out to support our findings here. What's more, future research on the safety and efficacy of *uPA*-BMSCs therapy in liver fibrosis is also needed to optimize this

approach for clinical applications that could be used to treat liver cirrhosis patients. Our current study may provide a foundation for designing therapeutic regimens for inhibiting the progression of chronic liver diseases in clinical settings.

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COMMENTS

Background

Bone marrow-derived mesenchymal stem cells (BMSCs) have been reported to be associated with the treatment of liver fibrosis. However, some reports indicated that the therapeutic effect of simple BMSCs therapy has been limited. Therefore, the genetic modification of BMSCs might be a more promising option for improving their therapeutic potential. Little is known about the therapeutic effects of human urokinase-type plasminogen activator (*uPA*) gene-modified BMSCs on liver fibrosis.

Research frontiers

Recently, it has been reported that *uPA* is a specific serine protease which plays an important role in extracellular matrix (ECM) degradation. Little is known about the therapeutic effects of human *uPA* gene-modified BMSCs on liver fibrosis. Therefore, *uPA* gene-modified BMSCs may be a novel therapy to treat liver fibrosis.

Innovations and breakthroughs

This study displayed that *uPA* gene modified BMSCs significantly improved liver function and attenuated CCl₄-induced liver fibrosis. Furthermore, it also resulted in decreased mRNA and protein expression of molecules involved in Wnt signaling, suggesting that it is antifibrotic partly due to the down-regulation of the Wnt signaling pathway.

Applications

This study provides a foundation for designing therapeutic regimens for inhibiting the progression of chronic liver diseases in clinical settings. Meanwhile, *uPA*-BMSCs therapy in liver fibrosis is hopeful to be optimized for clinical applications that could be used to treat liver cirrhosis patients.

Terminology

BMSCs, non-hematopoietic cells with multi-lineage potential, can differentiate into multiple mature cell phenotypes *in vitro*, including adipocytes, osteocytes, chondrocytes and so on. They are used for studies of stem cell differentiation, tissue engineering, cell and gene therapy, and have potential future clinical applications.

Peer-review

In this study, BMSCs transfected with Ad-*uPA* were transplanted into rats with CCl₄-induced liver fibrosis, to evaluate a possible therapeutic approach for treatment of liver fibrosis. The results revealed that *uPA* gene apparently was capable of BMSC modification by suppressing liver fibrosis through down-regulation of the Wnt signaling pathway. This well designed and executed study with an animal model of liver fibrosis, provides clear benefits of possible "gene therapy" in treatment of liver fibrosis. In general, the report is well written and the results are supported by the experimental data.

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Case Control Study

Prospective evaluation of the cause of acute pancreatitis, with special attention to medicines

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Abstract

AIM: To investigate the cause of acute pancreatitis (AP) by conducting a thorough investigation of drugs and their possible etiological role.

METHODS: We investigated the cause of AP in a large retrospective cohort of 613 adult patients admitted with AP at the Akershus University Hospital, Norway, from 2000 until 2009, who were evaluated with standard ward investigations. This group was compared with a prospectively evaluated group ($n = 57$) admitted from January 2010 until September 2010 who investigated more extensively using medical history and radiological assessment.

RESULTS: The groups were comparable with regards to gender, age, comorbidity and severity. The most common etiology was bile stones and alcohol, occurring in 60% in both groups. The prospective group was examined more thoroughly with regards to the use of alcohol and medicines. An increased number of radiological investigations during hospital stay and at follow-up were also performed. A more extensive use of radiological evaluation did not increase the detection frequency of bile stones. In the prospective group, more than half of the patients had two or more possible causes of pancreatitis, being mostly a combination of bile stones and drugs. No possible cause was found in only 3.5% of these patients, compared with 29.7% in the retrospective group.

CONCLUSION: A detailed medical history and extensive radiological evaluation may determine a possible etiology in almost all cases of AP. Many patients

have several possible risk factors, and uncertainty remains in establishing the definitive etiology.

Key words: Acute pancreatitis; Etiology; Medicines; Drugs; Bile stones; Alcohol

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Core tip: We conducted a study investigating a large cohort of patients admitted with acute pancreatitis to gain knowledge of the possible causes.

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INTRODUCTION

Acute pancreatitis (AP) is the most common disease of the pancreas, and is a significant cause of morbidity and mortality in patients admitted with abdominal pain. The etiology varies among countries. Bile stones and alcohol remain the main causes, accounting for about 70% of cases^[1]. The etiology is reported as unknown in 10%-20%, which is unfortunate, because patients with AP are at risk of new attacks^[2]. Diagnosis of bile stones might be missed if only first line imaging is conducted during the first acute admission^[3]. The aim of this study was to determine whether a more thorough medical history combined with more extensive radiological evaluation could increase the chance of finding the etiology of AP, thereby improving treatment and further prognosis. In Norway, there are 286 registered drugs with AP as a possible side effect, and their use is increasing^[4]. A causative relation between drugs and AP is difficult to establish in most cases. The pathophysiological pathways causing drug-induced pancreatitis are unknown and possible time-effect and dose-effect relations are also unknown. Using meticulous medical history and radiological examinations, several risk factors are often revealed. Many studies have attempted to find the causative action, stating both under-reporting as well as inaccuracies^[4,5].

In the present study, we compared a retrospective cohort of patients assessed with standard investigations with a cohort that was studied prospectively with a more extensive medical history, with special attention to the use of pharmacological agents, as well as broad radiological assessment. We presented circumstantial evidence for the possible role of drugs as causative factors in a defined geographical area.

Table 1 Patient characteristics *n* (%)

| | Prospective group (<i>n</i> = 57) | Retrospective group (<i>n</i> = 613) | <i>P</i> value |
|--------------------------|---------------------------------------|--|----------------|
| Age (yr) | 52.4 (17-94) | 58.9 (5-96) | 0.062 |
| Gender (M:F) | 35 (61): 22 (39) | 320 (52): 293 (48) | 0.179 |
| Comorbidity | | | |
| None | 14 (25) | 219 (36) | |
| Heart disease | 13 (23) | 127 (21) | |
| Hypertension | 19 (33) | 170 (28) | |
| Pulmonary disease | 5 (9) | 69 (11) | |
| Diabetes mellitus | 4 (7) | 57 (9) | |
| Previous pancreatitis | 15 (26) | 38 (6) | < 0.001 |
| Duration of pain (h) | 24 (0-504) | 20 (0-0.592) | |
| Severe pancreatitis | | | |
| CRP | 31 (54) | 276 (45) | 0.975 |
| CT | 3 (10) | 33 (10) | 0.945 |
| Complications | | | 0.660 |
| None | 45 (79) | 497 (81) | |
| Systemic | 8 (14) | 89 (14.9) | |
| Local | 4 (7) | 43 (7.2) | |
| Mortality | 1 (2) | 36 (6) | 0.356 |
| Severity of pancreatitis | | | 0.713 |
| Mild | 50 (88) | 521 (85) | |
| Moderate severe | 2 (4) | 54 (9) | |
| Severe | 5 (8) | 38 (6) | |

CT: Computed tomography.

PATIENTS AND METHODS

The study comprised two cohorts: (1) a retrospective analyses of clinical records of all patients (*n* = 613) admitted to the Akershus University Hospital with AP during a ten year period from January 2000 to December 2009; and (2) a prospective evaluation of 57 consecutive patients admitted with AP from January to September 2010. AP was defined as the acute onset of persistent epigastric pain in the presence of serum amylase level above three times the upper limit of normal (amylase > 200 IU/mL) or a computed tomography (CT) scan showing peripancreatic inflammation, with or without pancreatic necrosis. Organ failure and comorbidities were also documented to classify disease severity into mild, moderate or acute, according to the revised Atlanta classification system^[6]. In these patients, a thorough clinical history was taken, with special attention to alcohol and regular and/or new medication(s). Patient characteristics are shown in Table 1. When no cause was defined in the prospective group during first level radiological or laboratory investigation, further radiological evaluation was performed, either during the hospital stay, or at follow-up after 2-3 mo. Current and past regular and occasional medication use was registered. The sporadic nature of registration of non-prescribed medications in the retrospective cohort meant that this was not registered. Almost all medications in Norway must be prescribed by a doctor and are registered in a national

Table 2 Investigations during hospital stay and/or follow-up, *n* (%)

| | Prospective group (<i>n</i> = 57) | Retrospective group (<i>n</i> = 585 evaluable) | <i>P</i> value |
|-----------------------------------|------------------------------------|---|----------------|
| All patients with ultrasonography | 51 (89.5) | 420 (71.8) | 0.007 |
| All patients with CT | 45 (78.9) | 311 (53.1) | 0.001 |
| All patients with MRI | 32 (56.1) | 196 (33.5) | |
| US | 5 (8.8) | 142 (24.3) | |
| US + CT | 16 (28.1) | 143 (24.4) | |
| US + MRI | 7 (12.3) | 86 (14.7) | |
| US + CT+ MRI | 23 (40.3) | 49 (8.4) | |
| CT alone | 4 (7.0) | 88 (15.4) | |
| CT + MRI | 2 (3.5) | 31 (5.3) | |
| MRI alone | 0 | 30 (5.1) | |
| No imaging | 0 | 16 (2.7) | |

CT: Computed tomography; MRI: Magnetic resonance imaging; US: Ultrasonography.

pharmacological database^[7]. Akershus University Hospital Emergency Department serves around 500000 inhabitants, accounting for 10% of the Norwegian population, with a precise definition of geographical areas of responsibility and number of inhabitants in that area. This allows us to obtain precise figures of the number of users of different prescribed medications in our catchment area. Furthermore, there is a precise national publication describing all pharmacological agents and their side effects, such as AP^[8]. The frequency of side-effects are given as: frequent ($\geq 1/100$ -< 1/10), less frequent ($\geq 1/1000$ -< 1/100), seldom ($\geq 1/10,000$ -< 1/1000), very seldom (< 10,000) and case reports. We can therefore calculate the minimal and maximal expected cases of AP induced by medicines in our area, which is minimum 31 and maximum 310 cases of AP due to medicines in our catchment-area.

Statistical analysis

Student's two-tailed *t*-test and Pearson's χ^2 test were used to test differences between groups as appropriate, and Fisher exact test was used in cross-tables with numbers < 7.

RESULTS

Patient characteristics are shown in Table 1 and demonstrate that the retrospective and prospective groups were comparable with regards to gender, age, American Society of Anesthesiologists severity, CRP and CT-scores. As expected, the use of radiological investigations was more extensive in the prospective group compared with the retrospective group, as shown in Table 2. In the prospective group, all patients were investigated using some form of radiological examination, and many (40%) with three radiological tools: ultrasonography (US), CT and magnetic resonance imaging. Biliary and alcoholic pancreatitis was the most frequent etiology in the retrospective and

prospective group, at 61% and 58% respectively (Table 3). Table 4 summarizes details of the prospective group with regards to the use of medications with AP registered as a possible side effect. Fifty-eight percent (*n* = 33) of patients used these drugs, with 21 patients using one of these medications and 12 patients using more than two medicines associated with AP as a side effect (Table 5). In our prospective group, detailed description of medication use was noted and Table 5 summarizes the drugs used, showing statins and anti-hypertensive drugs to be the most commonly used medicines.

Our data shows a significantly higher number of patients reporting previous episodes of pancreatitis in the prospective groups compared with the retrospective group (26% vs 6%, respectively). This may be caused by a more thorough questioning of the patient at time of admission, and further stresses the importance of finding the causative factor, because this might increase the likelihood of optimizing treatment, improving prognosis and reducing risk of recurrence.

DISCUSSION

The most common cause of AP in Europe and North America is gallstones (50%) and alcohol (25%), whereas idiopathic pancreatitis is seen in around 10% of cases^[9]. The present study showed a lower frequency of bile-stones or alcohol (60%), whereas idiopathic pancreatitis was diagnosed in almost 30% in the retrospective group. In a large population-based analysis of 1224121 patient visits diagnosed with AP with a 75% admission rate, McNabb-Baltar *et al*^[10] found the etiology to be bile stones in 17.1%, alcohol in 14.6% and others/unknown in 67.8% of the patients. The discrepancy of these findings compared with our cohort might reflect the lower degree of investigations in outpatient cases with assumed lower severity and, therefore, less extensive radiological evaluation.

The present study revealed that a possible cause of AP can be defined in almost all cases if a meticulous history and radiological evaluation are performed. However, in a significant minority, uncertainty exists when several possible causes, such as bile stones, alcohol and medicines, are identified. In these cases, it is not possible to be certain which is the true cause.

In most patients, the etiology is determined during the first admission to hospital. First line evaluation consists of medical history and abdominal US, and often contrast-enhanced CT. In about 20% of patients where no definitive causative agent is found, a repeat meticulous history is also recommended^[11] and a secondary line of investigation is necessary, using magnetic resonance cholangiopancreatography (MRCP), endoscopic ultrasonography (EUS) and possibly endoscopic retrograde cholangiopancreatography (ERCP), as indicated. Repetition of EUS is also indicated, because this increases the sensitivity of

Table 3 Cause of acute pancreatitis in the prospective and retrospective group *n* (%)

| | Prospective group (<i>n</i> = 57) | | Retrospective group (<i>n</i> = 613) | | <i>P</i> value |
|----------------------------|------------------------------------|---------------------------------------|---------------------------------------|---------------------------------------|----------------|
| | Patients | Patients using medicines ¹ | Patients | Patients using medicines ¹ | |
| Bile stone | 23 (40.4) | 13 (57) | 271 (44.2) | 4 (0.1) | < 0.001 |
| Bile stone or alcohol | 4 (7.0) | 4 (100) | 14 (2.3) | | |
| Alcohol | 6 (10.5) | 1 (17) | 87 (14.2) | 1 (0.1) | |
| Hypertriglyceridemia | 3 (5.2) | 3 (100) | 8 (1.3) | | |
| ERCP | 1 (1.8) | 0 | 19 (3.1) | | |
| PTC | 1 (1.8) | 0 | 0 | | |
| Tumor/cancer pancreas | 1 (1.8) | 0 | 9 (1.5) | | |
| Pancreas divisum | 2 (3.5) | 1 (50) | 1 (0.2) | | |
| Stone in pancreatic duct | 1 (1.8) | 0 | 1 (0.2) | | |
| Postoperative pancreatitis | 0 | 0 | 3 (0.5) | | |
| Trauma | 0 | 0 | 1 (0.2) | | |
| Viral infection | 0 | 0 | 2 (0.3) | | |
| Medicines ¹ | 11 (19.3) | 11 (100) | 15 (2.4) | 15 (100) | |
| Unknown ² | 4 (7.0) | 0 | 182 (29.7) | 0 | |
| All | 57 (100) | 33 (58) | 613 (100) | | |

¹Medicines associated with pancreatitis as a side effect; ²Two of these had a previous cholecystectomy due to bile stones, suggesting small bile-stones as a possible cause. ERCP: Endoscopic retrograde cholangiopancreatography.

Table 4 Details of drugs used in patients with possible etiologies in the prospective group

| Etiology | Generic name | <i>n</i> |
|--|--------------------------------|----------|
| Bile stone (<i>n</i> = 23) | Azathioprine | 1 |
| of which medicine users (<i>n</i> = 13) | Simvastatin | 1 |
| | Diclofenac | 1 |
| | Amipril | 1 |
| | Enalapril | 1 |
| | Drospirenin/etinylostradiol | 1 |
| | Simvastatin, Amipril | 1 |
| | Atorvastatin, Ezetimib | 1 |
| | Amipril | 1 |
| | Simvastatin, Amipril, | 1 |
| | Diclofenac | |
| | Methotrexate | 1 |
| | Atorvastatin | 1 |
| | Simvastatin, | 1 |
| | Candesartan | 1 |
| | hydrochlorothiazide | |
| | Simvastatin, | 1 |
| | Losartan hydrochlorothiazide | |
| Bile stone or alcohol (<i>n</i> = 4) | Estradiol | 1 |
| of which medicine users (<i>n</i> = 4) | Ramipril, Prednisolone | 1 |
| | Estradiol | 1 |
| | Diclofenac | 1 |
| Alcohol (<i>n</i> = 6) | Prednisolone | 1 |
| of which medicine users (<i>n</i> = 1) | | |
| Hypertriglyceridemia (<i>n</i> = 3) | Gabapentin | 1 |
| of which medicine users (<i>n</i> = 3) | Simvastatin | 1 |
| | Venlafaxine | 1 |
| Medication (<i>n</i> = 11) | Azathioprine | 2 |
| of which medicine users (<i>n</i> = 11) | Simvastatin | 1 |
| | Atorvastatin | 1 |
| | Venlafaxine, Ramipril, | 1 |
| | Asparaginase | |
| | Atorvastatin | 1 |
| | Methotrexate, Prednisolone, | 1 |
| | Etanercept | |
| | Mycophenolic acid, Tacrolimus, | 1 |
| | Prednisolone | |
| | Cyclosporine, Pravastatin, | 1 |
| | Simvastatin, Ramipril | |
| | Diclofenac | 1 |

| | | |
|---|-----------------------|----|
| | Simvastatin, Losartan | 1 |
| | hydrochlorothiazide | |
| Pancreas divisum (<i>n</i> = 2) | Simvastatin | 1 |
| of which medicine users (<i>n</i> = 1) | | |
| Total number | | 34 |

diagnosing bile stones^[12]. If no cause is found, some authors suggest empirical treatment of the possible biliary cause using either cholecystectomy or ERCP and papillotomy^[13]. However, these procedures are surgical, with possible complications, including death^[13].

Bile stones and alcohol

In a Norwegian population study, ultrasound testing revealed gallbladder stones in 21.9% of the participants and the lifetime risk of biliary pancreatitis in the presence of gallstones has been reported to be around 2%^[14]. Therefore, with a much higher prevalence of bile stones in our study population, we assumed that bile stones were a definite risk factor and all patients admitted with biliary pancreatitis underwent cholecystectomy at our center.

Alcohol is a known independent risk factor in the development of both acute and chronic pancreatitis. Patients in the prospective group were specifically questioned with regards to type, amount and timing of consumption before admission. However, it is also well known that many of these patients have consumed alcohol many times before the attack of AP, without harmful effect on the pancreas. In our data, a dose-response curve could not be demonstrated, as patients with AP had ingested variable volumes and types of alcohol, on single or multiple occasions, without any direct correlation with disease severity. When alcohol was the suspected etiology, the patient was informed of its dangerous effect on the pancreas and recommended complete cessation of its intake. On

Table 5 Number of drugs used in patients with medicines as a possible etiology

| Use of a single drug | | Use of two drugs | | Use of three drugs | |
|-----------------------------|----------|--------------------------|----------|-------------------------------------|----------|
| Name | <i>n</i> | Name | <i>n</i> | Name | <i>n</i> |
| Azathioprine | 3 | Simvastatin, Amlodipine | 1 | Simvastatin, Ramipril, | 2 |
| Simvastatin | 4 | Simvastatin, Candesartan | 1 | Diclofenac | |
| | | hydrochlorothiazide | | | |
| Gabapentin | 1 | Simvastatin, Losartan | 2 | Atorvastatin, Ezetimib, Ramipril | 1 |
| | | hydrochlorothiazide | | | |
| Methotrexate | 1 | Simvastatin, Pravastatin | 1 | | |
| Estradiol | 1 | Ramipril, Prednisolone | 1 | Atorvastatin, Venlafaxine, Ramipril | 1 |
| Asparaginase | 1 | | | Methotrexate, Etanercept, | 1 |
| | | | | Prednisolone | |
| Diclofenac | 2 | | | Mycophenolic acid, Tacrolimus, | 1 |
| | | | | Prednisolone | |
| Atorvastatin | 2 | | | | |
| Venlafaxine | 1 | | | | |
| Furosemide | 1 | | | | |
| Enalapril | 1 | | | | |
| Drospirenen etinylostradiol | 1 | | | | |
| Estradiol | 1 | | | | |
| Prednisolone | 1 | | | | |
| Sum | 21 | | 6 | | 6 |

discharge, the general practitioner was also notified of the complete course of the hospital admission to provide a suitable follow-up.

Smoking in combination with alcohol has also been suggested to further increase the risk of pancreatitis^[15]. Therefore, we recommend informing patients of this both on a general health basis and specifically related to pancreatitis.

Drugs

The importance of drugs as a causative agent of AP is currently under discussion. Some authors question their relevance^[5], while others suggest that it is underreported^[4]. A causative link to the development of AP is difficult to document^[16]. Several case reports, as well as larger studies, have provided comprehensive data on these drugs and the limitation of published data^[5,17,18]. The most common drugs indicated include L-asparaginase, which may not be associated with elevated levels of amylase because inhibition of protein synthesis, as well as steroids, Valproic acid, Azathioprine and Mesalazine. AP caused by ciprofloxacin has also been reported in 3.1% of patient treated for infectious colitis, with cessation of disease on ciprofloxacin termination^[19]. This convincing drug-link to pancreatitis might reflect a possible amplifying drug side effect when combined with an acute inflammatory condition. In the present study, 33 (58%) of 57 patients used medication associated with AP. In a recent epidemiological report from the United States, statin use was as high as 50% for men and 36% for women between the ages of 65 and 74 years^[20]. The authors suggested that Simvastatin was associated with a reduced risk of AP in the cohort of users compared with non-users, and increasing doses of Simvastatin were associated with further risk reduction. According to the Norwegian Electronic Pharmacy Data,

the corresponding numbers in Norway were 39% for men and 36% for women (for Simvastatin, Lovastatin, Pravastatin, Fluvastatin, Atorvastatin, Rosorvastatin)^[7]. Simvastatin and other statins (*n* = 14) were the most commonly used pancreatitogenic drugs in our prospective cohort. Statins are also used commonly as a treatment for hypertriglyceridemia, which is also an independent risk factor for pancreatitis. Similarly, many patients with diabetes mellitus (DM) use statins to reduce cardiovascular risk. Patients with DM also have a higher risk of pancreatitis, which might be caused by the more common presence of gallstones and hypertriglyceridemia^[21]. Thus, these confounding variables must be considered, and a causative link is difficult to establish in individual patients because of the lack of studies investigating re-challenge and lifetime risk analysis. Many patients use the drugs intermittently and in combination with other drugs, which makes it difficult to determine accurately the causative role of the drug, which may have a direct toxic effect on the pancreas or an indirect effect on pancreas physiology^[22]. As with the introduction of any new medication, the patient should be asked to contact their physician if they experience any side effects. If the detailed medical history suggests that a drug is the most likely etiology, the necessity of drug continuation must outweigh the risk of recurrence of AP. The possibility of re-challenge and drug substitution must also be reviewed. These factors must be tailored according to the individual patient, and general recommendations are difficult to give.

Other causes of pancreatitis

Hypertriglyceridemia is a cause of AP secondary to fatty acid-induced acinar cell damage^[23], and might also be associated with a more severe course of the disease^[24]. Zeng *et al.*^[25] showed that a high level

of triglycerides was associated with a more severe disease in patients with bile stone pancreatitis. In the present study, three patients in the prospective cohort had hypertriglyceridemia as a possible cause of pancreatitis. All three patients used Simvastatin, which makes a certain determination of the true cause difficult.

Tumors in the pancreas were also considered a cause of pancreatitis in about 1.5% and 1.9% in our cohorts, which was similar to other studies. AP may be the presenting complication in up to 25% of patients with head of pancreas cancer.

In two patients in the retrospective cohort, viral infection was considered a causative agent. This has also been suggested by others describing AP caused by a rotavirus^[26]. The mechanism is thought to be either viral infection ascending to the pancreatic duct to cause infection, or edema of the Ampulla of Vater similar to the mechanism of bile stone-induced AP^[27-29].

Some reports are also emerging that suggest that genetic traits might be a disposition for the development of AP. In one study, the authors suggested that about 1/3 of patients with acute recurrent pancreatitis carried mutations associated with hereditary pancreatitis, including those in genes encoding trypsinogen (PRSS1), cystic fibrosis transmembrane conductor regulator (CFTR), achymotrypsinogen C (CTRC) and serine protease inhibitor Kazal type 1 (SPINK1)^[16,30]. Beger *et al.*^[2] found that the p.N34S mutation in the *SPINK1* gene was found more often in patients with AP (12%) than in the normal population (2.4%), regardless of etiology, suggesting that this mutation lowers the threshold for the development of AP. We speculated that such genetic traits might lower the threshold for AP caused by other factors, such as drugs.

Lastly, it is important to be aware of subgroups of patients, such as the elderly and children, who have a different spectrum of etiological factors, such as drugs, infections, trauma, anatomical abnormalities and secondary to *Ascaris* in the pancreatic duct^[1].

Limitations of the study

In this study, we only reported drug-use in patients presenting with AP requiring hospital admission. Registration of drug dosage, duration of use and drug rechallange were not performed when drug-induced pancreatitis was suspected as a possible cause; therefore, we emphasized how drugs may be a possible, but not definite, contributing factor to the reported cases in this study. Large case control studies would have an important role in furthering our knowledge compared with case reports that may be prone to bias towards newer drugs and more severe cases of AP.

Knowledge of etiology is crucially important to optimize treatment and, thereby, improve prognosis. Our data showed that in many cases of AP, we could find several possible etiologies. Bile stones and

alcohol remain the main causes of AP in about 60% of Norwegian patients, and these frequencies remained unchanged despite more detailed medical history and radiological investigations conducted in the prospective sample compared with the retrospective group. Drugs associated with AP used widely; however, a definite cause is difficult to prove in most cases. Therefore, we recommend meticulous assessment of the patient using detailed medical history and radiological investigation to establish the etiology.

COMMENTS

Background

Acute pancreatitis (AP) is a serious disease, resulting in considerable morbidity and mortality. Many causes have been identified; however, in many cases, the etiology is unknown.

Research frontiers

Several studies have been published showing a wide spectrum of drugs as possible causes of acute onset pancreatitis.

Innovations and breakthroughs

In this article, the authors compare a large cohort of retrospectively reviewed patients with AP with a prospective group investigated more broadly to identify the cause of disease onset.

Applications

A more detailed history and radiological evaluation might determine at least one possible etiology in almost all cases of AP. The clinician should therefore ensure that detailed investigation of the possible etiology has been performed to tailor treatment according to the patient's need.

Terminology

The revised Atlanta classification system is an international consensus classifying AP severity, allowing standardized reporting in research as well as in clinical practice.

Peer-review

The authors investigated possible causes of AP in retrospective and prospective cohorts of patients with a special focus on drug-induced disease. The most common etiologies were bile stones and AD alcohol in about 60% of cases, which is somewhat lower than the 80% reported elsewhere. No possible cause was found in 3.5% of the prospective cohort, which is also lower than previously reported data.

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Retrospective Study

Response to strict and liberalized specific carbohydrate diet in pediatric Crohn's disease

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Informed consent statement: As a retrospective review, a waiver of consent was granted by the Stanford University Human Subject Research Institutional Review Board for all study participants per the requirement that the research involved no more than minimal risk to the subjects, the waiver will not adversely affect the rights and welfare of the subjects, the research could not be practicably carried out without the waiver and pertinent information when appropriate will be provided after participation.

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Data sharing statement: A waiver of authorization was granted by the Stanford University Human Subject Research Institutional Review Board and all data was obtained from internal records review. Dataset available from the corresponding author at jburgis@stanford.edu.

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Abstract

AIM: To investigate the specific carbohydrate diet (SCD) as nutritional therapy for maintenance of remission in pediatric Crohn's disease (CD).

METHODS: Retrospective chart review was conducted in 11 pediatric patients with CD who initiated the SCD as therapy at time of diagnosis or flare. Two groups defined as SCD simple (diet alone, antibiotics or 5-ASA) or SCD with immunomodulators (corticosteroids and/or stable thiopurine dosing) were followed for one year and compared on disease characteristics, laboratory values and anthropometrics.

RESULTS: The mean age at start of the SCD was 11.8 ± 3.0 years (range 6.6-17.6 years) with five patients starting the SCD within 5 wk of diagnosis. Three patients maintained a strict SCD diet for the study period and

the mean time for liberalization was 7.7 ± 4.0 mo (range 1-12) for the remaining patients. In both groups, hematocrit, albumin and ESR values improved while on strict SCD and appeared stable after liberalization (P -value 0.006, 0.002, 0.002 respectively). The majority of children gained in weight and height percentile while on strict SCD, with small loss in weight percentile documented with liberalization.

CONCLUSION: Disease control may be attainable with the SCD in pediatric CD. Further studies are needed to assess adherence, impact on mucosal healing and growth.

Key words: Specific carbohydrate diet; Crohn's disease; Pediatrics; Nutrition therapy

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Core tip: Enteral nutrition is effective for both induction and maintenance therapy for pediatric Crohn's disease (CD), but adherence to a formula-based diet can be challenging. The specific carbohydrate diet (SCD) may offer a real-food nutritional therapy. Mild liberalization after response to a strict diet has not been described and may improve adherence while maintaining therapeutic effect. Laboratory parameters improved when following a strict SCD and were stable after liberalization. Despite this restrictive diet, growth was supported. The SCD may offer an alternative or adjunct to traditional medication therapy for pediatric CD.

Burgis JC, Nguyen K, Park KT, Cox K. Response to strict and liberalized specific carbohydrate diet in pediatric Crohn's disease. *World J Gastroenterol* 2016; 22(6): 2111-2117 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i6/2111.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i6.2111>

INTRODUCTION

The western lifestyle and a diet high in refined sugars and processed foods have been implicated to induce an abnormal immunological response in the digestive tract and potentially contribute to the increased incidence of inflammatory bowel disease (IBD) over the last 60 years^[1-3]. Diets high in fruits, vegetables and fiber have shown in some studies to be protective against the risk of developing IBD^[4,5]. Proposed mechanisms of how nutrition impacts intestinal inflammation include modifying the intestinal microbiota, generating an immune response to dietary antigen exposure and alteration in inflammatory cytokine profiles^[6-8].

Manipulation of nutrition creates a therapeutic opportunity in IBD. Enteral nutrition using formula as therapy has clear efficacy for inducing remission in pediatric Crohn's disease (CD)^[9-11]. However, formula-based diets carry a risk of taste fatigue, may require a

nasogastric tube or change the social dynamic around meals, and long term adherence may be difficult^[12]. Diets with limited ingredients such as the specific carbohydrate diet (SCD) may be more accepted and easier to follow for patients and their families.

The SCD was introduced by Haas in the 1920s as a therapy for celiac disease and was popularized by Gottschall, a biochemist and mother of a patient with ulcerative colitis in the late 1980s as a therapy for various intestinal disorders^[13,14]. The basic premise of SCD is restriction to simple carbohydrates found in fruits, honey, yogurt, vegetables and nuts. No grains or starches including wheat, rice, corn or potatoes are permitted. The diet postulates that undigested complex carbohydrates are malabsorbed and then fermented in the colon, producing acid and inflammatory byproducts, which worsen diarrhea and lead to bacterial overgrowth. The SCD allows proteins such as meat, poultry, fish and lactose free natural cheeses. Processed meats and other dairy products are not permitted. Fresh fruits and vegetables and some legumes, such as specific soaked beans and lentils, are encouraged. There is a focus on food quality and elimination of processed foods, specifically sucrose. The multi-factorial challenges of adhering to and remaining "legal" on the SCD can not be understated, and include limited availability of legal ready-to-eat foods and the demands of home food preparation. Given these challenges, concerns regarding patient adherence have been raised, which parallel questions about long term enteral nutrition use^[15,16].

A recent case series of seven children with CD on the SCD without immunosuppressive medications demonstrated symptom improvement *via* pediatric CD activity index after three months and significant improvement of serum albumin, CRP, hematocrit (HCT) and stool calprotectin over a long follow-up period (average 14.6 ± 10.8 mo)^[17]. A second prospective report of nine pediatric patients with active CD on the SCD demonstrated significantly improved disease index scores over the initial 12 wk. Improvement was sustained in 7 children who completed 52 wk on the diet, including mucosal healing documented in two patients by capsule endoscopy. No changes or addition of medications occurred during the study period^[18]. There are limited case reports of other diets with varied carbohydrate restrictions in patients with CD with positive outcomes^[19-21]. Other diets restricting refined sugar and modified fiber intake have shown mixed results^[22,23].

Of all these restricted carbohydrate diets, the SCD is the most restrictive and thus, difficult to maintain long term even when a distinct positive response is observed while following the diet strictly. Studies have shown that partial enteral nutrition at greater than 50% of caloric intake may be effective maintenance therapy in CD^[11]. There is rationale that over time allowing a small amount of illegal foods or ingredients on the SCD may not alter the potential therapeutic

Table 1 Diagnostic and treatment characteristics of patients

| Gender | Age at diagnosis (yr) | Prior treatment | Disease length (mo) | Concurrent treatment | Time liberalized (mo) | Follow-up (mo) | Added food |
|---------------------------|-----------------------|-----------------|---------------------|------------------------|-----------------------|----------------|--|
| SCD Simple | | | | | | | |
| F | 11.9 | None | 0 | None | 5.2 | 8.2 | Wheat daily, then corn, yeast, potatoes |
| M | 11 | None | 1.2 | None | 9.5 | 15.1 | Illegal meal on major holidays |
| M | 6.5 | None | 1.2 | None | Strict | 9.0 | Strict |
| M | 17 | Antibiotic 5ASA | 7.2 | 5ASA ¹ | Strict | 14.2 | Strict |
| F | 7.7 | Antibiotic | 6.6 | None ² | 10 | 12.4 | Illegal meal per week |
| SCD with Immunomodulators | | | | | | | |
| M | 11.8 | None | 0 | Prednisone × 6 wk | 2.5 | 8.8 | Rice daily, "rare" corn + Illegal meal per month |
| M | 14.4 | None | 1.2 | Prednisone × 4 wk | 12 | 13.7 | Illegal meal per week + rare illegal snacks |
| M | 12.2 | Budesonide | 3.0 | Budesonide × 4 wk | 14 | 17.5 | Illegal snacks daily then daily potatoes |
| F | 12.2 | 6MP | 21.6 | Prednisone × 4 wk, 6MP | 5 | 9.9 | Daily rice + Illegal meal per month |
| M | 10.5 | 5ASA, 6MP | 15.6 | 5ASA, 6MP | 3 | 9.5 | Illegal meal every 2 wk |
| M | 6.3 | 5ASA, 6MP | 39.6 | 5ASA, 6MP | Strict | 12.1 | Strict |

¹Antibiotic course for fistula with seton in place; ²Antibiotic taper for *Clostridium difficile* infection.

effect. Liberalization represents a real-world application of the SCD to facilitate longer adherence and possibly broader use.

No studies to date have evaluated the impact of mild liberalization from a strict SCD on disease control and growth parameters. We completed a retrospective chart review of pediatric patients with CD on the SCD to help evaluate clinical response while on a strict and on a liberalized SCD.

MATERIALS AND METHODS

Retrospective chart review was conducted of pediatric patients with CD followed in the pediatric gastroenterology practice at Lucile Packard Children's Hospital between 2003 and 2012. These patients were diagnosed with CD based on standard criteria including symptomatology, laboratory values, radiologic studies and endoscopy with pathologic confirmation. These patients chose to follow the SCD as primary therapy on their own accord. The SCD was started at the time of diagnosis or at the time of a disease flare. Some patients added the SCD to their medication therapy.

Data including age at diagnosis, disease location, medication use, dietary adherence, anthropometrics, and laboratory and imaging findings were extracted from the medical record (Table 1). Patients were stratified into two groups defined by overall treatment approach for induction and maintenance therapy including: SCD alone or in combination with only 5ASA or antibiotics vs SCD in combination with immunomodulating medications including corticosteroids or thiopurines. Corticosteroids were tapered over a maximum 6 wk time period and thiopurine dose was stable.

Patients followed the diet strictly for various time periods, and the SCD was liberalized by patients based on personal preference. Time at liberalization was defined as any significant variance from the SCD including one illegal meal more than once every month or the addition of an illegal ingredient on a regular basis. End of follow-up was defined as clinic visit or laboratory testing at approximately 12 mo while still using the SCD as primary therapy, whether strict or liberalized.

Descriptive statistics including mean, SD and range are reported for patient characteristics. Wilcoxon rank sum tests were used to compare laboratory values. Visual representation of median, quartiles and 95%CI are given in Figures 1-3. Test of statistical inference was deferred for weight and height due to the nature of covariance with patient age. The statistical methods of this study were reviewed by KT Park, MD from Stanford University. Technical appendix, statistical code, and dataset are available from the corresponding author. This study was reviewed and approved by the Stanford University Human Subjects Research Institutional Review Board. As a retrospective review, a waiver of consent was granted for all study participants. Technical appendix, statistical code, and dataset are available from the corresponding author at jburgis@stanford.edu. A waiver of consent was granted for this study; the presented data are anonymized and the risk of identification is low. This retrospective chart review served as a pilot study to NCT01749813 which is registered as a clinical trial. There are no conflicts of interest to disclose. No animal studies were conducted. Funding provided by private donation from George Serrurier through the Lucile Packard Foundation for Children's Health.

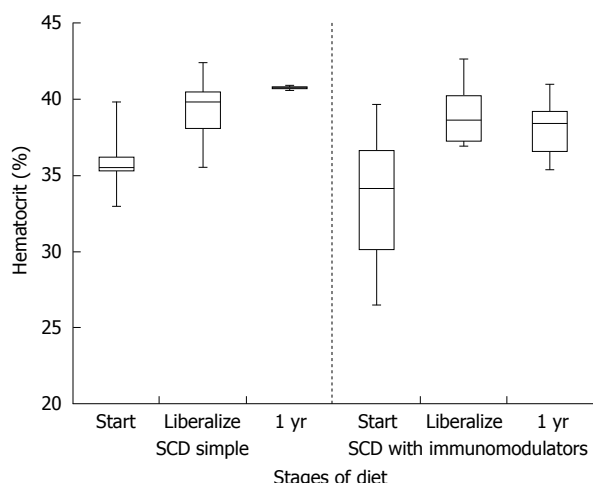


Figure 1 Change in hematocrit on the specific carbohydrate diet. SCD: Specific carbohydrate diet.

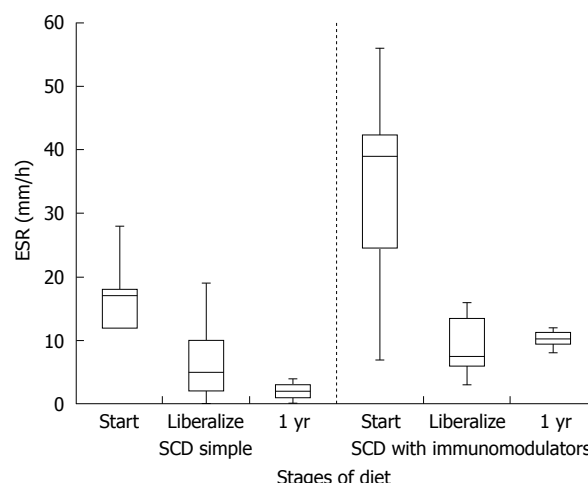


Figure 3 Change in erythrocyte sedimentation rate on the specific carbohydrate diet. SCD: Specific carbohydrate diet.

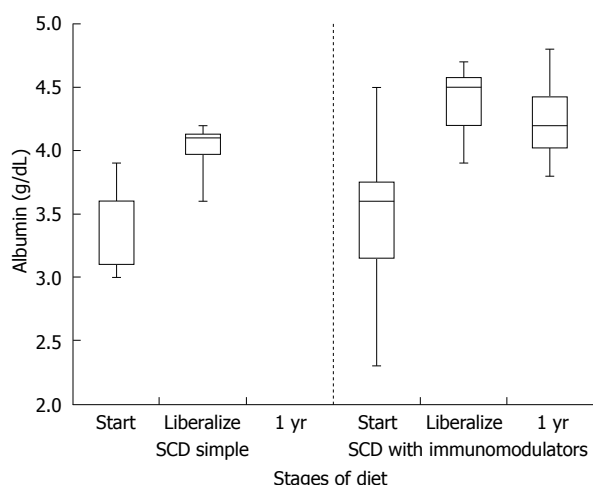


Figure 2 Change in albumin on the specific carbohydrate diet. SCD: Specific carbohydrate diet.

used the SCD alone without other medications; one patient completed an 8 wk vancomycin taper due to concurrent *Clostridium difficile* infection; one patient continued a 5-aminosalicylic acid and antibiotics for a fistula with seton in place (Ciprofloxacin for 4 wk and vancomycin for 12 wk). Of the six patients in the SCD with immunomodulator group, three patients started the SCD with a brief (4-6 wk course of corticosteroids) and one patient weaned off budesonide after 4 wk. Three patients in this group were on a stable dose of 6-mercaptopurine and continued 5-aminosalicylic acid. No patients were on biologics. No new medications were started during the study period (Table 1).

Liberalization

Three patients maintained a strict SCD diet for mean 11.8 ± 2.1 mo (range 9-14.2). The remainder of patients liberalized their diet at various times. The mean time for liberalization was 7.7 ± 4.0 mo (range 1-12). End of follow-up for all patients occurred at a mean 11.9 ± 3.0 mo (range 8.2-17.5) after starting SCD. For patients who liberalized, mean follow-up time on a liberalized diet was 4.2 ± 1.7 mo (range 1.7-6.5). Half of the patients added an illegal ingredient to their daily diet and the other patients added illegal meals or snacks at varying frequency, some as often as daily.

Changes in laboratory values

HCT values improved significantly while on a strict SCD for all patients (mean at start of SCD 35.6% vs mean at liberalization 39.7%, P -value 0.006). Improvement was similar between the simple SCD group and the SCD with immunomodulator group (P -value 0.855). After liberalization, HCT values appear to remain stable (Figure 1). The box plots for all figures are defined by 1st and 3rd quartile with median as middle line and error bars defined by minimum and maximum values. Significant improvement in albumin levels also occurred between start of SCD and time of

RESULTS

Patient characteristics

There were 11 patients who met eligibility criteria and underwent detailed medical record review including eight males and three females. One patient had isolated colonic disease and the remainder had small bowel and colonic disease at diagnosis. Including pathologic descriptions of gastritis, five patients had upper gastrointestinal tract involvement at diagnosis. Three patients had history of perianal disease; one with rectal fissures, one with fistula with seton in place, and another with history of perirectal abscess. The mean age at diagnosis of CD was 11.0 ± 3.2 years (range 6.3-17 years) and mean age at start of the SCD 11.8 ± 3.0 years (range 6.6-17.6 years). Mean duration of disease until initiation of the SCD 8.8 ± 11.8 mo (range 0-44.4 mo) with five patients starting the SCD within 5 wk of diagnosis (Table 1).

Of five patients in the simple SCD group, three

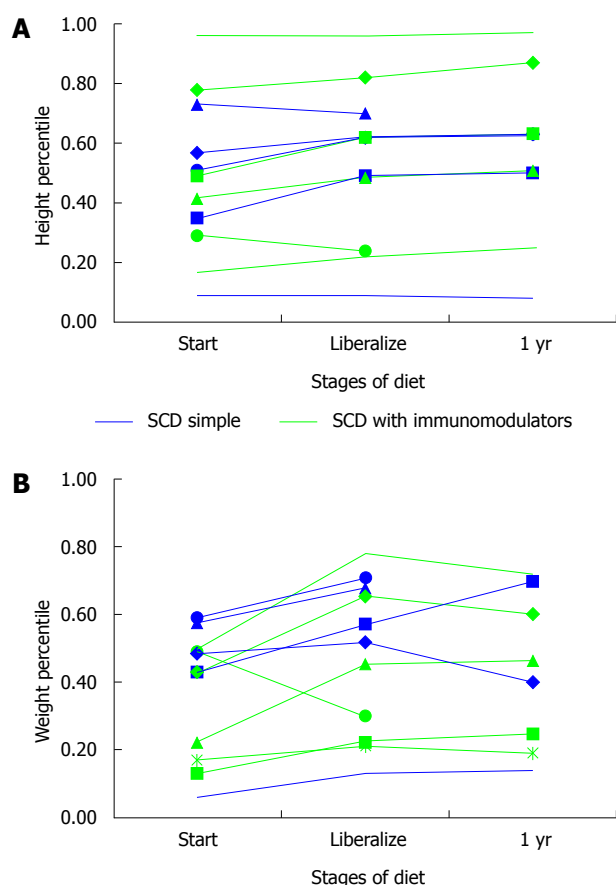


Figure 4 Change in height (A) and weight (B) on the specific carbohydrate diet. SCD: Specific carbohydrate diet.

liberalization (3.4 g/dL vs 4.2 g/dL respectively, P -value 0.002). There was a larger change in albumin in the SCD with immunomodulator group compared to the SCD simple group (0.9 g/dL vs 0.6 g/dL, respectively, P -value < 0.001). One patient in the SCD simple group had an albumin level of 3.8 g/dL after liberalization, which was stable. Albumin appeared to show a small drop in SCD with immunomodulator group after liberalization (Figure 2). Inflammation as measured by erythrocyte sedimentation rate (ESR) significantly decreased in all patients while on strict SCD (mean at start of SCD 26.5 mm/h vs mean at liberalization 8.27 mm/h, P -value 0.002). There was no significant difference in the change in ESR between the SCD simple and SCD with immunomodulator groups (P -value 0.14). ESR appeared relatively stable after liberalization for both groups (Figure 3).

Changes in anthropometrics and other metrics

Ten (90%) children gained in weight percentile while following a strict SCD diet (Figure 4A Height percentile-for-age and 4B Weight percentile-for-age). Nine (82%) patients had stable or increased height percentiles on a strict SCD. In the eight children who liberalized, weight loss was seen in 50% with a mean decrease of less than 1 percentile. Height percentiles during liberalization increased by 1 percentile or more in

all by one patient. Among all patients, only one had weight and height below the 10th percentile while on SCD, and she was using the SCD alone without other medications.

DISCUSSION

Despite preliminary data from two case series on the effectiveness of the SCD in pediatric patients with CD, it is currently unknown whether mild liberalization after initial response to a strict SCD could be detrimental. Building on the benefit of enteral nutrition in pediatric patients with CD, the SCD - even with mild liberalization - may offer a more sustainable real food therapeutic intervention. With the prospect of life-long medication therapy with intimidating side effect profiles, this nutritional therapy - even if difficult and restrictive - fills a need for patients and families willing to embrace the challenge.

Our retrospective study demonstrates improvements in anemia, albumin and inflammatory markers for patients in both cohorts when following a strict SCD. The majority of patients also demonstrated gains in both weight and height percentiles while on strict diet. After mild liberalization, most of these clinical laboratory changes were maintained for the follow-up period. Over time with liberalization, growth was relatively stable with a small mean decline in weight percentile and increased height percentile for most patients. A more thorough assessment of remission on the SCD, whether strict or liberalized, by disease index scores or measures of mucosal healing was not possible in this series due to the retrospective nature and varied provider practice. Evaluation at specific time points appeared to be limited by patients in this cohort as many had infrequent clinic visits and wanted to minimize surveillance, even blood draws.

Appropriate nutritional surveillance is paramount when dietary therapy is undertaken for CD, and adequate provision of calories is an obvious concern for a disease process inherent with inflammation, malabsorption and growth failure^[24]. As a retrospective review, we were unable to directly assess calorie provision, but an improved or stable weight and height percentile for most patients is a highlight of our study. Longer term follow-up would be needed to assess improvements in weight and height velocity over time. Close follow-up and detailed dietary logs reviewed by a registered dietician would be necessary to monitor caloric intake and macro and micronutrient needs for patients on dietary therapy in a more comprehensive prospective study.

Anecdotally, our patients are attracted to the SCD vs enteral nutrition as it offers an opportunity to eat conventional palatable foods as their main caloric source, avoidance of tube feedings and a less disruptive social dynamic around meals. Adolescents face additional challenges as they gain independence in preparing meals, encounter peer pressure and

consume more meals outside the home. Self-management, patient activation and adherence to therapy for IBD will continue to be a goal^[25]. We have seen improved acceptance among children whose parents have proficient cooking skills and dedicated time in the kitchen. Success has also been tied to social networks and connections with other families on the SCD for additional support and resources. Practical concerns have been raised in the literature about the SCD such as financial burdens on the family, long term adherence, physician knowledge of and comfort with patients and families who want to avoid standard medication therapy^[16,26]. These again are similar barriers and issues reported by pediatric gastroenterologists with the use of enteral nutrition as primary therapy for CD^[15]. Use of complementary therapies in children with IBD is frequent practice, and nutritional supplements and vitamins are commonly used^[27-29]. Many patients and families facing this challenging diagnosis are motivated to find an alternative approach, and dietary therapies are frequently sought out. Provider awareness is paramount to maintain a therapeutic alliance with the patient and offer appropriate clinical monitoring.

Overall, limited research exists on the use of the SCD as monotherapy or adjuvant to enteral or medication therapy. Our retrospective review provides additional support for the SCD to control inflammation as demonstrated by improvements in anemia, albumin and inflammatory markers and supporting stable growth parameters. To help understand its' possible therapeutic mechanism, we are currently completing a prospective pilot study of pediatric patients with CD on the SCD investigating the impact on disease activity, inflammatory markers including fecal calprotectin, cytokine profiles and intestinal microbiota populations.

COMMENTS

Background

Crohn's disease (CD) is thought to be triggered by autoimmunity, an exaggerated inflammatory cascade and interactions with gut microbiota. Standard therapy involves immunosuppressive medications but enteral nutrition (EN) can also modify the inflammatory response and alter the intestinal flora, providing an alternative therapeutic modality. Although EN has been shown effective to improve patient symptoms and control inflammation in pediatric CD, adherence to this formula-based diet can be challenging. The specific carbohydrate diet (SCD), which excludes complex carbohydrates such as refined sugar, gluten, starches and lactose, may provide a more palatable and flexible real-food alternative. In this study we evaluated the efficacy of a strict and liberalized SCD to improve markers of inflammation and growth parameters in pediatric CD.

Research frontiers

Patient driven interest in nutritional therapies is on the rise as a more holistic approach to avoid potential side effects of immunosuppressive medications. The risk for malnutrition and growth failure characteristic of pediatric CD makes nutritional decisions critical. Limited studies have been conducted on the effectiveness of various dietary modifications for disease control in pediatric CD. This study provides additional experience and expertise to help guide patient treatment decisions.

Innovations and breakthroughs

In this study, a strict SCD was effective to improve laboratory values and maintain growth parameters in pediatric patients with CD. These results are similar to limited prior studies. Our study also demonstrated the feasibility of strict adherence to the SCD for a mean of 7.7 ± 4.0 mo, much longer than standard EN regimens of 8-12 wk. In addition, patient laboratory values and growth parameters were stable despite mild diet liberalization.

Applications

This study demonstrates that the SCD offers therapeutic benefit as a nutritional modification to control inflammation in pediatric CD. Mild liberalization of the SCD, after a period of strict adherence, may provide an option for maintenance therapy which can promote long term adherence. More research is needed to help understand how restricted carbohydrate content can impact disease activity.

Terminology

CD is a chronic inflammatory condition of the gastrointestinal tract with a relapsing and remitting course. EN: Enteral nutrition refers to a diet restricted to a nutrient rich liquid formula. SCD: SCD avoids complex carbohydrates, excluding grains and most dairy products, focusing on meats, fruits, fresh fruits, vegetables, oils, nuts and honey.

Peer-review

The authors aim to investigate the SCD as nutritional therapy for maintenance of remission in pediatric CD. This is a quite non-complicated study using retrospective chart review. The authors studied 11 pediatric patients with CD who initiated the SCD as therapy at time of diagnosis or flare. Two groups defined as SCD simple (diet alone, antibiotics or 5-ASA) or SCD with immunomodulators (corticosteroids and/or stable thiopurine dosing) were followed for one year and compared on disease characteristics, laboratory values and anthropometrics. Conclusions of the authors are that disease control may be attainable with the SCD in pediatric CD. Enteral nutrition is indeed effective for both induction and maintenance therapy for pediatric CD.

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Retrospective Study

Healthcare and economic impact of diarrhea in patients with carcinoid syndrome

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Institutional review board statement: We conducted a retrospective cohort study using the Truven Health Analytics MarketScan® Database, a commercial health insurance claims database for employer-insured beneficiaries in the United States. The database is fully compliant with the Health Insurance Portability and Accountability Act and meets the criteria for a limited-use dataset. Since the patient and provider data included in this analysis were fully de-identified, this study was exempt from the Institutional Review Board review.

Informed consent statement: This study involved analyses of a Health Insurance Portability and Accountability Act-compliant secondary database, Truven Health Analytics MarketScan® Database, thus no informed consent was feasible or necessary.

Conflict-of-interest statement: Funding for this study was provided by Novartis Pharmaceuticals Corporation, One Health Plaza, East Hanover, NJ 07936-1080, United States. Maureen P. Neary is an employee of Novartis Pharmaceuticals Corporation. Michael S. Broder, Eunice Chang, and Dasha Cherepanov are employees of the Partnership for Health Analytic Research, LLC (PHAR, LLC), a health services research company paid by Novartis to conduct this research; Dorothy Romanus is a former employee of PHAR, LLC.

Data sharing statement: The study statistician, Eunice

Chang, conducted all statistical analysis for this study using a Health Insurance Portability and Accountability Act-compliant commercial-insurance secondary database, Truven Health Analytics MarketScan® Database.

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Abstract

AIM: To examine healthcare resource utilization patterns and costs accrued by carcinoid syndrome (CS) patients with and without diarrhea.

METHODS: We conducted a retrospective cohort study using MarketScan® data from 1/1/2002-12/31/2012. Newly diagnosed CS patients had 1 medical claim for CS (ICD-9-CM code 259.2) plus either ≥ 1 additional claim for CS or for carcinoid tumors (ICD-9-CM 209.x), and had no evidence of CS for 1 year prior to index

CS diagnosis, in commercially-insured patients < 65 years old. Patients were required to have continuous enrollment one year prior and after index date (first claim with CS diagnosis in the ID period). We identified patients with evidence of non-infectious diarrhea (ICD-9-CM codes 564.5 and 787.91) within one year from the index date. Overall and CS-related healthcare resource utilization and costs were compared between patients with and without non-infectious diarrhea during the one year period after the index date.

RESULTS: There were 2822 newly diagnosed CS patients; 534 (18.9%) had evidence of non-infectious diarrhea. Compared to patients without non-infectious diarrhea, non-infectious diarrhea patients more commonly had at ≥ 1 CS-related hospitalization (13.7% *vs* 7.2%), ≥ 1 CS-related ED visit (11.0% *vs* 4.4%), and CS-related office visits in one year (6.9 *vs* 4.1; all $P < 0.001$). After adjusting for demographics, region, number of chronic conditions and the Charlson Comorbidity Index, the proportions of patients with any and with CS-related hospitalizations were 9.7% and 6.8% higher, respectively, among non-infectious diarrhea patients compared to those with without non-infectious diarrhea ($P < 0.001$). Unadjusted costs were significantly higher among non-infectious diarrhea patients *vs* those without non-infectious diarrhea. The non-infectious diarrhea group was also more costly, with adjusted mean annual costs of \$81610, compared to \$51719 in the group without non-infectious diarrhea ($P < 0.001$).

CONCLUSION: Diarrhea is burdensome and costly in CS patients. Reduction of CS-related healthcare expenditures may be achievable through preventive treatment and appropriate management of diarrhea in CS.

Key words: Carcinoid; Neuroendocrine tumor; Diarrhea; Cost; Healthcare resource utilization

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Core tip: Healthcare resource utilization patterns and costs accrued by carcinoid syndrome (CS) patients with and without diarrhea have not been well described. We examined newly diagnosed CS patients using MarketScan® commercial claims data from 2003-2012 and found that non-infectious diarrhea (NID) is particularly burdensome and costly in CS patients. The adjusted proportions of patients with any and with CS-related hospitalizations were 9.7% and 6.8% higher in patients with NID than in those with no NID, respectively ($P < 0.001$). The NID group was also significantly more costly, with adjusted mean annual healthcare costs of \$81610, compared to \$51719 in the no NID group ($P < 0.001$).

MP. Healthcare and economic impact of diarrhea in patients with carcinoid syndrome. *World J Gastroenterol* 2016; 22(6): 2118-2125 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i6/2118.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i6.2118>

INTRODUCTION

Neuroendocrine tumors (NETs), historically known as carcinoids, are rare lesions that originate in clusters of secretory cells in the gastrointestinal, respiratory, and urogenital tracts and are typically indolent in nature^[1]. These neoplasms produce peptides and neuroamines that induce characteristic hormonal syndromes, including carcinoid syndrome (CS)^[2,3]. NETs comprised 0.66% of malignancies in the United States from 1973 to 2004^[1]. An analysis of the Surveillance, Epidemiology, and End Results (SEER) database found that the incidence of NETs has increased from 1.09 per 100000 individuals in 1973 to 5.25 per 100000 in 2004, with a prevalence of NETs in the United States may exceed 100000^[4].

The classic description of CS by Oberndorfer^[5,6] in 1907 included the triad of diarrhea, flushing, and bronchospasm. In a prospective single-institution database of over 900 patients with NETs, Ter-Minassian *et al*^[7] found that diarrhea, abdominal pain, and flushing were the most common presenting symptoms. Of the myriad of symptoms that afflict patients with CS, diarrhea appears to have a particularly profound impact on patients' sense of well-being. Based on surveys using the RAND version of the Short Form-36 (SF-36) and the Patient-Reported Outcomes Measurement Information System (PROMIS-29), patients with CS experience worse quality of life (QOL) compared to the general population and to other cancer patients and survivors. Those with uncontrolled diarrhea have reported even poorer QOL^[8].

The healthcare and economic burden of diarrhea in CS patients has not been previously quantified. The goal of the current study was to examine healthcare resource utilization (HRU) patterns and healthcare costs accrued by CS patients with and without diarrhea in an insured United States population.

MATERIALS AND METHODS

Data source

We conducted a cross-sectional, retrospective cohort study of newly diagnosed patients with CS using the Truven Health Analytics MarketScan® Database, using data between January 1, 2002 and December 31, 2012. In 2007, this claims database contained data for approximately 23 million employer-insured beneficiaries in the United States. The database includes patient-level medical and pharmacy claims submitted by large employers, managed care organizations, hospitals,

and Medicare and Medicaid programs. It contains longitudinal records of reimbursable services by insurance, including medical claims (hospital stays, outpatient visits, emergency department visits, home care services, laboratory and imaging services) and pharmacy claims (outpatient prescription drug claims). Claims include information on each physician visit, medical procedure, hospitalization, drugs dispensed, dates of service, number of days of medication supplied, test performed, and complete payment information. Each medical claim has a principal diagnosis and secondary diagnoses codes associated with it. Available patient demographic information includes age, gender, and geographic region. The database also includes enrollment information such as date of enrollment and disenrollment. The database is fully compliant with the Health Insurance Portability and Accountability Act (HIPAA) and meets the criteria for a limited-use dataset. Since the patient and provider data included in this analysis were fully de-identified, this study was considered exempt from approval by the Institutional Review Board.

Cohort selection

The study cohort comprised newly diagnosed patients with CS. Patients with a diagnosis of CS were identified if they had a medical claim for CS [International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM) code 259.2] between January 1, 2003 and December 31, 2011 [the identification (ID) period]^[9]. A single diagnosis code in claims may represent services provided to rule out a diagnosis. To increase the specificity of our cohort selection algorithm, we further required that eligible patients have either at least one additional claim for (1) CS; or (2) carcinoid tumors (ICD-9-CM 209.x), in any diagnostic field during the ID period. The date of the first claim with a diagnosis code for CS in the ID period was defined as the index date. To ensure the study population only included newly diagnosed cases of CS, patients were required to have no claims with a diagnosis code for CS during the year prior to the index date (disease-free period). All patients were followed for one year after the index date (study period). Patients were excluded if they were not continuously enrolled both during the disease-free period and for one year following the index date.

Study measures

Among all identified newly diagnosed CS patients, we identified patients with at least one claim for non-infectious diarrhea (ICD-9-CM codes 564.5 and 787.91) in any diagnosis field. We grouped CS patients according to whether or not they had experienced non-infectious diarrhea.

Patient demographic characteristics (age, gender, United States census region) were derived from the enrollment files. Baseline measures of disease burden were also included in the analysis. First, we calculated

the Charlson Comorbidity Index by examining the ICD-9-CM diagnostic codes appearing in medical claims within one-year prior to the index date^[10]. The Charlson Comorbidity Index is calculated using weights for comorbidities derived from medical claims through a validated prediction formula for 1-year mortality. Second, the number of chronic conditions experienced by each patient within one-year prior to the index date was calculated using the Healthcare Cost and Utilization Project Chronic Condition Indicator (CCI)^[9,11]. The CCI is a validated index which captures chronic conditions that last at least one year, and place limitations on self-care, independent living, and social interactions, or require ongoing medical care or special equipment.

HRU, including hospitalizations, emergency department (ED) visits, and physician office visits, was identified in medical claims during the study period. We examined the overall annual HRU related to any diagnosis occurring in the same period. In addition, we identified HRU related to CS based on claims with a primary diagnosis associated with carcinoid syndrome, CS-related symptoms, or carcinoid tumor progression. CS-related symptoms were identified with claims for non-infectious diarrhea (564.5, 787.91), nausea/vomiting (787.0x), flushing (782.62), asthma (493.x), dyspnea/wheezing (786.0x), cardiac palpitations (785.1), hypotension (458.0x), asthenia/fatigue (728.87, 780.71, 780.79), and dizziness (780.4). Carcinoid tumor progression was identified with claims for intestinal obstruction (560.0, 560.2, 560.9).

Cumulative annual healthcare costs were summed up for each patient from the index date. Costs were reported as total costs. In addition, we disaggregated the costs into medical costs (defined as costs related to medical claims), pharmacy costs (defined as related to pharmacy claims), inpatient hospitalization costs, ED visit costs, and outpatient (non-ED) costs.

Analyses

Descriptive statistics were conducted for all study measures. We reported means and SDs for continuous variables, and patient counts and percentages for categorical variables. To compare differences between groups with and without non-infectious diarrhea, χ^2 tests or *t*-tests were used for categorical and continuous variables, respectively. Our three key outcomes of interest were overall and CS-related hospitalizations, and total healthcare costs. We conducted multivariate analyses to compare the risk of overall and CS-related hospitalizations, and total healthcare costs between patients with and without non-infectious diarrhea. To model number of inpatient hospitalizations and number of ED visits, we used negative binomial models. All models were adjusted for age, gender, CCI, Charlson comorbidity index, and census region.

Costs were adjusted to 2012 United States dollars (the latest year of data in the study database) using

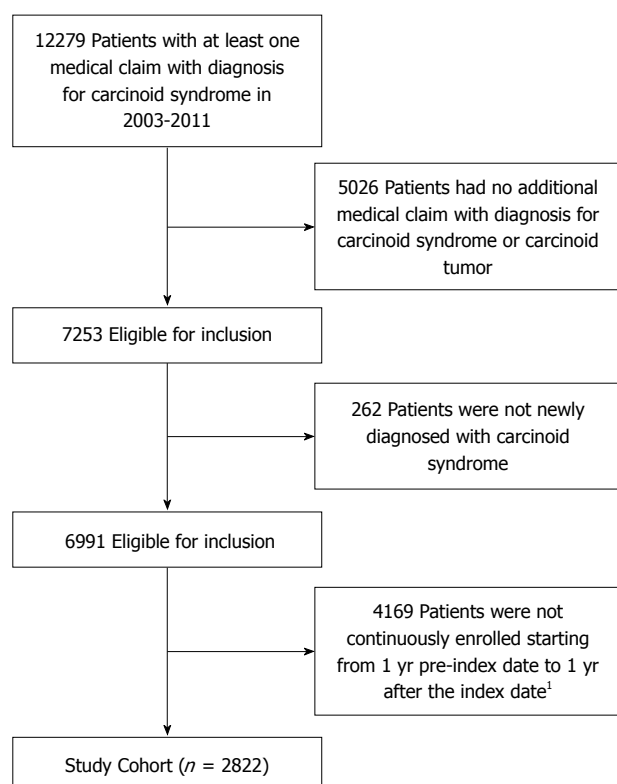


Figure 1 Flow diagram of study cohort. ¹The first claim with a diagnosis code for carcinoid syndrome during 2003-2011 was defined as the index date.

the medical care components of the Consumer Price Index. All reported *P* values are two-sided with a significance level of 0.05. To compare costs between patients with and without non-infectious diarrhea, we used multivariate analyses to adjust for baseline characteristics, including age, gender, region, number of chronic conditions and the Charlson Comorbidity Index. We used linear regression models to estimate overall healthcare costs, pharmacy costs, non-pharmacy costs, outpatient non-ED costs, and number of office visits, and logistic regression models for risk of inpatient hospitalization and ED visits. As the majority patients had no hospitalization or ED visit, we used a 2-step approach to estimate adjusted inpatient and ED costs. We first conducted logistic regression models to estimate the risk of an event then used linear regression to estimate the adjusted costs among patients who had such event. Data transformations and statistical analyses were performed using SAS[®] version 9.4 (SAS Institute, Cary, NC).

RESULTS

Baseline characteristics

The overall study cohort included 2822 patients newly diagnosed with CS (Figure 1). Mean age among patients was 51.5 years, 56.9% were women and 43.0% lived in the southern United States. Patients had a mean Charlson Comorbidity Index of 3.6 and were diagnosed with a mean 3.5 chronic conditions.

Table 1 Baseline characteristics of patients with carcinoid syndrome, stratified by evidence of non-infectious diarrhea

| | With non-infectious diarrhea <i>n</i> = 534 | Without non-infectious diarrhea <i>n</i> = 2288 | All newly diagnosed CS patients <i>n</i> = 2822 | <i>P</i> value |
|----------------------------|--|--|--|----------------|
| Age, yr | 51.3 ± 9.9 | 51.6 ± 10.1 | 51.5 ± 10.1 | 0.639 |
| Charlson comorbidity index | 3.7 ± 3.9 | 3.6 ± 3.8 | 3.6 ± 3.8 | 0.643 |
| No. of chronic conditions | 4.0 ± 2.4 | 3.4 ± 2.0 | 3.5 ± 2.1 | < 0.001 |
| Age group, yr | | | | 0.120 |
| ≤ 17 | 1 (0.2) | 22 (1.0) | 23 (0.8) | |
| 18-24 | 8 (1.5) | 30 (1.3) | 38 (1.3) | |
| 25-34 | 33 (6.2) | 114 (5.0) | 147 (5.2) | |
| 35-44 | 80 (15.0) | 279 (12.2) | 359 (12.7) | |
| 45-54 | 157 (29.4) | 752 (32.9) | 909 (32.2) | |
| 55-64 | 255 (47.8) | 1091 (47.7) | 1346 (47.7) | |
| Sex | | | | 0.005 |
| Female | 333 (62.4) | 1273 (55.6) | 1606 (56.9) | |
| Region | | | | 0.961 |
| North Central | 134 (25.1) | 563 (24.6) | 697 (24.7) | |
| Northeast | 86 (16.1) | 358 (15.6) | 444 (15.7) | |
| South | 224 (41.9) | 989 (43.2) | 1213 (43.0) | |
| West | 90 (16.9) | 378 (16.5) | 468 (16.6) | |

Data are expressed as absolute numbers (percentage) or mean ± SD. CS: Carcinoid syndrome.

Of the total study cohort, 534 patients (18.9%) had at least one claim associated with non-infectious diarrhea. There were no significant differences between those with non-infectious diarrhea and those without when considering age, geographic region and Charlson Comorbidity Index. A significantly higher number of chronic conditions was observed among those with diarrhea compared to those without (4.0 vs 3.4, *P* < 0.001). Additionally, a higher percentage of patients with diarrhea were female compared to those without (62.4% vs 55.6%, *P* = 0.005) (Table 1).

Unadjusted healthcare resource utilization and costs

CS patients with diarrhea had significantly higher rates of unadjusted healthcare resource utilization compared to patients without diarrhea (Table 2). In comparison to those without diarrhea, those with diarrhea more commonly had at least one hospitalization (49.6% vs 39.6%, *P* < 0.001), at least one ED visit for any cause (37.6% vs 20.9%, *P* < 0.001), as well as more all-cause office visits in one year (25.5 vs 18.7, *P* < 0.001). Moreover, the mean duration of all-cause hospitalization among patients with diarrhea was longer than in those without diarrhea (11.6 d vs 8.0 d, *P* < 0.001).

Similar trends were observed in unadjusted CS-related healthcare resource utilization. Compared to patients without diarrhea, patients with diarrhea more commonly had at least one CS-related hospitalization (13.7% vs 7.2%, *P* < 0.001), at least one CS-related ED visit (11.0% vs 4.4%, *P* < 0.001), as well

Table 2 Annual unadjusted healthcare utilization and costs in patients with carcinoid syndrome, stratified by evidence of non-infectious diarrhea

| | With non-infectious diarrhea <i>n</i> = 534 | Without non-infectious diarrhea <i>n</i> = 2288 | <i>P</i> value |
|---|--|--|----------------|
| Overall healthcare utilization | | | |
| Patients with hospitalizations | 265 (49.6) | 907 (39.6) | < 0.001 |
| Average LOS among hospitalized patients (d) | 11.6 ± 13.4 | 8.0 ± 9.2 | < 0.001 |
| Patients with ED visits | 201 (37.6) | 479 (20.9) | < 0.001 |
| Outpatient physician visits | 25.5 ± 18.4 | 18.7 ± 15.8 | < 0.001 |
| CS-related utilization ¹ | | | |
| Patients with hospitalizations | 73 (13.7) | 165 (7.2) | < 0.001 |
| Average LOS among hospitalized patients (d) | 7.4 ± 7.1 | 5.5 ± 3.6 | 0.029 |
| Patients with ED visits | 59 (11.0) | 101 (4.4) | < 0.001 |
| Outpatient physician visits | 6.9 ± 7.8 | 4.1 ± 6.1 | < 0.001 |
| Healthcare costs (USD) | | | |
| Total costs | 82032 ± 90181.7 | 51621 ± 63890.8 | < 0.001 |
| Total medical costs | 74654 ± 86742.3 | 47083 ± 61214.0 | < 0.001 |
| Total pharmacy costs | 7378 ± 13949.8 | 4538 ± 10314.2 | < 0.001 |

¹Claims with primary diagnosis of CS, CS-related syndrome, or carcinoid tumor progression. Data are expressed as absolute numbers (percentage) or mean ± SD. All costs were adjusted to 2012 United States dollars using the medical care components of the Consumer Price Index. CS: Carcinoid syndrome; ED: Emergency department; LOS: Length of stay.

Table 3 Multivariate analyses of annual overall and carcinoid syndrome-related hospitalization and total costs

| | Any hospitalization | | Any CS-related hospitalization ¹ | | Total costs | |
|----------------------------|---------------------|----------------|---|----------------|-------------------|----------------|
| | OR (95%CI) | <i>P</i> value | OR (95%CI) | <i>P</i> value | mean (SE) | <i>P</i> value |
| Age group, yr | | | | | | |
| ≤ 17 vs 55-64 | 0.84 (0.35-2.02) | 0.702 | 2.38 (0.78-7.22) | 0.127 | \$-8004 (13971.7) | 0.567 |
| 18-24 vs 55-64 | 1.33 (0.69-2.57) | 0.389 | 0.81 (0.24-2.70) | 0.726 | \$-8328 (10946.6) | 0.447 |
| 25-34 vs 55-64 | 0.93 (0.65-1.33) | 0.687 | 0.52 (0.25-1.10) | 0.087 | \$-10875 (5867.4) | 0.064 |
| 35-44 vs 55-64 | 0.97 (0.76-1.23) | 0.785 | 0.80 (0.52-1.24) | 0.314 | \$-5474 (3993.0) | 0.171 |
| 45-54 vs 55-64 | 0.93 (0.78-1.11) | 0.412 | 0.85 (0.63-1.16) | 0.307 | \$-2433 (2855.8) | 0.394 |
| Sex | | | | | | |
| Female vs Male | 0.88 (0.75-1.02) | 0.093 | 0.89 (0.68-1.17) | 0.401 | \$-7165 (2539.7) | 0.005 |
| Region | | | | | | |
| North Central vs West | 1.01 (0.79-1.28) | 0.951 | 0.88 (0.58-1.35) | 0.562 | \$-6623 (3954.7) | 0.094 |
| Northeast vs West | 0.91 (0.69-1.19) | 0.477 | 1.18 (0.76-1.85) | 0.458 | \$-7458 (4393.6) | 0.090 |
| South vs West | 1.02 (0.82-1.27) | 0.842 | 0.88 (0.60-1.29) | 0.521 | \$-11343 (3601.1) | 0.002 |
| No. of chronic conditions | 1.03 (0.99-1.08) | 0.107 | 0.98 (0.91-1.05) | 0.521 | \$1115 (665.6) | 0.094 |
| Charlson comorbidity index | 1.02 (1.00-1.04) | 0.099 | 0.99 (0.95-1.03) | 0.658 | \$5231 (354.5) | < 0.001 |
| Non-infectious diarrhea | | | | | | |
| Yes vs No | 1.48 (1.22-1.80) | < 0.001 | 2.12 (1.57-2.86) | < 0.001 | \$29890 (3204.5) | < 0.001 |

¹Claims with primary diagnosis of CS, CS-related syndrome, or carcinoid tumor progression. CS: Carcinoid syndrome.

as more CS-related office visits in one year (6.9 vs 4.1, $P < 0.001$). The mean duration of CS-related hospitalization among patients with diarrhea was also longer than in those without diarrhea (7.4 d vs 5.5 d, $P = 0.029$).

Unadjusted healthcare costs - both in total, and divided into medical and pharmacy costs-were also significantly higher among patients with diarrhea compared to those without (Table 2). CS patients with diarrhea incurred \$82032 in annual total costs, 58.9% higher than the \$51621 among those without diarrhea ($P < 0.001$). In the one-year post index date, those with diarrhea also had higher medical (\$74654 vs \$47083, $P < 0.001$) and pharmacy costs (\$7378 vs \$4538, $P < 0.001$) compared to those without diarrhea. Components of medical cost also differed

significantly between groups. Inpatient costs were \$27018 in patients with diarrhea compared to \$16609 in those without ($P < 0.001$). Outpatient medical costs were \$46917 vs \$30140 ($P < 0.001$), and ED costs were \$719 vs \$334 ($P < 0.001$) in CS patients with vs without diarrhea.

Adjusted healthcare resource utilization and costs

After adjusting for age, gender, geographic region, number of chronic conditions and the Charlson Comorbidity Index, the differences in our primary outcomes of interest were still significant in the one-year post index date. The odds of any hospitalization were 1.48 (95%CI: 1.22-1.80, $P < 0.001$) (Table 3) times greater among those with diarrhea compared to those without. The adjusted proportion of patients with

Table 4 Adjusted multivariate analyses of annual overall and carcinoid syndrome-related hospitalization and total costs

| Non-infectious diarrhea | Adjusted proportion of patients with any hospitalization ¹ % (95%CI) | Adjusted proportion of patients with any CS-related hospitalization ^{1,2} % (95%CI) | Adjusted total cost ¹ mean (95%CI) |
|-------------------------|--|---|--|
| Yes | 49.4 (45.1-53.6) | 13.8 (11.1-17.1) | \$81610 (\$75962-\$87258) |
| No | 39.7 (37.7-41.7) | 7.0 (6.0-8.2) | \$51719 (\$49007-\$54432) |

¹Adjusted by age, gender, region, number of chronic conditions and the Charlson Comorbidity Index; ²Claims with primary diagnosis of CS, CS-related syndrome, or carcinoid tumor progression. CS: Carcinoid syndrome.

any hospitalization was 9.7% higher among those with diarrhea [49.4% (45.1%-53.6%) vs 39.7% (95%CI: 37.7%-41.7%)] (Table 4, Figure 2). Patients with diarrhea had 24.2 (95%CI: 22.9-25.5) office visits in the study period compared to 19.0 (95%CI: 18.4-19.7) in those without ($P < 0.001$).

The difference in odds was also significantly greater for CS-related hospitalizations. Those with diarrhea were at 2.12 (95%CI: 1.57-2.86, $P < 0.001$) (Table 3) times greater odds of CS-related hospitalization compared to those without diarrhea. The adjusted proportion of patients with any CS-related hospitalization was 6.8% higher among those with diarrhea [13.8% (95%CI: 11.1%-17.1%) vs 7.0% (95%CI: 6.0%-8.2%)] (Table 4, Figure 2).

The adjusted total healthcare costs were significantly higher, by \$29890 ($P < 0.001$) for those with diarrhea at \$81610 (95%CI: \$75962-\$87258) compared to those without diarrhea at \$51719 (95%CI: \$49007-\$54432) (Tables 3 and 4, Figure 2). Pharmacy costs were \$2557 ($P < 0.001$) more than in patients without diarrhea. Medical costs were higher by \$27334 ($P < 0.001$). Medical costs comprised outpatient visits (cost of which was \$16695 higher) and inpatient hospitalization (\$11431 higher) ($P = 0.003$).

DISCUSSION

Beyond its deleterious influence on quality of life, our results demonstrate that diarrhea in patients with CS has a significant medical and economic impact. Our findings suggest that diarrhea associated with CS accounts for 1.5-fold higher total healthcare spending and almost a 2-fold higher risk of CS-related hospitalizations compared to when diarrhea is not present. The adjusted mean total healthcare costs in our analysis were \$81610 compared \$51719 per year among patients with CS who suffered from diarrhea compared to those who did not have diarrhea ($P < 0.001$). The adjusted risk of CS-related hospitalizations increased from 7.0% among CS patients with no evidence of diarrhea to 13.8% who were diagnosed

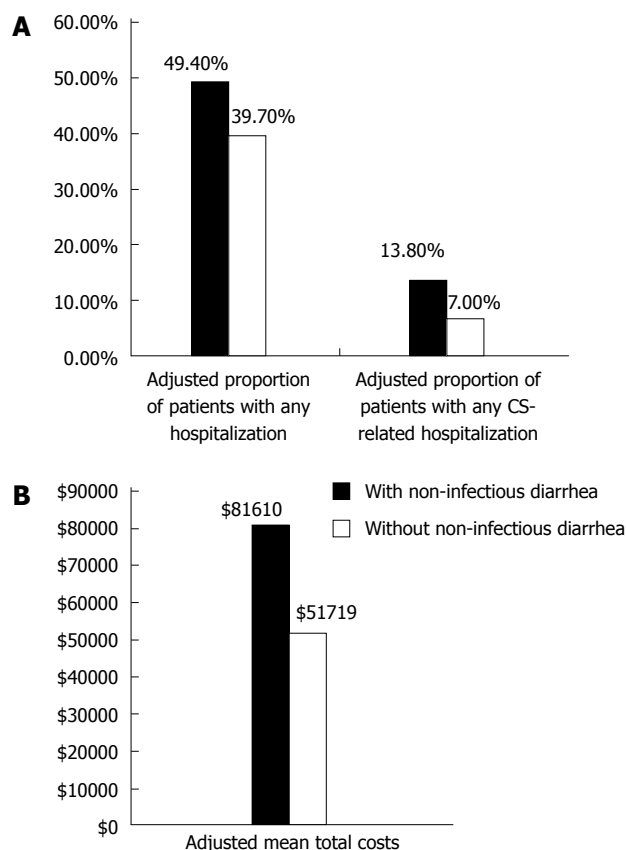


Figure 2 Adjusted proportions of overall and carcinoid syndrome-related hospitalization and mean total costs. A: Adjusted proportion of patients with any hospitalization and carcinoid syndrome-related hospitalization by evidence of non-infectious diarrhea; B: Adjusted mean total annual costs by evidence of non-infectious diarrhea. Results were adjusted by age, gender, region, number of chronic conditions and the Charlson Comorbidity Index.

with diarrhea symptoms ($P < 0.001$). Effective preventive treatment of diarrhea in patients suffering from CS would be a reasonable approach to reducing healthcare resource utilization and costs in this population.

Our results indicate that diarrhea symptoms are common in patients with CS. In the present analysis, we found 18.9% of cases with at least one claim for the diagnosis of non-infectious diarrhea in the one-year study period. These findings are comparable to an analysis of commercially insured patients with NETs in which 17.6% of patients had symptoms of diarrhea and up to 72% of patients had CS within the same time period^[12]. In that analysis the annualized total healthcare costs for patients with NETs were estimated at approximately \$106000 (2011 United States \$)^[12]. While those cost estimates were not disaggregated by CS symptoms, both analyses highlight the substantial economic burden associated with treatment of patients with CS.

Patients with NETs have significantly higher rates of mortality and hepatic and gastrointestinal morbidities compared to patients without NETs or other cancers matched by age, sex, and year of diagnosis^[13]. They also have worse health-related QOL than general

population controls, with evidence that this is attributable to symptoms such as diarrhea, fatigue, and depression^[14,15]. Fröjd *et al.*^[14] used the EORTC QLQ-C30 instrument to demonstrate that diarrhea, along with fatigue, had particularly prominent adverse impact on physical, emotional, and social well-being in a cohort of Swedish patients with NETs.

Chronic secretory diarrhea in patients with NETs results from imbalances in intestinal absorptive and secretory processes, leading to dehydration, renal insufficiency, and various serum electrolyte imbalances, and improper digestion of food^[16]. Nutritional deficiencies caused by inadequate digestion may further exacerbate weight loss and fatigue caused by fluid and electrolyte loss^[16]. NETs are among eight different neoplastic diseases known to cause chronic diarrhea, but because these conditions as a group comprise less than 1% of all chronic diarrhea, they are often ignored in the differential diagnosis, which may lead to delay in care^[17]. Preventive treatment and management of non-infectious diarrhea in patients with CS could directly reduce health service use and cost, and it may also help resolve fatigue and other secondary consequences of the condition previously associated with CS. Effective management of diarrhea also could potentially contribute to a reduction in emotional distress, which has been shown repeatedly to be associated with greater resource utilization in other populations^[18-21]. The NCCN Clinical Practice Guidelines in Oncology on NETs recommend the use of the long-acting somatostatin analogues, octreotide and lanreotide, should result in improvement of diarrhea and flushing symptoms of carcinoid syndrome^[22]. Octreotide LAR dose and frequency may be further increased for symptom control as needed^[22].

The results of this study need to be interpreted in the context of several limitations. First, in our analysis we attributed all diagnoses of non-infectious diarrhea to CS. We mitigated the possibility of misdiagnosis by excluding certain ICD-9-CM codes that were not clearly indicative of non-infectious diarrhea, such as gastroenteritis (*e.g.*, 558.9: other and unspecified noninfectious gastroenteritis and colitis). However, claims do not attribute diarrhea to a cause, they merely note the presence of the condition. Some cases of non-infectious diarrhea could have been from causes other than CS, resulting in an overestimate of the presence of CS-related diarrhea. On the other hand, codes for infectious diarrhea may have been applied to CS-related diarrhea simply because clinicians were more familiar with them. Studies of diarrhea in other conditions have been inconsistent in the ICD-9-CM codes used for non-infectious diarrhea^[23-25]. Nonetheless, our estimate of an 18.9% annual prevalence of diarrhea was remarkably close to another published estimate of 17.6% in a cohort of NET patients in which the majority had CS (72%)^[12]. Second, we considered HRU to be CS-related if codes

for a variety of conditions associated with CS were identified in the primary position on a claim. This may have overestimated utilization, although limiting the definition of CS-related to only those claims with CS in the primary position would have almost certainly have underestimated utilization (*e.g.*, a patient admitted for management of intestinal obstruction from a growing tumor would be likely to have obstruction, rather than CS, coded as the primary diagnosis). Third, our patient identification algorithm allowed a relatively long interval to pass between the first and confirmatory diagnosis. This decision may have reduced the specificity of our algorithm, although such reduction should have affected both groups equally. Fourth, we adjusted for a variety of potential confounders in our comparisons, but we did not adjust for pre-diagnosis health care resource use or cost. We felt that although prior utilization can predict future utilization, the patients in this analysis were all newly diagnosed with CS and thus controlling for pre-diagnosis resource use would be of limited value. Finally, this study included only patients with commercial insurance coverage. Our cohort of incident cases with CS was younger (mean age: 52 years) than a population-representative sample of incident NET cases from the Surveillance, Epidemiology, and End Results (SEER) database (mean age: 62 years)^[4]. Our results may not be generalizable to the United States population at large, but they are representative of a commercially-insured population.

Diarrhea is a particularly burdensome and costly symptom suffered by patients with CS. Our study demonstrates that health care costs and resource utilization in newly diagnosed CS patients with diarrhea are consistently and significantly higher than in those without diarrhea. Reduction of healthcare expenditures attributable to diarrhea may be achievable through preventive treatment and appropriate management of diarrhea in patients with CS.

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COMMENTS

Background

Non-infectious diarrhea (NID) is particularly burdensome and costly in carcinoid syndrome (CS) patients. The authors found that the proportions of patients with any and with CS-related hospitalizations were 9.7% and 6.8% higher, respectively, among NID patients than in those with no NID ($P < 0.001$). The NID group was also significantly more costly, with adjusted mean annual healthcare costs of \$81610, compared to \$51719 in the no NID group ($P < 0.001$).

Research frontiers

The healthcare and economic burden of diarrhea in CS patients has not been previously quantified.

Innovations and breakthroughs

This study shows diarrhea is burdensome and costly in CS patients.

Applications

Reduction of CS-related healthcare expenditures may be achievable through preventive treatment and appropriate management of diarrhea in CS.

Terminology

Neuroendocrine tumors that produce peptides and neuroamines induce characteristic hormonal syndromes, including CS. Most common presenting symptoms of CS are diarrhea and flushing. Diarrhea appears to have a particularly profound impact on CS patients' well-being. Given the lack of evidence about the economic burden of diarrhea in CS patients, we examined healthcare resource utilization patterns and healthcare costs accrued by CS patients with and without NID.

Peer-review

This research is interesting, and it is well executed methodologically and clearly described.

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Retrospective Study

Role of a liver-first approach for synchronous colorectal liver metastases

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Author contributions: Wang K and Liu W contributed equally to this work; Wang K and Liu W designed and performed the research and wrote the paper; Xing BC designed the research and supervised the report; Liu W designed the research and contributed to the analysis; Yan XL provided clinical advice; Xing BC supervised the report.

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Abstract

AIM: To evaluate the feasibility and survival outcomes of a liver-first approach.

METHODS: Between January 2009 and April 2013, 18 synchronous colorectal liver metastases (sCRLMs) patients with a planned liver-first approach in the Hepatopancreatobiliary Surgery Department I of the Beijing Cancer Hospital were enrolled in this study. Clinical data, surgical outcomes, morbidity and mortality rates were collected. The feasibility and long-term outcomes of the approach were retrospectively analyzed.

RESULTS: Sixteen patients (88.9%) completed the treatment protocol for primary and liver tumors. The main reason for treatment failure was liver disease recurrence. The 1 and 3 year overall survival rates were 94.4% and 44.8%, respectively. The median survival time was 30 mo. The postoperative morbidity and mortality were 22.2% and 0%, respectively, following a hepatic resection, and were 18.8% and 0%, respectively, after a colorectal surgery.

CONCLUSION: The liver-first approach appeared to be feasible and safe. It can be performed with a comparable mortality and morbidity to the traditional treatment paradigm. This approach might offer a curative opportunity for sCRLM patients with a high

liver disease burden.

Key words: Liver metastases; Resection; Colorectal cancer; Synchronous

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Core tip: This is a retrospective study to investigate the feasibility and survival outcome of the liver-first approach for synchronous colorectal liver metastases. The postoperative morbidity and mortality were acceptable. The 1 and 3 year overall survival rates were 94.4% and 44.8%, respectively. The approach should be performed in patients with synchronous colorectal liver metastases with a high liver disease burden.

Wang K, Liu W, Yan XL, Xing BC. Role of a liver-first approach for synchronous colorectal liver metastases. *World J Gastroenterol* 2016; 22(6): 2126-2132 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i6/2126.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i6.2126>

INTRODUCTION

The liver is the most common organ for distant metastases from colorectal cancer^[1]. Up to 15%-42% of patients present with synchronous colorectal liver metastases at the time of diagnosis of their primary cancer^[2,3]. The synchronous presentation has been associated with poor survival outcomes^[4,5]. Nevertheless, surgical resection of all tumor sites is considered the only curative therapy for long-term survival from colorectal liver metastases (CRLMs)^[4,6]. Several large case series from tertiary centers have reported 5 year survival rates of 21%-58% and 10 year survival rates of 22%-26%^[4,7,8].

The traditional surgical strategy for resectable synchronous colorectal liver metastases (sCRLMs) is a two-stage approach that includes colorectal cancer resection followed by chemotherapy and a delayed hepatic resection of a CRLM. This approach might result in liver disease progression between the time of colorectal and hepatic resection and render the CRLM unresectable^[9]. This is a particular concern in patients who develop postoperative complications after colorectal cancer resection before the administration of chemotherapy and the hepatic resection of CRLMs^[10].

Upon the realization that liver metastases define the prognosis of a patient, the concept of a liver-first approach in patients with locally advanced rectal cancer and synchronous liver metastases was proposed^[11]. However, there has been limited data published on the feasibility and safety of the liver-first approach for sCRLMs. Therefore, the present study aims to describe the experience with the liver-first approach in a tertiary referral center. The feasibility, security and long-term outcomes of the liver-first

approach were also investigated.

MATERIALS AND METHODS

Study population

Between January 2009 and April 2013, 168 CRLM patients underwent hepatic resection in the Hepato-pancreatobiliary Surgery Department I of Beijing Cancer Hospital. All of the sCRLM patients were identified. Eighteen of these patients with a planned liver-first approach were included in the present study.

Preoperative evaluation

All the patients underwent a complete colonoscopy for colorectal cancer, abdominal and thoracic computed tomography scan and liver and pelvic (only rectal cancer patients) magnetic resonance imaging. The Response Evaluation Criteria for Solid Tumors were applied to the serial imaging studies obtained during a preoperative therapy to determine a chemotherapy response^[12]. The definition of advanced metastatic disease was based on a clinical risk score (CRS) described by Fong *et al.*^[13]. A CRS of 3 or higher has been validated as defining more severe disease.

Preoperative chemotherapy

Preoperative chemotherapy was considered in patients with initially unresectable disease or a high liver disease burden. Patients received oxaliplatin or irinotecan-based chemotherapy. In some recent cases, they also received cetuximab or bevacizumab. The response to chemotherapy was assessed after two or three cycles (more than four cycles for conversion chemotherapy) by MRI and carcinoembryonic antigen levels. When the liver metastases were resectable, a laparotomy was planned more than three weeks after the last course of systemic chemotherapy. Bevacizumab had to be excluded from the last course of chemotherapy to ensure an interval of at least six weeks.

Hepatic resection

All the patients underwent a hepatic resection with curative intent to achieve R0 and preserve as much normal functional liver parenchyma (with adequate vascular inflow, outflow and biliary drainage) as possible. A resection of three or more segments was considered a major hepatectomy^[14]. The normal liver parenchyma remnant volume was more than 40% if a patient received preoperative chemotherapy.

Chemoradiation and primary surgery

Preoperative chemoradiation was used in only two situations: (1) mid-to-low rectal cancer, defined as ≤ 10 cm distance from the lower edge of the tumor to the anal verge; and (2) a pre-treatment staging by MRI was T3/T4, or any T category, and N positive^[15]. Radiation therapy consisted of either a long course (total dose of 50 Gy) therapy or a modified short

Table 1 Patients and tumor characteristics

| Variable | No. of patients, <i>n</i> = 18 |
|---|--------------------------------|
| Patient characteristics | |
| Age (yr), median (range) | 54 (21-74) |
| Sex (male) | 10 |
| Pre-operative CEA level (μg/L), median (range) | 26.3 (1-860) |
| Primary tumor site | |
| Colon | 2 |
| Rectum | 16 |
| Symptoms caused by the primary tumor | 13 |
| Symptoms at the time of presentation | |
| None | 5 |
| Rectal blood loss | 7 |
| Changes in bowel habits | 6 |
| AJCC T-stage on pathology | |
| ypT1/ypT2 | 1 |
| ypT3/ypT4 | 15 |
| Lymph node status on pathology | |
| ypN1/ypN2 | 14 |
| ypN0 | 2 |
| Hepatic metastasis | |
| Size of largest metastasis (cm), median (range) | 4 (2-16) |
| No. of metastasis, median (range) | 4 (1-12) |
| Location (unilobular) | 11 |
| CRS score | |
| < 3 | 2 |
| ≥ 3 | 16 |
| Preoperative chemotherapy | 14 |
| Cycles, median (range) | 3 (0-5) |
| Indication | |
| Conversion | 4 |
| Locally advanced liver metastases | 10 |
| Regimens of preoperative chemotherapy | |
| Oxaliplatin | 10 |
| Irinotecan | 4 |
| Cetuximab | 4 |
| Bevacizumab | 2 |
| Response of preoperative chemotherapy | |
| PR | 9 |
| SD | 4 |
| PD | 1 |

course (total dose of 30 Gy) therapy with capecitabine 825 mg/m² twice per day only on radiotherapy days. A total mesorectal/complete mesocolic excision was performed in all patients.

Follow-up

All the patients had a follow-up visit every 3 mo for the first 2 years, with a physical examination, CEA and CA19-9 serum measurement and abdominal ultrasonography. The patients had a computed tomography scan and colonoscopy every 6 mo. No patients were lost to follow-up.

Statistical analysis

Continuous variables were summarized as a mean. Categorical variables were summarized as a frequency and percentage. A Kaplan-Meier survival was calculated from the date of initial treatment. Statistical analyses were performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, United States).

Table 2 Details of the surgical procedures and early outcomes

| Variable | No. of patients, <i>n</i> = 18 |
|--|--------------------------------|
| Type of hepatic resection | |
| Major | 14 |
| Minor | 4 |
| Extent of hepatic resection | |
| Partial | 10 |
| Hemihepatectomy | 3 |
| Extended hepatectomy | 5 |
| Type of colorectal resection | |
| Low anterior resection | 12 |
| Abdominoperineal resection | 2 |
| Left hemicolectomy | 2 |
| Resected lymph nodes, median (range) | 11 (6-20) |
| Complications | |
| Hepatectomy-related | |
| Hydrothorax | 3 |
| Abdominal abscess | 1 |
| Minor (Clavien grade < 3) | 4 |
| Major (Clavien grade ≥ 3) | 0 |
| Post-operative mortality (within 90 d) | 0 |
| Surgery on primary cancer | |
| Anastomotic leakage | 1 |
| Abdominal abscess | 2 |
| Minor (Clavien grade < 3) | 3 |
| Major (Clavien grade ≥ 3) | 0 |
| Post-operative mortality (within 90 d) | 0 |

RESULTS

Patient characteristics

Between January 2009 and April 2013, 48 sCRLM patients were identified. The liver-first approach was planned for 18 of them (37.5%). There were 10 male and 8 female patients. The median age was 54 years (range: 21-74; mean: 51.9). At the time of presentation, 13 (72.2%) patients had clinical symptoms. The median size of the liver metastases was 4 cm (range: 2-16; mean: 5.33). The median number of metastases was 4 (range: 1-12; mean: 4.06). The median preoperative CEA blood level was 26.3 ng/mL (range: 1-861; mean: 87.37). The median CRS was 3 (range: 2-4; mean: 3.17). The most common site of the primary tumor was the rectum (*n* = 16; 88.9%). The characteristics of these patients are detailed in Table 1.

Surgery details and early postoperative outcomes

Of the 18 patients in whom a liver-first approach was planned, a major hepatectomy was performed in 14 patients (77.8%). Due to liver recurrence after the hepatectomy, only 2 patients did not undergo surgery for the primary tumor. The operative characteristics of primary and liver metastases are detailed in Table 2. The complication rates after the hepatic and primary resections were 22.2% (*n* = 4) and 18.8% (*n* = 3), respectively. According to the Clavien-Dindo classification system^[16], all the complications were minor (Clavien grade < 3). Importantly, there was no postoperative mortality after the liver or primary

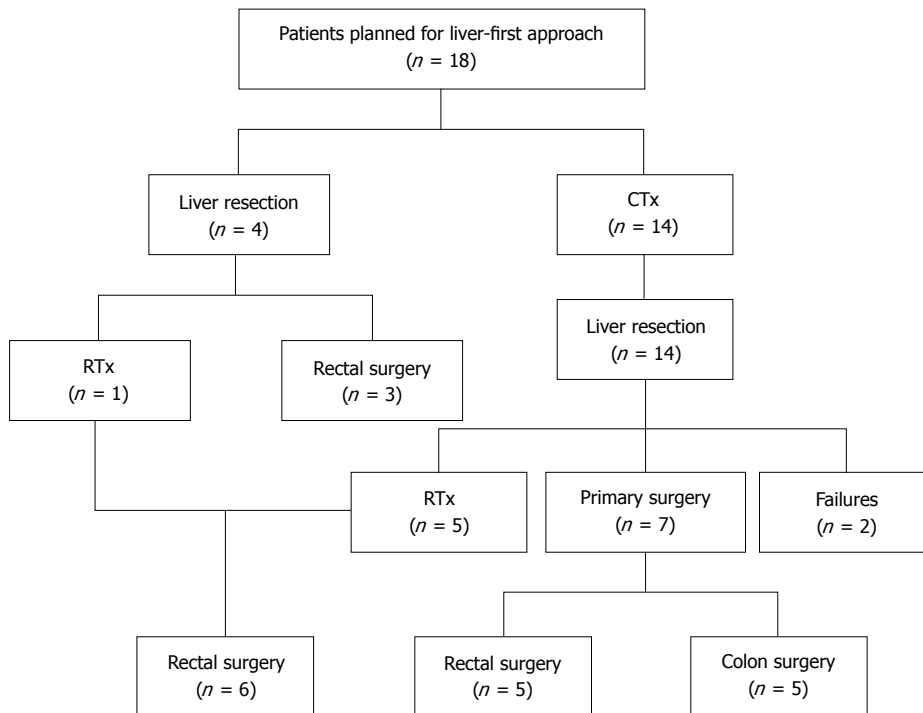


Figure 1 Flow chart of the 18 patients enrolled in the study.

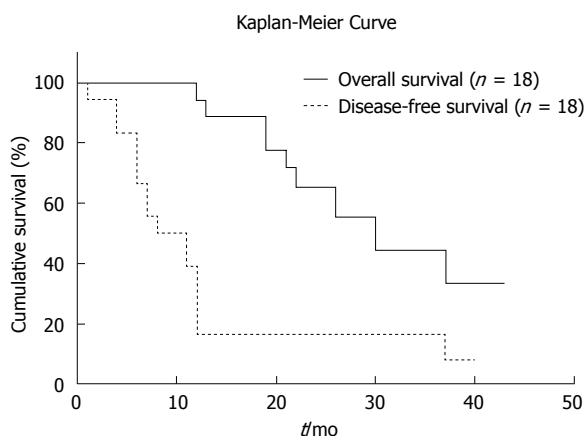


Figure 2 Kaplan-Meier Curve showing the overall and disease-free survivals of the 18 patients who underwent the liver-first approach.

surgeries. The specifics are detailed in Table 2.

Preoperative chemotherapy and chemoradiation

A flow diagram of the treatment overview of all 18 patients is shown in Figure 1. At the time of the initial presentation, 4 patients had unresectable CRLMs and received conversion chemotherapy. Ten patients had locally advanced liver metastases and received neoadjuvant chemotherapy. Two patients refused any neoadjuvant therapy and the other 2 patients had a CRS of less than 3. All of them immediately underwent a hepatic resection. The median preoperative chemotherapy cycle was 3 (range: 0-5; mean: 2.5). It included an oxaliplatin-based chemotherapy in 10 patients and an irinotecan-based chemotherapy in 4

patients. During the first courses of the preoperative chemotherapy, cetuximab was added to 4 patients and bevacizumab was added to 2 patients. Between the window of the hepatic and colorectal surgeries, six patients received radiation therapy, 3 patients a short course of radiation therapy and 3 patients a long course of radiation therapy. The specifics are detailed in Table 3.

Survival analysis

At the time of the last follow-up, 16 (88.9%) patients completed a curative paradigm. The median follow-up was 30 mo (range: 12-43; mean: 30.54). The 1 and 3 year overall survival rates were 94.4% and 44.8%, respectively (Figure 2). The median disease-free survival after surgery was 11 mo (range: 1-40; mean: 13.4). After the hepatic resection, 16 patients had a recurrence during the follow-up. Nine died of disease recurrence. The patterns of recurrence were intrahepatic only (10, 62.5%) and combined intra and extrahepatic (6, 37.5%).

DISCUSSION

In the current series, 18 patients who were scheduled to undergo the liver-first approach were included in this study. Sixteen (88.9%) of them completed the treatment protocol for liver and primary tumors. The percentage of feasibility is in concordance with those reported in assorted cohorts of sCRLM^[17,18]. The remaining two patients deviated from the protocol as a result of recurrence of liver metastasis after resection. For the patients who underwent the liver-

Table 3 Characteristics of 18 patients who underwent the formalized treatment plan

| Patient | Largest size (cm) | CEA level (μg/L) | cTN | No. of mets | CTx | Response on CTx | Liver surgery | RTx | Primary surgery |
|---------|-------------------|------------------|-------|-------------|----------------|-----------------|---------------------|--------------|-----------------|
| 1 | 16 | 861.4 | cT3N1 | 1 | Xelox | PD | Hemihep | None | Left Hemicol |
| 2 | 2 | 1 | cT4N2 | 4 | Folfox | PR | Extended hemihep | None | LAR |
| 3 | 3.5 | 13.4 | cT4N1 | 1 | None | None | Partial | 30 Gy/Xeloda | LAR |
| 4 | 6 | 113.4 | cT3N1 | 5 | Folfox | SD | Partial | None | Left Hemicol |
| 5 | 2.5 | 16.8 | cT3N1 | 6 | Xelox | PR | Extended hemihep | 30 Gy/Xeloda | None |
| 6 | 7.9 | 30.4 | cT4N1 | 4 | Xelox | PR | Extended hemihep | None | LAR |
| 7 | 4 | 3.2 | cT2N1 | 3 | Folifiri + Cet | PR | Hemihep | None | LAR |
| 8 | 2.4 | 2.4 | cT3N2 | 3 | Folifiri + Cet | PR | Partial | 50 Gy/Xeloda | LAR |
| 9 | 5 | 78.1 | cT4N2 | 1 | None | None | Partial | 50 Gy/Xeloda | APR |
| 10 | 3.8 | 160.9 | cT3N2 | 1 | Folfox | SD | Partial | None | APR |
| 11 | 4 | 3.7 | cT3N0 | 6 | Folifiri + Cet | PR | Partial | 30 Gy/Xeloda | LAR |
| 12 | 1.5 | 19.7 | cT3N1 | 4 | Folfox + Bev | PR | Partial | None | LAR |
| 13 | 8.5 | 87.2 | cT3N1 | 5 | None | None | Partial | None | LAR |
| 14 | 12 | 22.1 | cT4N1 | 6 | Folfox + Cet | PR | Hemihep | None | LAR |
| 15 | 6 | 79.4 | cT3N1 | 3 | Folfoxiri | SD | Extended hemihep | None | LAR |
| 16 | 4.3 | 6.1 | cT3N0 | 12 | Xelox + Bev | PR | Partial | None | LAR |
| 17 | 3 | 34.7 | cT3N1 | 2 | None | None | Partial | None | LAR |
| 18 | 3.5 | 39.0 | cT3N2 | 6 | Xelox | SD | Extended hemihep | 50 Gy/Xeloda | None |

LAR: Low anterior resection; APR: Abdominal perineal resection; Hemihep: Hemihépatectomy; Hemicol: Hemicolectomy; Partial: Partial hepatectomy; Cet: Cetuximab; Bev: Bevacizumab; xelox: Oxaliplatin plus capecitabine; Folfox: Oxaliplatin, leucovorin and 5-FU; Folfiri: Irinotecan, leucovorin and 5-FU; Folfoxiri: Oxaliplatin, irinotecan, leucovorin and 5-FU.

first approach, the 1 and 3 year overall survival rates were 94.4% and 44.8%, respectively. The median disease free survival time after surgery was 10 mo (range: 1-40; mean: 13.3). The complication rate after hepatic resection and primary resection was 22.2% ($n = 4$) and 18.8% ($n = 3$), respectively. These surgical outcomes were comparable with other results associated with the liver-first approach^[19,20]. In addition, our results may need to be confirmed in a prospective, randomized clinical trial with a larger sample size.

Numerous surgical series have demonstrated that a hepatic resection for CRLM may offer the possibility of long-term survival^[5,6]. Additionally, except for the hepatic resection, no other treatment has shown a survival plateau. These results support that a hepatic resection is the standard practice and only curative treatment for CRLM. Apparently, metastatic disease, rather than primary colorectal cancer, has been proposed to be the main determinant of patient survival. Thus, treating a CRLM should be the first priority^[11,17]. It has been suggested that liver disease burden rather than the primary cancer leads to subsequent systemic metastatic disease^[6,21].

The optimal timing and sequence of surgical resection for sCRLM has been a topic of much debate. The timing of when to undergo a "classic", "simultaneous" or "liver-first" approach remains controversial^[22]. Following the EORTC trial^[23], many centers still favor the classical approach. The rationale for this approach was that the colorectal primary

tumor was the usual source of symptoms and thus should be removed first^[24]. Recent studies have demonstrated that a primary resection in patients with metastatic colorectal cancer significantly increased the 30 d mortality by 10% when compared with a non-metastatic setting^[25]. Therefore, a CRLM might progress beyond resectability during the primary tumor resection (especially in patients with postoperative complications after the colorectal resection).

In the past decade, a simultaneous resection for sCRLMs has been performed more often. The strategy for the simultaneous resection was to avoid missing the surgical opportunity^[26]. Equivalent perioperative morbidity and mortality and survival outcomes were achieved if the colorectal resection was combined with a minor hepatic resection^[19,27]. Compared with a staged resection, a simultaneous resection in patients was accompanied with much milder complications^[28]. Thus, a simultaneous resection was preferred in highly selected patients^[29,30].

The alternative paradigm for the management of sCRLMs is the reverse, or so-called liver-first approach. This modern procedure has evolved as a result of the increasing complexity of care of primary colorectal cancer with the development of preoperative chemoradiotherapy and colonic stenting^[31]. It allows the ability to first control the CRLM and optimizes the chance of a potentially curative hepatic resection, which improves the long-term survival in these patients^[32]. The approach also evaluates the biological behavior of the neoplasm, treats the occult disease

and avoids an operation in patients with rapidly progressing tumors^[33].

De Rosa *et al.*^[34] summarized the indications for the liver-first approach or patients with a high or low liver disease burden with a locally advanced primary tumor. In fact, the ideal patient is likely to be someone who has advanced synchronous liver metastatic disease and rectal cancer^[35]. In our study, 12 patients had locally advanced liver metastases and 4 patients had initially unresectable liver tumors. All of them had a high liver disease burden, which was largely in accordance with the attitude of van der Pool *et al.*^[27] who reported that the appropriate patients for the liver-first approach had a heavier tumor size, diameter and distribution for liver disease burden.

Knowledge of the natural history and pattern of metastatic dissemination in patients with colorectal cancer has revolutionized the understanding and management of this disease. It may be more appropriate to first use chemotherapy to provide early systemic treatment^[18]. Current evidence indicates that colorectal cancer is a chemosensitive disease. Thus, it is logical to start early systemic treatment^[31,36]. Additionally, in patients with a high liver tumor burden, it is crucial to control the disease with down-staging chemotherapy^[37].

Generally, candidates for the liver-first approach include those with a heavy liver disease burden and/or required down-staging therapy with a hepatic resection containing more than three segments.

COMMENTS

Background

The liver is the most common organ for distant metastases from colorectal cancer. Up to 15%-42% of patients present with synchronous colorectal liver metastases (sCRLMs) at the time of a primary cancer diagnosis. However, a standard surgical approach for sCRLM remains undetermined. There were three surgical strategies, including the traditional or classic resection, liver-first resection and simultaneous resection. In this study, the authors retrospectively analyzed the feasibility and survival outcome of the liver-first approach.

Research frontiers

In fact, liver metastases define the prognosis of CRLM patients. The liver-first approach was performed and compared with patient outcomes in the last decades. There were only 4 studies that analyzed the feasibility and survival outcomes of the approach.

Innovations and breakthroughs

Based on present results and daily work experience, the liver-first approach is an appropriate surgical strategy for sCRLM patients with a high liver disease burden. We proposed exact indications for the liver-first approach.

Applications

The candidates for the liver-first approach included those with a heavy liver disease burden and/or who required down-staging therapy with a hepatic resection containing more than three segments.

Terminology

The classic approach is a primary cancer resection followed by a hepatic resection. The liver-first approach is a colorectal liver metastases hepatic resection followed by a primary cancer resection. The simultaneous approach

is a resection for a primary cancer and liver metastasis that is performed simultaneously.

Peer-review

This is an interesting study about the liver first approach for synchronous colorectal liver metastases.

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Retrospective Study

Minimizing tacrolimus decreases the risk of new-onset diabetes mellitus after liver transplantation

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Abstract

AIM: To investigate the impact of minimum tacrolimus (TAC) on new-onset diabetes mellitus (NODM) after liver transplantation (LT).

METHODS: We retrospectively analyzed the data of 973 liver transplant recipients between March 1999 and September 2014 in West China Hospital Liver Transplantation Center. Following the exclusion of ineligible recipients, 528 recipients with a TAC-dominant regimen were included in our study. We calculated and determined the mean trough concentration of TAC (cTAC) in the year of diabetes diagnosis in NODM recipients or in the last year of the follow-up in non-NODM recipients. A cutoff of mean cTAC value for predicting NODM 6 mo after LT was identified using a receptor operating characteristic curve. TAC-related complications after LT was evaluated by χ^2 test, and the overall and allograft survival was evaluated using the Kaplan-Meier method. Risk factors for NODM after LT were examined by univariate and multivariate Cox

regression.

RESULTS: Of the 528 transplant recipients, 131 (24.8%) developed NODM after 6 mo after LT, and the cumulative incidence of NODM progressively increased. The mean cTAC of NODM group recipients was significantly higher than that of recipients in the non-NODM group (7.66 ± 3.41 ng/mL *vs* 4.47 ± 2.22 ng/mL, $P < 0.05$). Furthermore, NODM group recipients had lower 1-, 5-, 10-year overall survival rates (86.7%, 71.3%, and 61.1% *vs* 94.7%, 86.1%, and 83.7%, $P < 0.05$) and allograft survival rates (92.8%, 84.6%, and 75.7% *vs* 96.1%, 91%, and 86.1%, $P < 0.05$) than the others. The best cutoff of mean cTAC for predicting NODM was 5.89 ng/mL after 6 mo after LT. Multivariate analysis showed that old age at the time of LT (> 50 years), hypertension pre-LT, and high mean cTAC (≥ 5.89 ng/mL) after 6 mo after LT were independent risk factors for developing NODM. Concurrently, recipients with a low cTAC (< 5.89 ng/mL) were less likely to become obese (21.3% *vs* 30.2%, $P < 0.05$) or to develop dyslipidemia (27.5% *vs* 44.8%, $P < 0.05$), chronic kidney dysfunction (14.6% *vs* 22.7%, $P < 0.05$), and moderate to severe infection (24.7% *vs* 33.1%, $P < 0.05$) after LT than recipients in the high mean cTAC group. However, the two groups showed no significant difference in the incidence of acute and chronic rejection, hypertension, cardiovascular events and new-onset malignancy.

CONCLUSION: A minimal TAC regimen can decrease the risk of long-term NODM after LT. Maintaining a cTAC value below 5.89 ng/mL after LT is safe and beneficial.

Key words: Liver transplantation; Minimum tacrolimus; New-onset diabetes mellitus; Immunosuppressants; Allografts failure

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Core tip: New-onset diabetes mellitus (NODM) is a common and severe metabolic complication that develops after liver transplantation. It is more prominent in recipients with tacrolimus (TAC)-dominant regimens. In this study, we found that the incidence of NODM is TAC concentration (cTAC)-dependent. Using a receiver operating characteristic curve, we identified that a cutoff cTAC of 5.89 ng/mL was predictive of NODM development after 6 mo after LT. And recipients exposed to low mean cTAC developed less other TAC related complications. The strategy of maintaining cTAC below 5.89 ng/mL after 6 mo after LT is therefore safe and beneficial.

Song JL, Gao W, Zhong Y, Yan LN, Yang JY, Wen TF, Li B, Wang WT, Wu H, Xu MQ, Chen ZY, Wei YG, Jiang L, Yang J. Minimizing tacrolimus decreases the risk of new-onset diabetes

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INTRODUCTION

Liver transplantation (LT) has become a standard curative treatment for end-stage liver disease, and the 5-year survival rate of recipients has reached over 70%^[1]. However, improved long-term survival is accompanied by increasingly prevalent post-operative metabolic complications^[2]. Recent studies have shown that the prevalence of new-onset diabetes mellitus (NODM) after transplantation is approximately 16%-61%, depending on the medical center^[3,4]. The development of post-LT NODM is associated with an increased risk of cardiovascular disease, rejection, infection, neuropsychiatric problem, allograft failure and even death^[5,6]. Previous studies have found that old age, obesity, non-Caucasian ethnicity, family history of diabetes, hepatitis C virus infection and certain immunosuppressive agents are risk factors for the development of post-LT NODM in Western populations^[7].

Tacrolimus (TAC), a calcineurin inhibitor, has become the most commonly used immunosuppressive agent worldwide over the past two decades^[8]. Compared to cyclosporine, TAC effectively reduces acute rejection (AR) and increases allograft survival in liver recipients^[8,9]. However, prolonged exposure to TAC leads to significant adverse events, including nephrotoxicity, neurotoxicity, and diabetogenic effects^[10]. Some studies have suggested that higher trough concentrations of TAC (cTAC) after transplantation are associated with increased risk of complications^[11-13], and many LT centers have recommended different minimal TAC regimens^[14-16]. According to the current practice, target TAC level falls within the range of 10-15 ng/mL in the first month after transplantation, then is maintained at 5-10 ng/mL^[17]. A prospective study has reported that reducing cTAC within the range of 5-8 ng/mL combined with mycophenolate mofetil (MMF) administration early did not increase the risk of rejection within 26 wk^[18]. Jia *et al*^[14] proposed that an early cTAC of 5-7 ng/mL would be safe and effective. A previous study performed in our center suggested that cTAC < 8 ng/mL after 1 mo and cTAC < 6 ng/mL after 3 mo are protective against chronic kidney disease (CKD) after LT^[19]. However, all target cutoffs or ranges for cTAC are arbitrary, and there are no studies concerning the long-term maintenance of cTAC level after LT and its impact on NODM development. In this study, we aim to identify the risk factors for NODM and to determine the ideal long-term range of cTAC for preventing chronic complications.

MATERIALS AND METHODS

Patient population

We performed a retrospective study of 973 Chinese patients who received liver transplantation between March 1999 and September 2014 in the West China Hospital Liver Transplantation Center. All recipients were followed until June 2015 or until death or withdrawal. We excluded patients who had been diagnosed as diabetics before transplantation; those aged younger than 18 years old at transplantation; and those followed up for less than 6 mo, who died within 6 mo, and who received a cyclosporine-dominant regimen after liver transplantation. Finally, we collected demographic and clinical data of 528 recipients for this study. All liver grafts were voluntarily donated after cardiac death or by living donors. All donations were approved by the West China Hospital Ethics Committee and were in accordance with the ethical principles of the Declaration of Helsinki. Both the West China Hospital Liver Transplantation Center and the China Liver Transplant Registry approved and supported this study and its methods.

Definition of NODM and other clinical terms

NODM was defined as a composite endpoint consisting of the first occurrence of at least one of four parameters: two occurrences of a fasting plasma glucose level ≥ 7.0 mmol/L more than 30 d apart; oral hypoglycemic agent use for more than 30 consecutive days; insulin therapy for more than 30 consecutive days; or hemoglobin A1c $\geq 6.5\%$ ^[20]. Arterial hypertension was defined as systolic blood pressure over 140 mmHg or diastolic pressure over 90 mmHg occurring twice at different time points^[21]. Dyslipidemia was defined as total plasma cholesterol ≥ 6.22 mmol/L (*i.e.*, hypercholesterolemia), triglyceride ≥ 2.26 mmol/L (*i.e.*, hypertriglyceridemia) or high density lipoprotein cholesterol (HDL-C) < 1.04 mmol/L^[21]. Chronic kidney disease (CKD) was defined as an estimated glomerular filtration rate (eGFR) < 60 mL/min per 1.73 m² for at least 3 consecutive months^[22]. AR was defined either by liver biopsy or recovery of liver function *via* high-dose methylprednisolone pulse therapy. If chronic rejection (CR) was suspected, liver biopsy was performed for confirmation. The Model for End-stage Liver Disease (MELD) score was calculated according to the United Network for Organ Sharing (UNOS) formula for each recipient before LT^[23].

Immunosuppression protocol

The mode of initial immunosuppressive therapy was a triple-drug regimen after transplantation consisting of corticosteroids, TAC and MMF. Methylprednisolone was given intravenously at a 200 mg dose on the first day after transplantation, then gradually decreased daily and discontinued after one week. Alternative oral prednisone was also generally discontinued within 3

mo after transplantation. The initial dose of TAC was 0.05–0.10 mg/kg per day and was adjusted according to liver function and TAC trough concentration. MMF was individualized between 1.0 g/d and 1.5 g/d initially and was discontinued when severe side effects occurred and in long-term survivors with stable graft function after 6 mo after LT. Rapamycin was given as an alternative to MMF or an auxiliary for liver tumor at a dose of 1 mg/d.

Monitoring TAC trough concentrations and other clinical parameters

TAC trough concentrations were monitored daily during the first week following transplantation, weekly during the first month after LT, monthly within 3 mo and every 3–6 mo thereafter. The ideal serum trough level of TAC was 5–10 ng/mL during the first 3 mo after LT. Allograft function and cTAC were monitored closely while adjusting the TAC dose. If AR occurred, the prior dosage was reinstated, together with an increase in prednisone or the administration of high-dose methylprednisolone. After 6 mo post-LT, we reduced the TAC dosage very slowly and carefully while closely monitoring allograft function to maintain cTAC as low as possible. After transplantation, the recipients' fasting plasma glucose level was monitored at 3, 6 and 12 mo, then annually thereafter according to international consensus guidelines^[24]. A 2-h 75 g glucose tolerance test was performed in recipients with impaired fasting glucose. We also recorded the weight, blood pressure, serum lipid level, renal function, and chronic complications such as moderate to severe infections, cardio-cerebral vascular events, new-onset malignancy and allograft failures of each recipient at each visit after transplantation.

Statistical analysis

Quantitative descriptive data were expressed as the mean \pm SD or median (minimum to maximum). Qualitative descriptive data were expressed as percentages. Univariate analysis using the χ^2 and, when appropriate, Fisher's exact test was performed for qualitative descriptive variables. Quantitative descriptive variables were analyzed by independent sample Student's *t* test if the data were normally distributed or by the rank-sum test if the data were non-normally distributed. Survivor curves were analyzed using the Kaplan-Meier method and were compared using the log-rank test. The best cutoff mean cTAC after 6 mo was determined using a receiver operating characteristic (ROC) curve. Independent risk factors for NODM were identified by a stepwise forward Cox regression model. Candidate risk factors with a *P* value < 0.05 in univariate analysis were included in the multivariate analysis. Statistical analysis was performed using SPSS version 21.0 statistical software (SPSS Company, Chicago, IL, United States). *P* values of less than 0.05 were considered statistically significant. The statistical

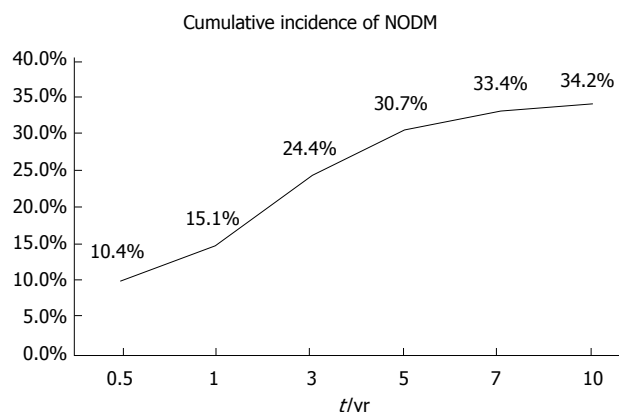


Figure 1 Cumulative incidence of new-onset diabetes mellitus over a 10-year period after liver transplantation. NODM: New-onset diabetes mellitus.

methods used in this study were reviewed by Ji-Zheng Qin from West China School of Public Health, Sichuan University.

RESULTS

Recipient and donor characteristics

A total of 973 recipients underwent LT between March 1999 and September 2014 in West China Hospital Liver Transplantation Center. Following the exclusion of ineligible recipients, 528 recipients were included in this study. The demographical and clinical records of recipients meeting the inclusion criteria were reviewed retrospectively. Recipients were followed up for a median of 46 mo (range, 6-173 mo). Recipients were 44.93 ± 9.41 years (range, 18-70 years) old and were predominantly male (87%). HBV (79.5%) was the most common etiology of liver disease; only six recipients had HCV (1.1%), and approximately half of the recipients (50.9%) had liver tumors. The pre-LT baseline included overweight/obesity ($\text{BMI} \geq 25$) in 110 (20.8%) recipients, hypertension in 12 (2.3%) recipients, and dyslipidemia in 41 (8.2%) recipients. The median MELD score of all recipients was 13 (range, 6-40). MMF was administered in 322 (61%) recipients, and 129 (24.4%) recipients were also treated with Rapamycin. Donors were aged 34.01 ± 8.75 years (range, 5-65 years) old and were more likely to be male (84.5%). The living donor liver transplantation rate was 29.9%.

Prevalence of NODM and other post-LT complications

Eventually, 24.8% of the study population (131 cases) developed NODM during the follow-up period. The cumulative incidence of NODM increased progressively, and the 1-, 3-, 5- and 10-year incidence rates were 15.1%, 24.4%, 30.7% and 34.2%, respectively (Figure 1). We compared the 26 demographical and clinical parameters between recipients with and without NODM, as shown in Table 1. Common post-LT TAC-related complications included overweight/obesity

($\text{BMI} \geq 25$) in 128 (24.2%) recipients, hypertension in 67 (12.7%) recipients, dyslipidemia in 175 (33.1%) recipients, and CKD in 91 (17.2%) recipients. There were 58 (11%) and 20 (3.8%) recipients with AR and CR, respectively. Predictably, we found that NODM recipients experienced more cardio-cerebral vascular events (7.6% vs 2.0%, $P < 0.05$), moderate to severe infections (36.7% vs 25.2%, $P < 0.05$), and allograft failures (15.3% vs 8.1%, $P < 0.05$) than non-NODM recipients. The 1-, 5-, and 10-year overall survival rates (86.7%, 71.3%, and 61.1% vs 94.7%, 86.1%, and 83.7%, $P < 0.05$) and allograft survival rates (92.8%, 84.6%, and 75.7% vs 96.1%, 91%, and 86.1%, $P < 0.05$) in the NODM group were significantly lower than in the non-NODM group, as shown in Figure 2.

Definition of the cutoff mean cTAC after 6 mo

In our center, cTAC was measured and recorded at each visit. The mean cTAC was calculated and determined in the year when diabetes was diagnosed in the NODM group and in the last year of follow-up in the non-NODM group. Our study suggested that the mean cTAC was higher in the NODM group (7.66 ± 3.41 ng/mL) than in the non-NODM group (4.47 ± 2.22 ng/mL, $P < 0.05$; Table 1). A cutoff cTAC of 5.89 ng/mL was identified as predictive of post-LT NODM using an ROC curve (Figure 3). The diagnostic value showed that the area under the curve (AUC) was 0.815 (95%CI: 0.770-0.859, $P < 0.05$) with a sensitivity of 0.733 and a specificity of 0.809. All liver recipients were divided into two groups: a low mean cTAC (< 5.89 ng/mL) group ($n = 356$) and a high mean cTAC (≥ 5.89 ng/mL) group ($n = 172$).

To evaluate the impact of different mean cTAC levels on the long-term survival of the recipients after LT, we compared the common post-LT complications between the two cTAC groups (Table 2). We found that recipients in the high mean cTAC group were more frequently overweight/obese (30.2% vs 21.3%), and were more likely to develop dyslipidemia (44.8% vs 27.5%), CKD (22.7% vs 14.6%), and moderate to severe infection (33.1% vs 24.7%) than recipients in the low mean cTAC group ($P < 0.05$). However, there was no significant difference in other complications between the two groups. Kaplan-Meier survive curves suggested that recipients in the low mean cTAC group had higher 1-, 5-, and 10-year allograft survival rates (96.8%, 92.3%, and 87.4%) than recipients in the high mean cTAC group (92.0%, 82.9%, and 72.0%, $P < 0.05$; Figure 4A). The low mean cTAC group also exhibited higher 1-, 5-, and 10-year overall survival rates (93.7%, 83.8%, and 78.3% vs 90.5%, 78.6%, and 71.8%), but the difference was not statistically significant ($P = 0.129$; Figure 4B).

Risk factors for post-LT NODM

We examined more than 20 parameters to identify risk

Table 1 Demographic and clinical characteristics of recipients with and without new-onset diabetes mellitus after liver transplantation (*n* = 528) *n* (%)

| Characteristics | Total (<i>n</i> = 528) | NODM group (<i>n</i> = 131) | Non-NODM group (<i>n</i> = 397) | <i>P</i> value |
|--------------------------------|-------------------------|------------------------------|----------------------------------|----------------|
| Recipient characteristics | | | | |
| Age (yr) | 44.93 ± 9.41 | 46.24 ± 9.54 | 44.50 ± 9.34 | 0.068 |
| Gender (male) | 446 (84.5) | 144 (87.0) | 332 (83.6) | 0.352 |
| Child-Pugh (A/B/C) | 136/223/169 | 39/44/48 | 97/179/121 | 0.069 |
| MELD Score | 13 (6-40) | 15 (6-40) | 13 (6-40) | 0.010 |
| BMI ≥ 25 pre-LT | 110 (20.8) | 36 (27.5) | 74 (18.6) | 0.006 |
| Hypertension pre-LT | 12 (2.3) | 7 (5.3) | 5 (1.3) | 0.017 |
| Dyslipidemia pre-LT | 41 (8.2) | 15 (11.5) | 26 (6.5) | 0.069 |
| Indications for LT | | | | |
| Hepatitis B virus disease | 420 (79.5) | 102 (77.9) | 318 (80.1) | 0.582 |
| Hepatitis C virus disease | 6 (1.1) | 1 (0.8) | 5 (1.3) | > 0.990 |
| Alcoholic cirrhosis | 16 (3.0) | 7 (5.3) | 9 (2.3) | 0.137 |
| Tumors | 269 (50.9) | 56 (42.7) | 213 (53.7) | 0.030 |
| Mean cTAC (ng/mL) | 5.26 ± 2.91 | 7.66 ± 3.41 | 4.47 ± 2.22 | < 0.001 |
| Rapamycin administration | 129 (24.4) | 30 (22.9) | 99 (24.9) | 0.638 |
| MMF administration | 322 (61.0) | 78 (59.5) | 244 (61.5) | 0.696 |
| Complications post-LT | | | | |
| BMI ≥ 25 post-LT | 128 (24.2) | 40 (30.5) | 88 (22.2) | 0.053 |
| Hypertension post-LT | 67 (12.7) | 22 (16.8) | 45 (11.3) | 0.104 |
| Dyslipidaemia post-LT | 175 (33.1) | 63 (48.1) | 112 (28.2) | < 0.001 |
| Cardio-cerebral events post-LT | 18 (3.4) | 10 (7.6) | 8 (2.0) | 0.005 |
| CKD post-LT | 91 (17.2) | 28 (21.4) | 63 (15.9) | 0.148 |
| AR post-LT | 58 (11.0) | 20 (15.3) | 38 (9.6) | 0.071 |
| CR post-LT | 20 (3.8) | 9 (6.9) | 11 (2.8) | 0.062 |
| Infection post-LT | 165 (28.7) | 65 (36.7) | 100 (25.2) | 0.042 |
| Graft failure | 52 (9.8) | 20 (15.3) | 32 (8.1) | 0.016 |
| Donor characteristics | | | | |
| Age (yr) | 34.01 ± 8.75 | 33.62 ± 8.33 | 34.13 ± 8.89 | 0.559 |
| Gender (male) | 443 (84.5) | 108 (82.4) | 335 (84.4) | 0.600 |
| Donor type (LDLT) | 158 (29.9) | 34 (26.0) | 124 (31.2) | 0.252 |

NODM: New-onset diabetes mellitus; Age: Age at transplantation; MELD: Model for end-stage liver disease; BMI: Body mass index; LT: Liver transplantation; cTAC: Tacrolimus trough concentration; MMF: Mycophenolate mofetil; CKD: Chronic kidney disease; AR: Acute rejection; CR: Chronic rejection; LDLT: Living donor liver transplantation.

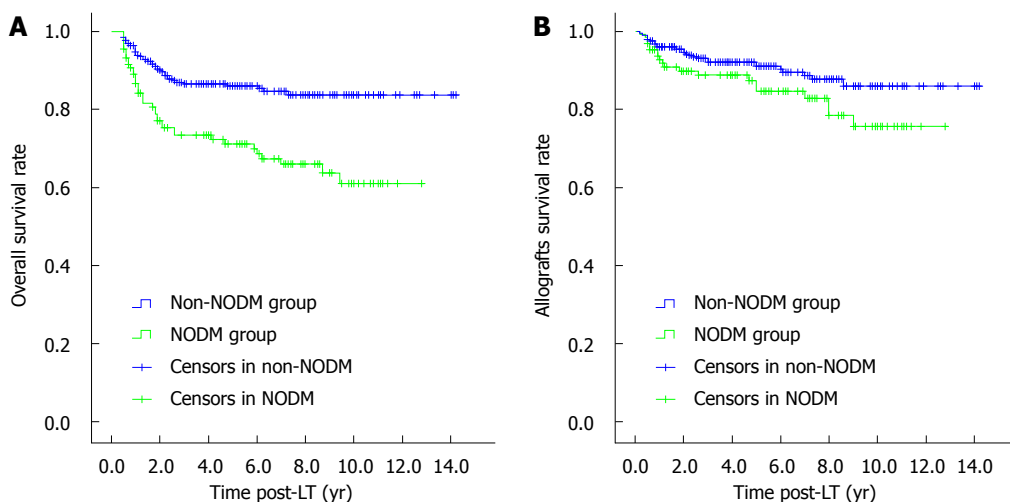


Figure 2 Survival rates of liver recipients in non-new-onset diabetes mellitus and new-onset diabetes mellitus groups. A: Overall survival rates (*P* < 0.05); B: Allograft survival rates (*P* < 0.05). NODM: New-onset diabetes mellitus; LT: Liver transplantation.

factors for NODM by univariate Cox regression analysis (Table 3). We chose all statistically significant factors as candidates for multivariate Cox regression analysis.

As a result, recipient' age at the time of LT (age > 50 years), pre-LT hypertension, and high mean cTAC (≥ 5.89 ng/mL) after 6 mo were deemed independent

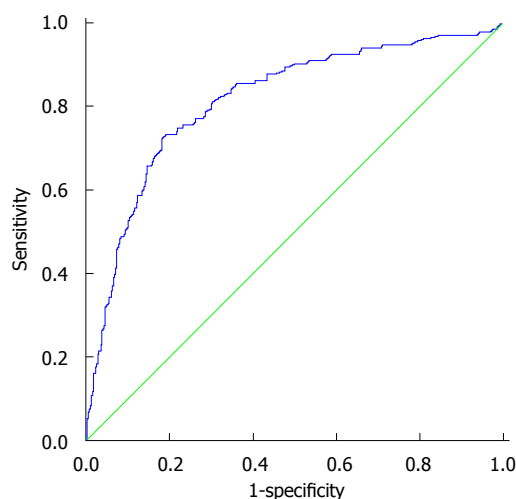


Figure 3 Receiver operating characteristic curve for mean cTAC after 6 mo to predict new-onset diabetes mellitus after transplantation.

Table 2 Clinical complications associated with mean tacrolimus trough concentration *n* (%)

| Complications post-LT | Low-cTAC group (<i>n</i> = 356) | High-cTAC group (<i>n</i> = 172) | <i>P</i> value |
|----------------------------------|-------------------------------------|--------------------------------------|----------------|
| Overweight/obesity (BMI ≥ 25) | 76 (21.3) | 52 (30.2) | 0.026 |
| Hypertension | 48 (13.5) | 19 (11.0) | 0.431 |
| Dyslipidaemia | 98 (27.5) | 77 (44.8) | < 0.001 |
| Cardio-cerebral events | 12 (3.4) | 6 (3.5) | 0.944 |
| CKD | 52 (14.6) | 39 (22.7) | 0.021 |
| AR | 34 (9.6) | 24 (14.0) | 0.129 |
| CR | 10 (2.8) | 10 (5.8) | 0.090 |
| Infection | 88 (24.7) | 57 (33.1) | 0.042 |
| New-onset malignance | 8 (2.2) | 1 (0.6) | 0.304 |

cTAC: Tacrolimus trough concentration; BMI: Body mass index; LT: Liver transplantation; CKD: Chronic kidney disease; AR: Acute rejection; CR: Chronic rejection.

risk factors for post-LT NODM (Table 4).

DISCUSSION

With improved long-term survival after transplantation, post-operative NODM in recipients has become more prevalent^[25]. Our analysis of 528 liver transplant recipients showed that the cumulative incidence of new-onset DM increased after LT. The recipients with NODM were more likely to develop dyslipidemia, cardio-cerebral vascular events, moderate to severe infections, and allograft loss, which often reduced recipient survival time^[26,27]. Inevitably, recipients with NODM had poorer long-term overall and allograft survival than non-NODM recipients^[5].

The immunosuppressive regimen employed after LT is important in decreasing the incidence of NODM. Corticosteroids could cause increased gluconeogenesis by inducing insulin resistance^[28]. Previous studies have shown that the diabetogenic risks of corticosteroids

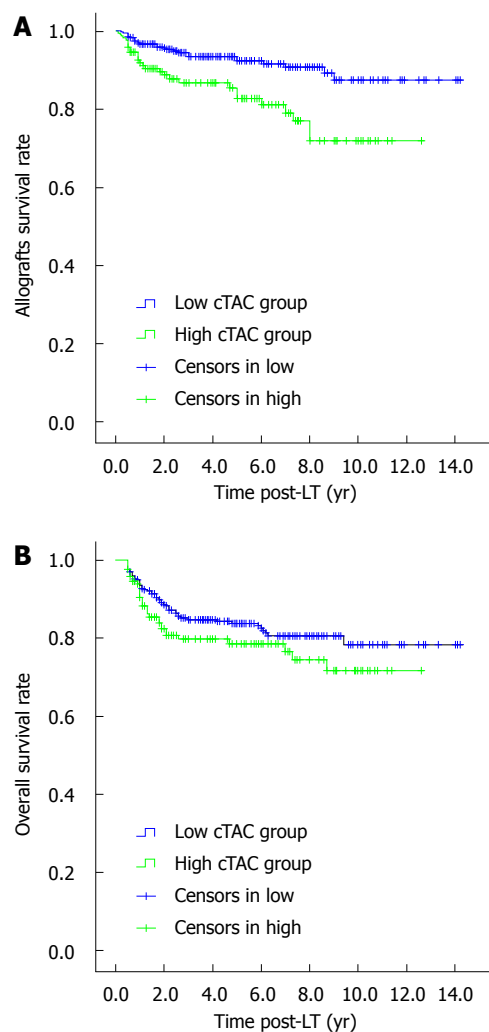


Figure 4 Survival rates of recipients in low and high mean tacrolimus trough concentration groups ($P < 0.05$). A: Allograft survival rates; B: Overall survival rate ($P = 0.129$). Low mean cTAC group: mean cTAC < 5.89 ng/mL; High cTAC group: mean cTAC ≥ 5.89 ng/mL. cTAC: Tacrolimus trough concentration; LT: Liver transplantation.

are cumulative and dose-dependent and that early tapering of corticosteroids decreased the incidence of diabetes at 1 year after LT^[29]. In our center, we therefore attempted to discontinue the use of corticosteroids within the first 3 mo of LT. Therefore, we analyzed blood glucose data after 6 mo to avoid the residual effects of corticosteroids on recipient metabolic profiling^[30].

TAC dominant therapies remain the first-line immunosuppressive regimen indicated for liver recipients. By inhibiting *IL-2* gene transcription, TAC decreases post-LT acute and chronic rejection. However, this mechanism may also contribute to insulin resistance and direct toxicity in pancreatic β -cells^[31]. Previous studies have reported that TAC-associated chronic complications, such as metabolic disorders^[2], renal dysfunction^[11], and hepatocellular carcinoma recurrence^[13], are related to TAC concentration. To reduce the TAC related complications, it is recommended that cTAC is reduced to 5–10 ng/mL during the first month^[14]. However, the

Table 3 Univariate analysis of risk factors for new-onset diabetes mellitus after liver transplantation

| Clinical factor | HR | 95%CI | P value |
|--|-------|--------------|---------|
| Recipient characteristics | | | |
| Elder recipient (age > 50 yr) | 1.568 | 1.096-2.245 | 0.014 |
| Male recipient gender | 0.690 | 0.414-1.150 | 0.155 |
| Child-Pugh (A/B/C) | 0.985 | 0.788-1.232 | 0.895 |
| MELD Score | 1.107 | 0.997-1.037 | 0.088 |
| BMI \geq 25 pre-LT | 1.616 | 1.100-2.373 | 0.014 |
| Hypertension pre-LT | 4.458 | 2.058-9.659 | < 0.001 |
| Dyslipidaemia pre-LT | 2.064 | 1.201-3.549 | 0.009 |
| Hepatitis B virus disease | 0.955 | 0.632-1.443 | 0.828 |
| Hepatitis C virus disease | 0.699 | 0.098-5.007 | 0.722 |
| Alcoholic cirrhosis | 2.307 | 1.076-4.948 | 0.032 |
| Tumors | 0.961 | 0.676-1.304 | 0.822 |
| With Rapamycin | 1.168 | 0.744-1.761 | 0.459 |
| With MMF | 0.979 | 0.690-1.387 | 0.903 |
| High mean cTAC (cTAC \geq 5.89 ng/mL) | 8.709 | 5.873-12.915 | < 0.001 |
| BMI \geq 25 post-LT | 1.345 | 0.927-1.951 | 0.119 |
| Hypertension post-LT | 1.278 | 0.808-2.021 | 0.294 |
| Dyslipidaemia post-LT | 2.014 | 1.429-2.838 | < 0.001 |
| CKD post-LT | 1.140 | 0.925-1.405 | 0.218 |
| AR post-LT | 1.701 | 1.056-2.742 | 0.029 |
| CR post-LT | 2.068 | 1.050-4.074 | 0.036 |
| Donor characteristics | | | |
| Donor age at LT (per year) | 0.994 | 0.975-1.015 | 0.590 |
| Male donor gender | 1.202 | 0.766-1.886 | 0.423 |
| Donor type (LDLT) | 0.859 | 0.581-1.270 | 0.446 |

LT: Liver transplantation; MELD: Model for end-stage liver disease; MMF: Mycophenolate mofetil; cTAC: Tacrolimus trough concentration; CKD: Chronic kidney disease; AR: Acute rejection; CR: Chronic rejection; BMI: Body mass index; LDLT: Living donor liver transplantation.

cutoffs or the ranges of cTAC were limited within early stages (4-26 wk) after transplantation and arbitrarily identified with no statistical evidence. Our study focused on the impact of long-term (after 6 mo) cTAC level on post-LT NODM and used an ROC curve to determine the best cutoff mean cTAC to be 5.89 ng/mL. Multivariate analysis showed that exposure to cTAC \geq 5.89 ng/mL significantly increased the risk of post-LT NODM (HR = 9.474, 95%CI: 6.357-14.119). Similarly, exposure to a high mean cTAC also increased the risk of being overweight or obese, dyslipidemia, CKD, and moderate to severe infection after LT. Fortunately, recipients with a low mean cTAC after 6 mo did not suffer from more acute and chronic rejections. Surprisingly, recipients exposed to a low mean cTAC benefited from longer allograft survival. Thus, we suggest adjusting and maintaining the cTAC below 5.89 ng/mL after 6 mo to reduce chronic complications and improve the overall and allograft survival rates.

Additionally, Cox regression analysis indicated that recipient age (> 50 years) and pre-LT hypertension were independent risk factors in the incidence of post-LT NODM. As we know, increasing age is a significant risk factor for type 2 diabetes in the general population^[32]. Correspondingly, diabetes has been a major cause of chronic complications, reduced quality of life and increased incidence of cardiovascular

Table 4 Multivariate analysis of risk factors for new-onset diabetes mellitus after liver transplantation

| Clinical factor | HR | 95%CI | P value |
|--|-------|--------------|---------|
| Elder recipient (age > 50 yr) | 1.925 | 1.335-2.776 | < 0.001 |
| Hypertension pre-LT | 4.220 | 1.931-9.226 | < 0.001 |
| High mean cTAC (cTAC \geq 5.89 ng/mL) | 9.474 | 6.357-14.119 | < 0.001 |

cTAC: Tacrolimus trough concentration; LT: Liver transplantation.

adverse events in the elderly. A UNOS study by Kuo *et al.*^[33] reported older age (> 50 years) to be an independent predictor of post-LT NODM, with a 24.1% risk increase in 15463 adult recipients. Otherwise, the prevalence of hypertension is usually high (> 50%) in diabetes patients^[34], and hypertension causes a quadruply increase in cardiovascular risk in people with diabetes^[35]. It is assumed that insulin resistance and the consequent hyperinsulinemia interacted with increased renal sodium retention, sympathetic tone and renin-angiotensin-aldosterone system activity^[36].

Many studies have reported that BMI \geq 25^[33,37,38], dyslipidemia^[38], and alcoholic cirrhosis^[33,39] were independent risk factors for NODM after transplantation, but they were significant only in univariate analysis. HCV-associated liver disease was a high risk factor in previous studies^[33,37], but was negative in our study. We assumed that this was due to the low percentage of HCV patients in our center (1.1%), unlike in western countries, where a large number of HCV patients received liver transplants.

In conclusion, some factors are positively related to diabetes progression after LT. Interestingly, mean cTAC is the only controllable factor, so adjusting the dose and trough concentration of TAC is important for preventing post-LT NODM. In accordance with the minimum required tacrolimus dosage early after transplantation, we recommend a decrease in the mean cTAC to < 5.89 ng/mL after 6 mo, as has been practical in Chinese liver transplantation recipients. Limitations of this study are that the data were collected retrospectively and that there was no detailed minimum scheme for timing after transplantation. Therefore, a well-designed prospective clinical trial is needed to confirm our findings and to develop an accepted tacrolimus adjustment protocol.

COMMENTS

Background

New-onset diabetes mellitus (NODM) is a serious metabolic complication after liver transplantation (LT) and is associated with increased rates of cardiovascular disease, rejection, infection and decreased survival. Tacrolimus has strong diabetic effects vs other immunosuppressants and early minimum tacrolimus strategy has been reported to be protective against other complications. The author performed this study to analyze the relationship between tacrolimus concentration (cTAC) and NODM development after 6 mo and to explore the impact of low cTAC on common complications after LT.

Research frontiers

Due to the negative impact of NODM on the long-term outcome of LT, the study about NODM has been important. cTAC is a controlled risk factor for NODM and early (4-26 wk) minimum tacrolimus strategy is safe and beneficial for LT recipients. This retrospective study indicated that reducing cTAC to below 5.89 ng/mL lately (after 6 mo) could prevent recipients from developing NODM and other complications.

Innovations and breakthroughs

Early minimum tacrolimus strategy can decrease the risk of renal dysfunction, dyslipidemia and tumor recurrence. But the cutoffs or the ranges of cTAC were limited within early stages (4-26 wk) after transplantation and arbitrarily identified with no statistical evidence. This study focused on the impact of long-term (6 mo) cTAC level on post-LT NODM and used an ROC curve to determine the best cutoff mean cTAC to be 5.89 ng/mL. And further analysis showed that reducing cTAC to 5.89 ng/mL decreased the incidence of other TAC related complications without increasing rejection.

Applications

Minimizing TAC lately (after 6 mo) to below 5.89 ng/mL is safe and protective against NODM after LT, but multicenter prospective clinical trials are needed to confirm the findings obtained in this study and to develop an accepted tacrolimus adjustment protocol.

Terminology

NODM is defined as diabetes newly diagnosed after LT, occurring in 16%-61% of recipients. Mean cTAC is determined as the average value of cTAC in the year of diabetes diagnosis in NODM recipients or in the last year of the follow-up period in non-NODM recipients.

Peer-review

This manuscript revealed that the risk of the new onset diabetes mellitus after liver transplantation is dependent on high mean tacrolimus. The number of patients is remarkable from a single institute.

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Retrospective Study

Inferoposterior duodenal approach for laparoscopic pancreaticoduodenectomy

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Author contributions: Wang XM designed and performed the research and wrote the paper; Sun WD designed and supervised the report; Hu MH designed the research and contributed to the analysis; Wang GN, Jiang YQ, Fang XS, and Han M provided clinical advice.

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Abstract

AIM: To investigate the advantages of inferoposterior duodenal approach (IPDA) for laparoscopic pancreaticoduodenectomy (LPD).

METHODS: A total of 36 patients subjected to LPD were admitted to the Affiliated Yijishan Hospital of Wannan Medical College from December 2009 to February 2015. These patients were diagnosed with an ampullary tumour or a pancreatic head tumour through computed tomography, magnetic resonance imaging or endoscopic retrograde cholangiopancreatography preoperatively. The cases were selected on the basis of the following criteria: tumour diameter < 4 cm; no signs of peripheral vascular invasion; evident lymph node swelling; and distant metastasis. Of the 36 cases, 20 were subjected to anterior approach (AA; AA group) and 16 were subjected to IPDA (IPDA group). Specimen removal time, intraoperative blood loss and postoperative complications in the two groups were observed, and their differences were compared.

RESULTS: During the operation, 2 cases in the AA group and 2 cases in the IPDA group were converted to laparotomy; these cases were excluded from statistical analysis. The remaining 32 cases successfully completed the surgery. The AA group and IPDA group exhibited the specimen removal time of 205 ± 52 and 160 ± 35 min, respectively, and the difference was significant ($P < 0.01$). The AA group and IPDA group revealed the intraoperative blood loss of 360 ± 210 mL and 310 ± 180 mL, respectively, but these values were not significantly different. Postoperative pathological

results revealed 4 cases of inferior common bile duct cancer, 8 cases of duodenal papillary cancer, 6 cases of ampullary cancer, 13 cases of pancreatic cancer, 3 cases of chronic pancreatitis accompanied with cyst formation or duct expansion, and 2 cases of mucinous cystic tumour in the pancreatic head. The postoperative complications were pulmonary *Staphylococcus aureus* infection, incision faulty union, ascites induced poor drainage accompanied with infection, bile leakage, pancreatic leakage and delayed abdominal bleeding.

CONCLUSION: In IPDA, probing for important steps can be performed in early stages, surgical procedures can be optimised and operation time can be shortened.

Key words: Laparoscopic pancreaticoduodenectomy; Surgical approach

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Core tip: This study investigated the advantages of inferoposterior duodenal approach for laparoscopic pancreaticoduodenectomy. Results revealed that the inferoposterior duodenal approach can be performed not only to probe for important steps in early stages but also to optimise surgical procedures and shorten operation time.

Wang XM, Sun WD, Hu MH, Wang GN, Jiang YQ, Fang XS, Han M. Inferoposterior duodenal approach for laparoscopic pancreaticoduodenectomy. *World J Gastroenterol* 2016; 22(6): 2142-2148 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i6/2142.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i6.2142>

INTRODUCTION

In 1994, Gagner^[1] completed the first laparoscopic pancreaticoduodenectomy (LPD) worldwide; after 20 years of efforts, LPD has been gradually performed globally, and its safety has been confirmed^[2-6]. However, compared with other fields of abdominal surgery, LPD is in the exploratory stage, and numerous problems, such as surgical approaches, remain unresolved. In LPD, an open abdominal approach or the anterior approach (AA) is commonly used^[7,8]. In this approach, the hepatic flexure of the colon is freed and Kocher incision is implemented, and this procedure is performed from the front parts to the rear parts and from the top sections to the bottom sections. However, the freedom degree of LPD cannot be compared with that of open abdominal surgery because LPD is limited by visual angles and operating holes, as well as insufficient exposure effects by hands; as such, the open abdominal surgical approach cannot be fully applied into LPD. Despite these drawbacks, LPD

exhibits good characteristics and provides advantages. For instance, small spaces or surgical fields that cannot be reached through laparotomy can be accessed through LPD. LPD provides unique caudal and dorsal visual angles. Dissection and freeing towards specific parts can be completed from specific angles. LPD can also be applied to perform amplification, which can yield a clear surgical field and high-throughput operations. Therefore, studies have developed new suitable surgical approaches for endoscopic surgeries; such novel approaches have been considered as the basis to optimise the advantages of LPD and to improve LPD. We have acquired experiences in AA-LPD; on the basis of these experiences, we developed a new surgical approach, namely, inferoposterior duodenal approach (IPDA). Here we describe this surgical approach and its advantages in detail.

MATERIALS AND METHODS

General information

A total of 36 cases (21 males and 15 females, aged 35 to 75 years) subjected to LPD were admitted to our hospital from December 2009 to February 2015. These patients were diagnosed with an ampullary tumour or a pancreatic head tumour through computed tomography, magnetic resonance imaging or endoscopic retrograde cholangiopancreatography preoperatively. The cases were selected in accordance with the following criteria: tumour diameter < 4 cm; no signs of peripheral vascular invasion; evident lymph node swelling; and distant metastasis. Of the 36 cases, 20 were included in the AA group and 16 were included in the IPDA group.

Surgical method (IPDA)

Anaesthesia and position: The patients received general anaesthesia and tracheal cannulation. The patients were then placed in a supine position and the two legs were in a split position. A laparoscope was inserted through one small incision at the inferior navel ring edge (or 3-5 cm below the belly button); four small incisions were made as primary and secondary operating holes below the front rib margin of the left and right armpits and mildly over the umbilical level of the left and right clavicular midline. The surgeons remained on the patients' left side, and the assistant stood on the patients' right side.

Probing: The liver, abdominal cavity and omentum were conventionally explored for several situations, such as metastasis, cholestasis in the liver or bile duct dilation. The transverse mesocolon was lifted, and the inferior duodenal flexure was exposed from the right side of its root (Figure 1A). The rear part along the inferior duodenal flexure was freed, Toldt's gap was penetrated, the inferior vena cava was exposed, and whether the lesion invaded this site was probed.

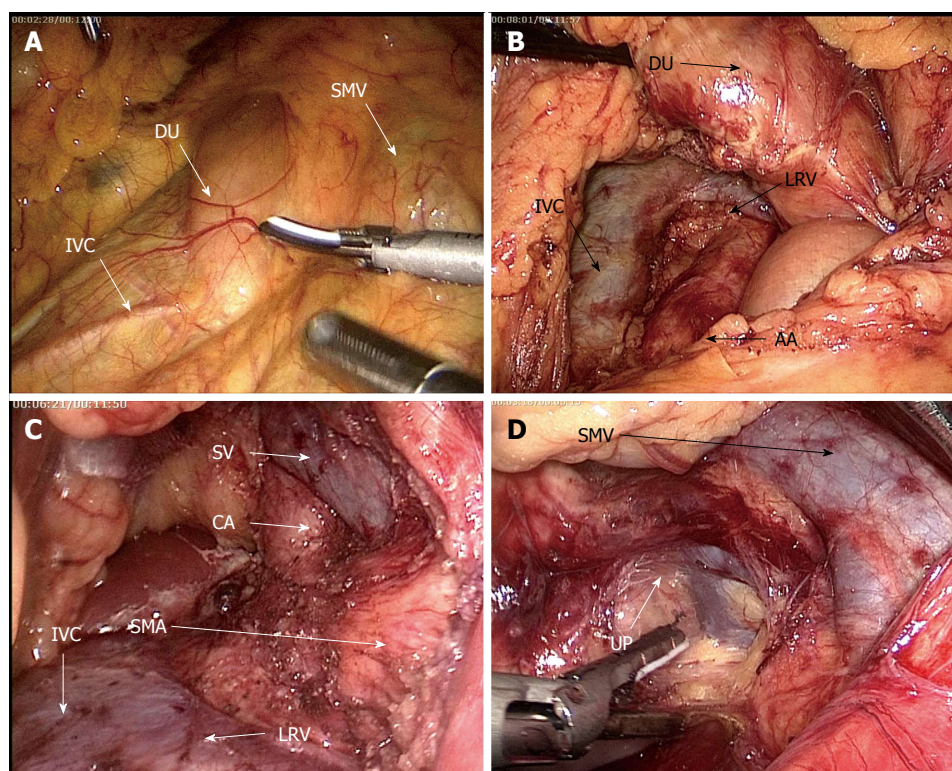


Figure 1 Surgical method - probing. A: After lifting the transverse mesocolon and exposing the inferior duodenal flexure from its root, the superomedial side of this section is SMV, and the posterolateral side is IVC, and in here, they are the "window" of LPD; B: After lifting the inferior duodenal flexure, freeing along its rear part, entering the Toldt's gap, revealing the inferior vena cava and left renal vein, and freeing leftwards to reveal the abdominal aorta, the para-abdominal aortic lymph nodes are obtained and sent for the intraoperative frozen section; C: After exposing SMA root from the upper site of LRV and dissecting along its distribution from the rear part, whether tumour invades the SMA or not is explored. The freeing is then continued towards the caput to reveal the VA root and clean its surrounding lymph nodes; D: After revealing the SMV from inferior duodena part, this segment has longer SMV, entirely located within the small bowel mesentery, and has no avascular association with the duodenum. SMV: Superior mesenteric vein; IVC: Inferior vena cava; DU: Duodenum; LPD: Laparoscopic pancreaticoduodenectomy; LRV: Left renal vein; AA: Abdominal aorta; CA: Celiac artery; UP: Uncinate process.

The abdominal aorta was then revealed and the para-aortic lymph nodes were harvested for the frozen section (Figure 1B). The surgery was terminated if lymph node metastasis occurred. Subsequently, The left renal vein (LRV) was revealed and the superior mesenteric artery (SMA) was exposed just above the LRV and dissected along its trunk under the unique dorsal view of laparoscopy until the horizontal part of duodenum, probing whether the tumor invaded the SMA or not. The root of celiac trunk was also revealed and the surrounding lymph nodes were cleaned (Figure 1C). The SMV was revealed at the inferior duodenal part (Figure 1D), the vascular sheath was opened and freed upwards, and the right gastroepiploic vein branches were anatomically dissected. The lower pancreatic edge was freed and lifted, sneak dissection was performed from the rear pancreas to the abouchement point of the splenic vein, and whether the tumour invaded the SMV was then determined.

Specimen dissection: After probing confirmed that the tumour was resectable, the visual field was shifted to the left of the transverse mesocolon root and the jejunum was transected 15 cm away from Treitz ligament. The proximal jejunum was pulled

to the right through the rear part of the mesenteric vessels. The gastroduodenal ligament was transected, the greater and lesser gastric curvatures were freed, and the gastric body was transected. The pancreatic neck was transected and the common hepatic artery was revealed on its upper edge. A tape, which was a "sling" from the pancreatic head and the inferior uncinate process, was suspended and pulled rightwards, while the SMV was pushed leftwards, and the right side wall of superior mesenteric artery was revealed. The arterial sheath was opened and the SMV and branches from the SMA to the pancreatic uncinate process from bottom to top were dissected, and the uncinate process was completely freed. The surrounding lymph adipose tissues were cleaned. The hepatoduodenal ligament was penetrated, and the GDA was dissected, freed towards the hepatic portal along the surface of the portal vein, and separated from the hepatic artery and the bile duct. The hepatic artery was fully anatomised and the surrounding fat lymphoid tissues were cleaned (Figure 2). The gallbladder was finally removed and the common hepatic duct was transected. The specimen was then removed.

Reconstruction: The digestive tract was recon-

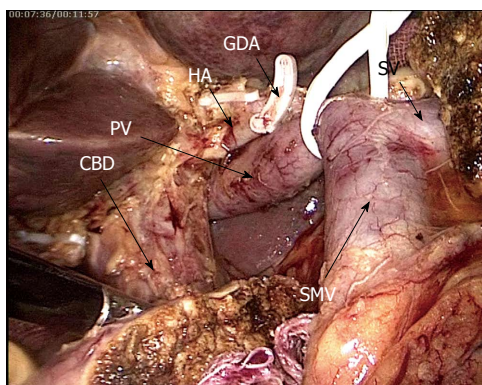


Figure 2 Surgical method - specimen dissection. After entering the hepatoduodenal ligament, dissecting GDA, and freeing towards the hepatic porta along the surface of hepatic portal vein, it is separated from the hepatic artery and common bile duct. The hepatic artery is fully dissected, and the surrounding fat lymphoid tissues are also dissected. Finally, the common bile duct is dissected. GDA: Gastric-duodenum artery; HA: Hepatic artery; PV: Portal vein; CBD: Common bile duct; SMV: Superior mesenteric vein; SV: Spleen vein.

structured in accordance with Child surgical procedures through endoscopy or with small incision assistance.

Observation indexes

Specimen removal time, intraoperative blood loss and postoperative complications in the two groups were observed.

Statistical analysis

Statistical analyses were carried out using SPSS 17.0 (SPSS Inc., Chicago, IL, United States). Data are presented as mean \pm SD. Comparisons between the two groups were performed using *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Intraoperative conditions

Of the 36 patients, 4 were converted to laparotomy; the lesions of the 3 cases were closely related to the vessels and difficult to be endoscopically separated, and the chronic pancreatic inflammation of 1 case exhibited heavy adhesion to the surrounding tissues and easily caused bleeding during separation. Of these 4 cases, 2 were in the AA group and 2 were in the IPDA group, and these 4 cases were not included in the statistical analyses. The remaining 32 cases successfully completed the surgery. The difference in the specimen removal time was significant ($P < 0.01$), with 205 ± 52 min in the AA group and 160 ± 35 min in the IPDA group. The blood loss was 360 ± 210 mL in the AA group and 310 ± 180 mL in the IPDA group, and this finding did not significantly differ ($P > 0.05$).

Postoperative pathological results

Of the 36 cases, the following conditions were observed: 4 cases of inferior common bile duct cancer;

8 cases of duodenal papillary cancer; 6 cases of ampullary cancer; 13 cases of pancreatic cancer (9 cases of adenocarcinoma, 2 cases of adenosquamous cancer, 1 case of neuroendocrine tumour, and 1 case of solid false papilloma); 3 cases of chronic pancreatitis accompanied with cyst formation or duct expansion; and 2 cases of mucinous cystic tumour in the pancreatic head.

Postoperative complications

Several postoperative complications were encountered. One case of postoperative pulmonary *Staphylococcus aureus* infection was observed; as such, the antibacterial treatment was strengthened. One case of incision faulty union was documented, and the phase II suture was performed to repair this problem. One case of ascites-induced poor drainage accompanied with infection was recorded; as such, re-surgical drainage was performed. Three cases of bile leakage occurred, but this condition spontaneously healed after drainage. Five cases of pancreatic leakage, including one case of secondary abdominal bleeding, were found. Therefore, re-operation was conducted, and a dehiscence wound was detected on the partial anterior wall of the pancreatic anastomotic site. The wound was then re-sutured, and the patient spontaneously healed after the wound was rinsed with double cannula. Four cases of pancreatic leakage were documented, and this condition spontaneously healed after drainage. One case of delayed abdominal bleeding was found, and surgical exploration revealed that this haemorrhagic condition was caused by a hepatic artery rupture; therefore, the patient was subjected to partial arterial resection and reconstruction. However, the anastomotic site bled postoperatively; as such, the patient was subjected to an artery ligation again. Afterwards, the patient recovered well and was discharged.

DISCUSSION

In this study, LPD was performed with the duodenum as the centre. In general, the traditional anterior approach is initiated by freeing the descending part of the duodenum. However, the intestinal segment is found deep into the retroperitoneum, in which the transverse colon and its mesenteria are covered; as such, these parts cannot be easily exposed or reached. Furthermore, the operation is difficult and limited by a trocar hole. However, we found that the location at the junction of the descending duodenum and its inferior part (*i.e.*, inferior duodenal flexure) was relatively superficial, and only one layer of the peritoneum was covered. The site was located on the right of transverse mesocolon root and thus could be exposed when the transverse mesocolon was lifted. The anteromedial side of the intestinal canal was the SMV, and the posterolateral side was the inferior vena cava,

which is the major hub of pancreaticoduodenectomy. We used this part as the initial step of the surgery, which was combined with the “window”, and the inferoposterior approach of LPD was developed. The outcomes were good, and the approach provided the following advantages.

First, the inferoposterior approach of LPD maximised the advantages of laparoscopy; in particular, small spaces could be reached. The probe could enter the dorsal part of the pancreatic head and the duodenum through the rear part of the “window”, and relevant exploration could be accomplished to determine the surgical methods. In the biopsy of the para-abdominal aortic lymph nodes, pancreatic cancer causes a high rate of lymph node metastasis; for instance, 54% to 86% of patients likely suffer from lymph node metastasis after they undergo surgery^[9,10]. Even a small pancreatic tumour (diameter < 2 cm) exhibits a lymphatic metastatic rate of up to 37.2%^[11]. In addition, the involvement of para-abdominal aortic lymph nodes is manifested as distant metastasis (M1), which often corresponds to poor prognosis. Although these patients are subjected to enlarged lymph node dissection, the long-term survival is significantly worse than those with negative lymphatic metastasis^[12-15], and this finding does not significantly differ from the patients who did not opt for the removal of their tumours^[16-18]. Therefore, the intraoperative biopsy towards the para-abdominal aortic lymph nodes helps guide this surgical approach; during surgery, a surgeon can further evaluate the patients’ prognosis and determine appropriate surgical methods. In AA-LPD, lymph nodes cannot be easily obtained in this region. For this reason, the hepatic flexure of the colon must be dissected, Kocher incision must be made and the pancreatic head and the duodenum must be fully freed. IPDA could be performed to directly enter the region after the hepatic flexure of the duodenum was freed; thus, the lymph nodes could be obtained with the shortest distance and the fastest speed to assess lymph node metastasis in early stages. Lymph node metastasis confirmed through rapid intraoperative pathological assessment could indicate poor prognosis, and surgical resection unlikely yielded positive outcomes and further trauma could be avoided. In the exploration to the superior mesenteric artery, the resection and reconstruction of SMV are safe and feasible through pancreaticoduodenectomy^[19,20]. By comparison, the invasion of tumour towards the SMA is a counterindication for surgery because the resection and reconstruction of the SMA likely cause a high mortality rate and induce complications after surgery; these procedures could not prolong the survival period of patients^[21]. In traditional PD surgery, the exploration towards the SMV and the portal venous system is set in early stages, and the SMA damaged by tumour is often determined in the last stage of resection. In particular, the SMA is determined during the disruption of the pancreatic neck, and the surgeon does not

have other options. Thus, the surgeon must resect the specimens. As a result, positive margins may be detected, and these patients likely show a poor prognosis. Furthermore, the long-term survival rate is very low. Pessaux *et al.*^[22] proposed the artery-leading surgical approach in 2006; SMA is developed and thus has allowed surgeons to find the damaged arteries in early stages; further resection is not performed, so it could avoid the embarrassment of not being able to regret. Since then, scholars have published similar reports; on the basis of different locations and conditions of tumours, these scholars proposed a number of artery-leading surgical approaches^[23-27]. In this study, the pancreatic head and the duodenum were lifted forward after we obtained the para-abdominal aortic lymph nodes, and the SMA root was then exposed from the site where the left renal vein spanned the upper edge of the abdominal aorta. The unique dorsal visual angle of laparoscopy was then used to dissect along its direction to confirm whether the SMA was invaded by tumours. Further freeing along this path and towards the caput could help elucidate the relationship of cancer with celiac trunk. Therefore, this approach could reveal the relationships between tumour and arteries in earlier stages than traditional surgical approaches; as such, appropriate surgical procedures can be determined and selected.

Second, in this approach, the SMV was dissected from the anteromedial side of the “window”; thus, the SMV probing could be completed safely. SMV probing is one of the difficulties in LPD. Traditional probing begins from the uncinate process segment of the blood vessels, and the lower edge of the pancreas is exposed; the mid colon vein or the right gastroepiploic vein is considered as an indicator^[28,29]. The branch vessels followed by the SMV trunk are processed. However, this SMV segment is the shortest and contains the highest number of branches, such as superoanterior pancreaticoduodenal vein and other branches besides the right gastroepiploic vein. These vessels are imported into the SMV with different vessels and from different levels; therefore, the anatomical levels are difficult to be determined during separation. Once the damage occurs, uncontrollable bleeding is inevitable; as such, this procedure likely causes bleeding. In this study, the SMV probing was initiated from the anteromedial side of the “window”, and this SMV segment was longer and located entirely within the small bowel mesentery. The segment did not show vascular association with the inferior duodenal part. Thus, the segment was convenient and safe for the exposure. Opening the vascular sheath from this site and freeing upwards along the intrathecal space, surgeons could quickly locate and process the blood vessel branches in the uncinate process and could reach the rear pancreatic vessels for further exploration. This trunk-first-branch-later approach could reduce the risk of bleeding and increase the safety of the surgery.

Lastly, this approach was more conducive to the lymph node dissection of the hepatoduodenal ligament, which is another technical difficulty in LPD. Traditional dissection methods begin from the top parts to the bottom parts of the hepatic portal, and this procedure is difficult to perform through endoscopy. When the common hepatic duct is transected or the hepatic artery is anastomosed, the risks of damaging the posterior portal vein and causing bleeding remain unknown. We believe that opening the portal vein sheath to completely expose the portal vein is an important measure to avoid injuries. However, in the three-tube structure of the liver ligament, the portal vein was located distally; as such, this vein cannot be easily revealed *via* the conventional method. Therefore, an appropriate approach should be determined. In this study, when the probing confirmed that the tumour could be removed, we firstly dissected the proximal jejunum and then transected the pancreatic uncinate process neck to expose the whole SMV and the portal vein. We anastomosed and dissected the hepatoduodenal ligament in the final stages of resection. At this time, the small branches that assemble into the portal vein system were processed when the uncinate process was freed; therefore, the clearance of the hepatoduodenal ligament could be completed only after anastomosing along the constant common hepatic artery. Such bottom-to-top anatomy would not only benefit the endoscopic operation but also "simplify" the complex skeletonisation of the hepatoduodenal ligament. As a result, operation efficiency could be improved and operation time could be reduced. This approach is safe and effective for patients with a history of cholangitis, which does not show a clear anatomy of the hepatoduodenal ligament; this approach is also beneficial for patients with liver tumours located in deep regions that cause difficulty in exposing the hepatoduodenal ligament.

In summary, this approach fully combined the duodenal anatomical characteristics and laparoscopic advantages; thus, probing, separation and sample resection could be finished in one step. With this approach, probing can be performed in early stages, surgical procedures can be optimised and operation time can be shortened. However, the number of cases in this study was small, and the advantages of the proposed procedure should be further confirmed through comparative studies.

COMMENTS

Background

Laparoscopic pancreaticoduodenectomy (LPD) is one of surgeries with the most difficulty in endoscopic surgery. This procedure is in the exploratory stage, and numerous problems, such as surgical approaches, remain unresolved. At present, laparotomy approach is often used for LPD, but this approach is not entirely suitable for laparoscopic operation. Studies should further discuss the mechanisms by which the characteristics and advantages of laparoscopy can be maximised and determine a suitable surgical approach.

Research frontiers

In 1994, LPD was completed for the first time. After 20 years of efforts, LPD has been gradually performed, and some surgical procedures, including pancreatic anastomosis and hook excision, have been improved. However, studies on surgical approaches of LPD are very rare.

Innovations and breakthroughs

On the basis of the duodenum anatomical characteristics and advantages of laparoscopy, we proposed the inferoposterior duodenal approach (IPDA). Compared with traditional anterior approaches, IPDA can be performed not only to probe in early stages but also to optimize the operation process and shorten the operation time.

Applications

IPDA is suitable for LPD. This procedure can optimise the surgical procedure, shorten the operation time and promote further applications of LPD.

Terminology

LPD is pancreaticoduodenectomy completed through laparoscopy.

Peer-review

This study investigated the advantages of the inferoposterior duodenal approach for laparoscopic pancreaticoduodenectomy. The results are significant and applicable to clinical practices and studies.

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Pancreatitis in hand-foot-and-mouth disease caused by enterovirus 71

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Abstract

Some viruses, including certain members of the enterovirus genus, have been reported to cause pancreatitis, especially Coxsackie virus. However, no case of human enterovirus 71 (EV71) associated with pancreatitis has been reported so far. We here report a case of EV71-induced hand-foot-and-mouth disease (HFMD) presenting with pancreatitis in a 2-year-old girl. This is the first report of a patient with acute pancreatitis in HFMD caused by EV71. We treated the patient conservatively with nasogastric suction, intravenous fluid and antivirals. The patient's symptoms improved after 8 d, and recovered without complications. We conclude that EV71 can cause acute pancreatitis in HFMD, which should be considered in differential diagnosis, especially in cases of idiopathic pancreatitis.

Key words: Pancreatitis; Enterovirus 71; Hand, foot and mouth disease

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Core tip: Acute pancreatitis associated with enterovirus 71 (EV71) infection is extremely rare. We here report a case of EV71-induced hand-foot-and-mouth disease (HFMD) presenting with pancreatitis in a 2-year-old girl. This is the first case report of acute pancreatitis associated with EV71 infection. EV71 can cause acute pancreatitis in HFMD, which should be considered in differential diagnosis, especially in cases of idiopathic pancreatitis.

Zhang YF, Deng HL, Fu J, Zhang Y, Wei JQ. Pancreatitis in hand-foot-and-mouth disease caused by enterovirus 71. *World*

INTRODUCTION

Enterovirus 71 (EV71) is a human enterovirus in the Enterovirus genus of the Picornaviridae family. Many of the EV71-infected cases have occurred in Asia-Pacific region, and posed a serious threat to children's health^[1]. EV71 primarily causes hand-foot-and-mouth disease (HFMD) in young children, and many neurological complications such as encephalitis, brain stem encephalitis and fatal pulmonary edema occur occasionally^[2]. However, no EV71-associated pancreatitis has been reported so far. We here describe a case of EV71-induced HFMD presenting with pancreatitis in a 2-year-old girl.

CASE REPORT

A 2-year-old girl was admitted to our hospital in June 2014 because of acute abdominal pain and vomiting for 2 d. Vomiting occurred about ten times a day. Moreover, 4 d before admission, maculopapular rashes had appeared on her hip and then spread to the palms of her hands and feet over the following 2 d, and she also had fever during the 4 d before admission. Her past medical history showed no record of pancreatitis and her family history was negative for pancreatic disease.

A physical examination on admission revealed a normal blood pressure, temperature, pulse rate, and breathing rate. Maculopapular rashes on her hip, palms and feet and vesicles in mouth cavity membrane were observed. Breathing sounds were clear on auscultation. Abdominal examination revealed only mild abdominal tenderness. No other abnormalities were found. Of note, she had not taken any drugs before admission.

On admission, her chest X-ray and electrocardiogram were both normal. A complete blood count, calcitonin and blood biochemical tests including C-reactive protein, glucose, bilirubin, triglycerides and calcium, were all within reference limits. The total levels of IgG, IgA and IgM in blood were normal. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were slightly elevated (ALT: 64 IU/L; AST: 68 IU/L; reference range: 10-35 IU/L), and serum amylase (385 IU/L; reference range: 0-220 IU/L) and urine amylase (5300 IU/L; reference range: 0-1200 IU/L) were also increased. Serological tests of various infectious agents including Epstein-Barr virus, varicella zoster virus, cytomegalovirus, HIV I and II, hepatitis A, B and C viruses, echoviruses, syncytial virus, flu virus A and B, parainfluenza 1, 2 and 3, and adenovirus were all negative. Tumor markers were also negative. Other relevant tests and examinations during



Figure 1 Adominal computed tomography showed acute pancreatitis with swelling of the pancreas and peri-pancreatic exudation. No cholelithiasis or tumor occluding the common bile duct or pancreatic duct was observed (arrow).

hospitalization were performed and the results were as follows: real-time reverse transcription PCR (RT-PCR) in a stool sample was positive for EV71, but negative for Coxsackie virus A16, and antibody titer against EV71 was markedly elevated and it was quadrupled during the recovery period. Ultrasonography of the abdomen revealed neither gallstones nor biliary sludge. Abdominal computed tomography (CT) showed acute pancreatitis with swelling of the pancreas, and peri-pancreatic exudation. No cholelithiasis or tumor occluding the common bile duct or pancreatic duct was observed (Figure 1). Magnetic resonance cholangiopancreatography (MRCP) performed in another hospital revealed peri-pancreatic exudation and no anatomical abnormalities in the pancreas or pancreatic duct. The girl was treated conservatively with nasogastric suction, intravenous fluid and antivirals. She was administered Cimetidine (0.15 g b.i.d.), antibiotics (Cefminox, 0.4 g q8h, iv), and antiviral agent (Leigh Bhav Lin, 0.15 g qd, iv).

The patient's symptoms improved after 8 d, and recovered without complications. On the day of discharge, all serum biochemical tests were normal. Two months later, findings on the abdominal CT scan were normal, all laboratory values were within the normal ranges, and the rashes had disappeared, and no further damage was observed. One year after follow-up, the patient was asymptomatic and showed no evidence of pancreatitis recurrence.

DISCUSSION

We have presented a case of EV71 infection associated with pancreatitis secondary to HFMD. EV71 was established as the causative pathogen of pancreatitis in this case based on the following evidence: EV71 positivity, negative history of alcoholic and drug use, no gallstones, no anatomical abnormalities in the pancreas or pancreatic duct, normal level of triglyceride and calcium, negative serological tests for other infectious agents, and presence of characteristic rash

on hip, palms and feet. Based on these observations, it is tempting to speculate that the EV71 is the most likely pathogenic factor for pancreatitis in this case.

Acute pancreatitis can be triggered by a variety of etiologies. Gallstones and alcohol are the most common causes of acute pancreatitis in adults^[3]. However, the etiology in children is often drugs, infection, trauma, genetic mutation, and congenital structural abnormalities such as choledochal cysts and abnormal union of the pancreatobiliary junction^[4,5]. The most common infections are mumps, viral hepatitis, Coxsackie virus and echovirus^[6]. At present, among anomalies of the pancreatobiliary system, choledochal cyst is the most common cause of acute pancreatitis^[7]. Some acute pancreatitis cases without a detectable cause are considered as "idiopathic"^[8]. Viral etiology may be involved in idiopathic acute pancreatitis.

The Enterovirus genus is part of the large Picornaviridae family, and is known to be highly cytolytic. The species are small non-enveloped RNA viruses and the most common viruses causing human diseases. EV71 belongs to the Enterovirus genus in the Picornaviridae family, and has been recognized as one of the most important viral pathogens. EV71 infection causes countless diseases ranging from mild HFMD or herpangina to fatal brain stem encephalitis complicated with pulmonary edema, and has become a serious threat to children's health. So far, no EV71-associated pancreatitis has been reported, but Coxsackievirus A and B have been reported to be causative pathogens of pancreatitis^[9-11] and type 1 diabetic mellitus^[12,13]. Studies have suggested the presence of enteroviruses in pancreatic tissue including the pancreatic islets of type 1 diabetic patients^[14]. Coxsackievirus has been shown to replicate and destroy human β -cells^[13]. But to the best of our knowledge, EV71-induced pancreatitis is quite rare, and our patient presented no other severe complications except for acute pancreatitis. A previous study analyzing the EV71 genome obtained from an immunocompromised host showed various EV71 lineages and indicated that a mutation in the VP1 BC loop region of EV71 (L97R) may play a critical role in cell tropism independent of the EV71 lineage^[15]. The mechanism of pancreatitis associated with EV71 is still unknown; EV71 might injure the pancreatic acinar cell membrane, leading to the leakage of intracellular enzymes, and at the same time, some other factors such as pancreatitis-related genetic susceptibility genes and virulence determinants in the genotype of the infecting strain should also be considered. Therefore, a multi-disciplinary approach is required to extend our understanding of this complex relationship.

In conclusion, EV71 can cause acute pancreatitis in HFMD, which should be considered in the differential diagnosis, especially in cases of idiopathic pancreatitis. It is important to screen the patients with acute pancreatitis for EV71 infections.

COMMENTS

Case characteristics

A 2-year-old girl presented with acute abdominal pain and vomiting for 2 d after the onset of rashes appearing on her hip, hands and feet.

Clinical diagnosis

Abdominal pain and vomiting, elevated level of urine and serum amylase concentration, magnetic resonance cholangiopancreatography (MRCP) and computed tomography (CT) findings of peri-pancreatic exudation and swelling.

Differential diagnosis

Gallstone-induced pancreatitis or pancreatic tumor.

Laboratory diagnosis

Serum amylase, 385 IU/L; urine amylase, 5300 IU/L.

Imaging diagnosis

MRCP and CT showed acute pancreatitis with swelling of the pancreas and peri-pancreatic exudation.

Treatment

The girl was treated conservatively with nasogastric suction, intravenous fluid, and antivirals.

Related reports

Reports of acute pancreatitis associated with enterovirus 71 (EV71) are rare, and only Coxsackie virus-related pancreatitis has been reported.

Experiences and lessons

EV71 can cause hand-foot-and-mouth disease (HFMD) with complications of acute pancreatitis, which should be noticed in differential diagnosis, especially in cases of idiopathic pancreatitis.

Peer-review

This interesting case is the first report of acute pancreatitis associated with EV71-related HFMD. The authors describe the clinical features, physical examination, laboratory findings, and MRCP and CT imaging in this case. Although the mechanism of pancreatitis associated with EV71 is unknown, suspected cases should be confirmed and treated as early as possible.

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Acquired double pylorus: Clinical and endoscopic characteristics and four-year follow-up observations

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Abstract

Double pylorus (DP), or duplication of the pylorus, is an uncommon condition that can be either congenital or acquired. Acquired DP (ADP) occurs when a peptic ulcer erodes and creates a fistula between the duodenal bulb and the distal stomach. The clinical features and endoscopic characteristics of four patients with ADP were reviewed and compared with previously reported cases. An accessory channel connects the lesser curvature of the prepyloric antrum with the duodenal bulb, and in all cases, a peptic ulcer was located in or immediately adjacent to the accessory channel. In one of the patients, the bridge between the double-channel pylorus disappeared, resulting in a single large opening and duodenal kissing ulcer after two years and three months. Finally, nonsteroidal anti-inflammatory drugs, *Helicobacter pylori* and other risk factors associated with ADP are assessed.

Key words: Acquired double pylorus; Peptic ulcer; Gastrointestinal hemorrhage; Nonsteroidal anti-inflammatory drugs; *Helicobacter pylori*

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Core tip: Double pylorus, which can be congenital or acquired, is a relatively rare condition consisting of two openings connecting the antrum to the duodenal bulb. This disease has a prevalence that ranges from 0.001%-0.4% of upper gastrointestinal endoscopies, and only a few reports have documented long-term endoscopic observations for this disease. In this report,

we present the clinical and endoscopic characteristics and four years of follow-up observations of four patients with acquired double pylorus complicated with gastric ulcer.

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INTRODUCTION

Double pylorus (DP), also called double-channel pylorus, is a rare condition involving a double communication between the gastric antrum and the duodenal bulb. DP is observed in 0.001% to 0.4% of upper gastrointestinal endoscopies^[1] and is twice as frequent in males than females^[2]. In most cases, DP is a complication of a penetrating ulcer, a condition that is called acquired double pylorus (ADP), pyloric duodenal fistula, antral duodenal fistula and peripyloric gastroduodenal fistula^[3]. DP occasionally occurs as a congenital abnormality, either isolated or in combination with other congenital abnormalities, such as heterotopic pancreatic tissue^[4], pancreatic divisum or gastric duplication^[5]. Herein, we describe four cases of gastric ulcer disease complicated by ADP and discuss risk factors for the occurrence of this disease.

CASE REPORT

Clinical features

The patients included three men and one woman ranging from 41 to 62 years of age. The disease durations ranged from 2 mo to 18 years. Two of the male patients presented to the emergency department with melena and/or coffee-ground vomitus suggestive of upper gastrointestinal hemorrhage; the other male patient was an outpatient who underwent an endoscopic examination due to epigastric pain for 2 mo. The only female patient was admitted to the hospital because of abdominal pain and dyspepsia with concomitant gouty arthritis. Physical examinations of the four patients indicated mild abdominal tenderness; the female patient exhibited gouty tophi in both hands and feet. For the two emergency patients, full blood counts revealed normocytic anemia, and blood biochemistry revealed enterogenous azotemia (*i.e.*, elevated blood urea nitrogen and normal creatinine). The other two (nonemergency) patients showed normal hemoglobin and urea nitrogen levels. All four patients exhibited normal platelet, prothrombin time, creatinine, liver enzyme, and calcium levels. *Helicobacter pylori* (*H. pylori*) urease breath test results were positive for all four patients. Three of the

patients abused nonsteroidal anti-inflammatory drugs (NSAIDs) for the treatment of concomitant diseases (Table 1).

Endoscopic characteristics

Esophagogastroduodenoscopies of all four patients revealed normal esophagi along the full length, without any pathological changes, and the bulbus and postbulbar duodenum were normal in appearance. However, each of the patients had DP and gastric ulcer disease, and the observed channel contractions suggested that their conditions might be related to the true pyloric rings. In all four patients, the accessory pylorus was located along the lesser curvature of the prepyloric antrum, connecting the lesser curvature with the duodenal bulb (Figure 1). A gastric ulcer was found in two of the patients: in one case on the lesser curve of the antrum and on the anterior wall adjacent to the accessory pylorus in the other case (Figure 1A and C). In contrast, the white bases of the ulcers were located in the accessory channel in the other two patients (Figure 1B and D). The duodenum could easily be entered *via* both of the pyloric channels. Additionally, the 58-year-old male patient exhibited mucosal erosion of the gastric fundus, active bleeding and irregular shallow ulcers (Figure 2) that were suggestive of acute erosive hemorrhagic gastropathy, which caused upper gastrointestinal bleeding in addition to the ADP and the accompanying gastric ulcer itself. This condition was the result of the patient's consumption of 8 bags of TouTongFen, which is also known as AkaFenSan (an over-the-counter analgesic consisting of acetaminophen, aspirin, and caffeine powder), to treat a long duration headache. No endoscopic interventions were performed in any of the patients. Real-time abdominal ultrasonography and computerized tomography findings were normal.

Treatment and follow-up

The two patients with gastrointestinal hemorrhages were immediately resuscitated with crystalloids and treated with intravenous pantoprazole (40 mg) twice daily until they left hospital. Bleeding did not recur, and epigastric pain was resolved. These patients were discharged on days 7 and 10. All patients were treated with *H. pylori* eradication therapy for 10 d. The 58-year-old male patient dropped out of the follow-up treatment regimen after discharge. The other three patients underwent urea breath tests after one month that indicated eradication of *H. pylori* in the two male patients. In addition, the 41-year-old male patient underwent an endoscopic examination at one month, which revealed that the gastric ulcer had healed, though the DP remained. The other two patients declined follow-up gastroscopies. The female patient's urea breath test remained positive, but she declined further therapy for the bacterial infection due to adverse reactions to the drugs. Both of these

Table 1 Clinical features of the four patients with acquired double pylorus¹

| Characteristic | Case number | | | |
|---------------------------------------|-----------------|-------------------------------------|------------------------|------------------------------|
| | 1 | 2 | 3 | 4 |
| Age (yr) | 41 | 61 | 58 | 62 |
| Sex | Male | Male | Male | Female |
| Disease duration | 2 mo | 1 yr | 8 yr | 2 mo |
| Symptoms at presentation | Epigastric pain | Melena | Coffee-ground vomitus | Abdominal pain |
| Previous history of gastric ulcer | - | - | + | - |
| Abdominal tenderness | + | + | + | + |
| NSAID use | - | Diclofenac | AKaFenSan ² | AKaFenSan ² |
| <i>Helicobacter pylori</i> infection | + | + | + | + |
| Hemoglobin (g/L) | - | 82 | 101 | 115 |
| Blood urea nitrogen (mmol/L) | - | 14.3 | 26.3 | 6.1 |
| Blood creatinine (μmol/L) | - | 80.46 | 98 | 75.11 |
| Blood uric acid (μmol/L) | - | 334.2 | 270.75 | 565.5 |
| Concomitant disease | - | Osteoarticular degenerative disease | Headache | Gout arthritis |
| Duration (yr) | - | 3 | 18 | 20 |
| Localization of the accessory pylorus | Lesser curve | Lesser curve | Lesser curve | Lesser curve |
| Localization of the gastric ulcer | Lesser curve | Within the accessory pylorus | Anterior wall | Within the accessory pylorus |

¹The plus signs denote the presence of a feature, and the minus signs denote its absence; ²AKaFenSan, also called TouTongFen, is an over-the-counter analgesic consisting of acetaminophen, aspirin, and caffeine powder. NSAID: Nonsteroidal anti-inflammatory drug.

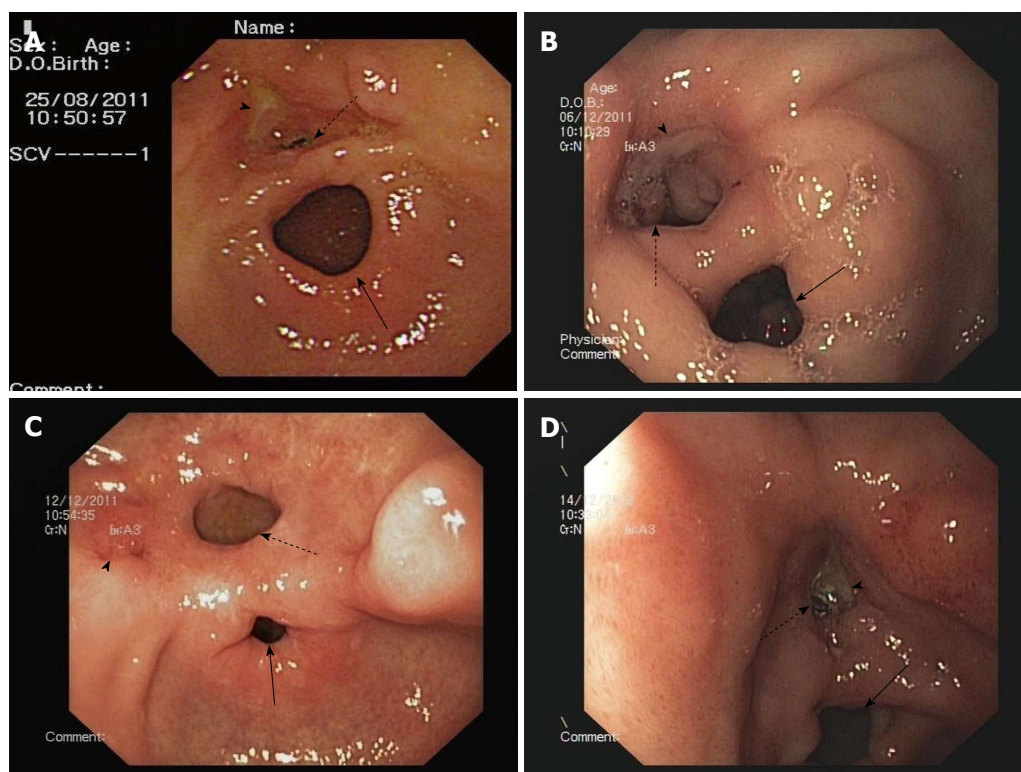


Figure 1 Double pylorus observed. A: A 41-year-old man undergoing endoscopy due to epigastric pain. A yellow-based irregular ulcer (arrowhead) is present in the antrum of the lesser curve over the accessory pylorus (dotted arrow). The solid arrow indicates the true pylorus; B: A 61-year-old man with osteoarticular degenerative disease who underwent endoscopy due to melena. A white-based ulcer (arrowhead) with edematous margins within the accessory pylorus (dotted arrow) on the lesser curve of the peri-pyloric region is present. The other opening is the normal pylorus (solid arrow); C: A 58-year-old man with headache who underwent endoscopy due to coffee-ground vomitus and melena. A white-based deep ulcer (arrowhead) is present in the anterior wall of the gastric antrum on the left side of the accessory pylorus (dotted arrow). The solid arrow indicates the true pylorus. D: A 62-year-old woman with gout who underwent endoscopy due to abdominal pain. A white-based ulcer (arrowhead) within the accessory pylorus (dotted arrow) is visible. The other opening is the true pylorus (solid arrow). Severe erythematous gastritis of the antrum is also present.



Figure 2 Gastric fundus mucosal congestion, erosion, active bleeding and an irregular hematin-based shallow ulcer within the thin white fur (arrow) in the 58-year-old man who underwent endoscopy due to coffee-ground vomitus and melena. Prior to the occurrence of the melena, the patient had ingested 8 bags of TouTongFen.

patients were asymptomatic and were instructed to avoid all NSAIDs and to use proton pump inhibitors (PPIs) if necessary to prevent ulcer recurrence and other complications. These two patients remained relatively healthy with the exception of occasional epigastralgia that was improved upon the use of over-the-counter drugs. Two years and three months after the initial endoscopy in March 2015, the female patient exhibited a recurrence of persistent epigastric pain. She consequently underwent a second endoscopic examination revealing that the bridge between the two channels had disappeared, resulting in a single large opening (Figure 3A) and a duodenal kissing ulcer (Figure 3B). A urea breath test indicated persistent *H. pylori* infection. This patient also admitted to the continued use of TouTongFen to treat gouty arthritis attacks. Because the ulcer remained, this patient was treated with triplex *H. pylori* eradication therapy and PPI maintenance therapy. The patient's abdominal pain rapidly improved, and she was healthy at the last follow-up.

DISCUSSION

DP can be congenital or acquired. The first case of congenital DP (CDP) was reported by Christien *et al.*^[6] in 1971, and only a few additional cases have been reported since that time. In CDP, a defect in canalization appears to occur during early embryonic development^[4]. Diagnosis is based on normal histology of both channels, the coexistence of another congenital abnormality, a bridge between the two channels with a normal muscle layer, a lack of a peptic ulcer disease history, and a lack of radiologic or endoscopic evidence of an ulcer^[7]. Conversely, ADP is a complication of a prepyloric or duodenal ulcer that perforates the gastric and duodenal walls to create a fistula. Although the first reported cases was in 1861, this condition was not considered a real entity until 1969^[8]. In our four patients, the presence of a peptic ulcer on endoscopic

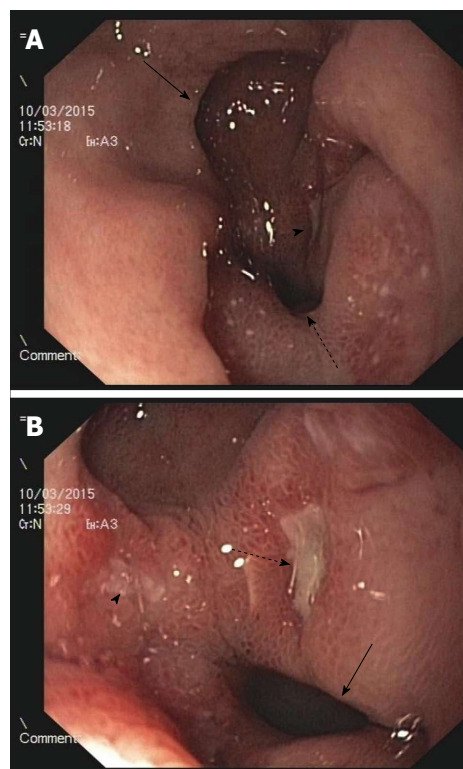


Figure 3 Two years and three months after the first endoscopic exam. A: The bridge between the two channels had disappeared, with a single large pylorus (solid arrow) observed in the woman with gouty arthritis. The arrowhead indicates the duodenal posterior wall ulcer; the dotted arrow indicates the descending duodenum; B: A clean-based duodenal kissing ulcer was observed in the woman with gouty arthritis. The arrowhead indicates the anterior wall ulcer. The dotted arrow indicates the posterior wall ulcer in the duodenum, and the solid arrow indicates the descending duodenum.

examination and/or a previous history of gastric ulcer indicated that the lesions were acquired.

ADP has no specific autonomous clinical manifestations and is not associated with upper abdominal pain or dyspepsia. ADP can present with chronic upper abdomen pain and/or discomfort, dyspepsia, vomiting and gastrointestinal bleeding^[9-11] due to an associated peptic ulcer or other diseases. For example, the 58-year-old male patient was admitted to the hospital due to upper gastrointestinal bleeding. Upon admission, acute gastroscopy confirmed that the cause of the bleeding was acute erosive hemorrhagic gastropathy (Figure 2) caused by the overuse of TouTongFen. In the 61-year-old man with osteoarticular degenerative disease, the cause of bleeding was a peptic ulcer within the accessory pylorus.

On endoscopy, the gastric antrum may appear normal^[1], inflamed, or ulcerated^[11] (as in the present four cases). The fistula may vary in size from a few millimeters to several centimeters, and in the majority of patients, these fistulae are located in the lesser curvature of the gastric antrum and the superior wall of the duodenal bulb^[2], as observed in all four patients described herein. However, the fistula can form an ulcer that penetrates from the posterior part of the

antrum to the third or fourth part of the duodenum^[12] or even the jejunum^[13]. Rarely, ADP can be found in patients with duodenal ulcers or gastric cancer^[10,14]. According to a follow-up study by Hu *et al*^[2], the accessory pylorus channel frequently persists for life (60%); nonetheless, in some patients, the accessory pylorus closes (25%) or connects with the true pylorus to form a single channel (5%)^[2,12,15]. The latter occurred in the female patient in this report. There are few reports of clinical improvement with fistula formation.

Several etiologies of ADP have been proposed. First, systemic diseases, such as diabetes, cirrhosis and chronic obstructive pulmonary disease, may be associated with ADP^[16-18]. We believe that damage to the gastric mucosal microcirculation can cause ADP^[16-18]. Second, long histories of treatment with drugs, including NSAIDs and corticosteroids, can affect peptic ulcer healing. Atiq *et al*^[19] reported a 54-year-old African woman with ADP and a clean-based ulcer in the accessory pylorus that resulted from the failure to follow medication instructions as well as the use of an over-the-counter analgesic consisting of acetaminophen, aspirin and caffeine. Peixoto *et al*^[9] described a 73-year-old man with multiple concomitant diseases who was admitted to their emergency department with first-episode melena. These authors believed that formation of the DP in these cases resulted from NSAID abuse. Moreover, *H. pylori* infection was absent in both of the above-mentioned cases^[9,19], suggesting that NSAIDs were responsible for the observed ADP. Yousuf *et al*^[20] reported a case of DP that was diagnosed endoscopically in a male patient with an adrenal adenoma; these authors believed that formation of the DP in this case resulted from a recurrent peptic ulcer that was likely induced by the hypersecretion of endogenous corticosteroids by the adrenal adenoma. Third, *H. pylori* plays a role in the pathogenesis of duodenal ulcer disease and the majority of gastric peptic diseases. Indeed, *H. pylori* is potentially responsible for refractory cases and the lack of healing. Akazawa^[15] reported a patient with none of the above-mentioned concomitant diseases or drug use, though this patient did have a continuous *H. pylori* infection for 14 years; recurrent gastric ulcers in this patient finally led to ADP. Fourth, poor compliance with medication regimens might be an important factor in ADP formation.

Among our four patients, all had *H. pylori* infections, and three abused NSAIDs. When the symptoms of the latter three patients were relieved, they continued using NSAIDs and refused regular recertification and follow-up gastroscopies. In addition, the female patient's NSAID abuse to treat her gouty arthritis and persistent *H. pylori* infection may have been related to formation of the fistula and subsequent duodenal kissing ulcer.

ADP is most frequently an incidental finding during investigations of other conditions. The diagnosis is

typically made based on endoscopic findings and is occasionally made based on radiologic findings. Endoscopy is generally the preferred method of visualization; however, ADP by endoscopy should be differentiated from gastric diverticulum, one of the rarest and controversial gastrointestinal pathologies. Very few cases of gastric diverticulum have been reported in the literature^[4,21]; such cases are typically asymptomatic, singular, saccular in shape, 1 to 4 cm in size and predominantly encountered in the 5th or 6th decade of life. Although DP has a characteristic appearance on upper gastrointestinal series, it may easily be misinterpreted as polyps, tumors, or large mucosal folds^[22,23].

Therapy should focus on the removal of the factors that impair mucosal healing. Ulcerogenic medications, such as NSAIDs and corticosteroids, should be avoided, and *H. pylori* infection should be eradicated. For patients who cannot stop using NSAIDs and those in whom *H. pylori* eradication fails, PPI maintenance therapy is necessary. ADP in the majority of patients responds well to medical treatments such as PPI, H₂-receptor antagonist and antacid therapies and gastric mucosal protective agents. The eradication of *H. pylori*, anti-acid treatments and the cessation of NSAID use are beneficial in terms of symptom relief, ulcer recurrence prevention, and fistula closure. For patients with symptoms of gastric outlet obstruction, endoscopic division of the tissue bridge with a sphincterotome should be considered first because the reestablishment of a normal pyloric aperture will alleviate symptoms^[24]. Indications for surgery include other complications, such as free perforations, obstructions that are refractory to endoscopic treatment, refractory bleeding, and failure to heal under maximum medical therapy with persistent symptoms not due to the fistula *per se*^[25].

COMMENTS

Case characteristics

Acquired double pylorus (ADP) can present with chronic upper abdomen pain and/or discomfort, dyspepsia, vomiting and gastrointestinal bleeding due to an associated peptic ulcer or other diseases.

Clinical diagnosis

The diagnosis of ADP is typically made based on endoscopic findings and is occasionally made based on radiologic findings. Endoscopy is generally the preferred method of visualization.

Differential diagnosis

ADP should be differentiated from gastric diverticulum on endoscopy; double pylorus (DP) has a characteristic appearance but may easily be misinterpreted as polyps, tumors, or large mucosal folds on upper gastrointestinal series.

Laboratory diagnosis

Full blood counts and blood biochemistry are often normal, but in patients with gastrointestinal bleeding, full blood counts may show anemia, and blood biochemistry may indicate enterogenous azotemia (*i.e.*, elevated blood urea nitrogen and normal creatinine). *Helicobacter pylori* (*H. pylori*) urease breath test results are often positive.

Imaging diagnosis

The real-time abdominal ultrasonography and computerized tomography findings were normal.

Pathological diagnosis

ADP arises from ulceration and fistulization between the gastric antrum and duodenal bulb; not all of the normal histological layers are present in this form.

Treatment

In the majority of patients, ADP responds well to medical treatments, such as proton pump inhibitor, H₂-receptor antagonist, and antacid therapies and gastric mucosal protective agents.

Related reports

DP, also called double-channel pylorus, is a rare condition involving a double communication between the gastric antrum and the duodenal bulb. DP is observed in 0.001% to 0.4% of upper gastrointestinal endoscopies and occurs at twice the frequency in males compared with females.

Term explanation

TouTongFen, which is also called AkaFenSan, is an over-the-counter analgesic consisting of acetaminophen, aspirin, and caffeine powder.

Experiences and lessons

PPI maintenance therapy is necessary in patients who cannot stop using NSAIDs and in those in whom *H. pylori* eradication fails.

Peer-review

This is a rare condition and an interesting report, and the manuscript is well written. It is a pity that follow-up endoscopy was performed on only one patient, and that the follow-up had to be based on clinical symptoms.

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