ISSN 1948-5182 (online)

World Journal of *Hepatology*

World J Hepatol 2015 October 18; 7(23): 2427-2496

Published by Baishideng Publishing Group Inc

World Journal of Hepatology

A peer-reviewed, online, open-access journal of hepatology

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2014-2017

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World Journal of Hepatology

Contents

Three issues per month Volume 7 Number 23 October 18, 2015

World Journal of Hepatology
Volume 7 Number 23 October 18, 2015

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NAME OF JOURNAL
World Journal of Hepatology

ISSN
ISSN 1948-5182 (online)

LAUNCH DATE
October 31, 2009

FREQUENCY
36 Issues/Year (8th, 18th, and 28th of each month)

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PUBLISHER
Baishideng Publishing Group Inc
8226 Regency Drive,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
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E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

PUBLICATION DATE
October 18, 2015

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DOI: 10.4254/wjh.v7.i23.2427

World J Hepatol 2015 October 18; 7(23): 2427-2431
ISSN 1948-5182 (online)

TOPIC HIGHLIGHT

2015 Advances in Hepatitis B virus

Antiviral therapy for chronic hepatitis B: Combination of nucleoside analogs and interferon

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Author contributions: Hagiwara S drafted the manuscript and wrote the final version; Nishida N revised the manuscript; Nishida N and Kudo M approved the final version of the manuscript.

Supported by Grant-in-Aid for Scientific Research (in part, KAKENHI: 24590997) from the Japanese Society for the Promotion of Science (to Nishida N); and a grant from the Smoking Research Foundation (to Nishida N).

Conflict-of-interest statement: There are no conflicts of interest to declare.

Data sharing statement: No additional data are available.

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Received: May 13, 2015
Peer-review started: May 15, 2015
First decision: June 2, 2015
Revised: June 18, 2015
Accepted: September 14, 2015
Article in press: September 21, 2015
Published online: October 18, 2015

Abstract

The ideal goal of chronic hepatitis B (CHB) treatment should be suppression of emergence of hepatocellular carcinoma through the disappearance of hepatitis B s antigen (HBsAg) rather than the control of serum hepatitis B virus-DNA level. For this purpose, various types of combination therapies using nucleoside analogs (NAs) and interferon (IFN) have been conducted. The therapeutic effects of combination of two different kinds of agents are better than those of the monotherapy using NAs or IFN alone, probably because different pharmaceutical properties might act in a coordinated manner. Recently, combination therapies with NAs and IFN and sequential therapies with NAs administration followed by IFN therapy have been routinely employed. We previously reported that combination therapy using entecavir (ETV) and pegylated (PEG)-IFN showed antiviral effects in 71% of CHB patients; the effect of this combination was better than that using lamivudine (LAM) and PEG-IFN. This is partially explained by the better antiviral effects of ETV than those of LAM. In our analysis, the cohort of CHB consisted of the patients who showed a flare-up of hepatitis before antiviral therapy, and their baseline HBsAg levels were relatively low. Therefore, in addition to the combination of the agents, the appropriate selection of patients is critical to achieve a good viral response.

Key words: Hepatitis B virus; Interferon; Sequential therapy; Combination therapy; Nucleoside analog

Core tip: For the suppression of emergence of hepatocellular carcinoma, disappearance of hepatitis B s antigen (HBsAg) is necessary, which is an important goal for the treatment of chronic hepatitis B. In order to achieve HBsAg clearance, combination therapies with nucleoside

analogs (NAs) and interferon (IFN) and sequential therapies with NAs administration followed by IFN therapy have been routinely employed. The combination of antiviral agents, and the appropriate selection of patients are critical to obtain a good response for HBsAg clearance.

Hagiwara S, Nishida N, Kudo M. Antiviral therapy for chronic hepatitis B: Combination of nucleoside analogs and interferon. *World J Hepatol* 2015; 7(23): 2427-2431 Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i23/2427.htm DOI: http://dx.doi.org/10.4254/wjh.v7.i23.2427

INTRODUCTION

Hepatitis B virus (HBV) is a DNA virus, and it is characterized by reverse transcription for replication in infected hepatocytes[1]. Various nucleoside analogs (NAs) have been employed as antiviral agents for chronic hepatitis B (CHB) patients. Lamivudine (LAM)[2,3], adefovir[4,5], and entecavir (ETV)[6,7] have been used to inhibit HBV replication by blocking DNA chain elongation. However, resistant viruses that appear after long-term administration of NAs[8-10] should be taken into consideration. HBV infects liver cells and forms stable circular double-stranded DNA [covalently closed circular DNA (cccDNA)] using DNA polymerase in the liver cell nuclei, providing a template for viral proliferation[11,12]. NAs do not directly act on cccDNA but inhibit HBV proliferation by blocking reverse transcription. Thus, discontinuing the administration may well cause viral rebound that lead to the reactivation of hepatitis. On the other hand, interferon (IFN) induces natural killer (NK) cell and reduces cccDNA through the elimination of infected liver cells. However, IFN is rarely indicated for the cirrhotic patients with deteriorated liver functions. In addition, it actually exerts a weak inhibitory effect on virus proliferation compared to the NAs[13]. In general, unlike NAs, IFN shows different therapeutic effects depending on the HBV genotypes; patients with the genotypes C and D are more resistant to IFN than those with the genotypes A and B. Instead of IFN, pegylated-IFN, whose metabolism and elimination are suppressed by linear polyethylene glycol (PEG) modification, was developed, achieving stronger antiviral effects[14].

In order to utilize the synergic effects of these agents, NAs have been combined with IFN. Combination therapies with IFN and NAs and so-called "sequential therapies", which are characterized by the NAs administration followed by the IFN therapy, have been routinely employed. In this article, we focus on the recent advancement of antiviral therapy for CHB in the context of combination therapy using NAs and IFNs.

CONCURRENT ADMINISTRATION OF IFN AND NAS AND THEIR COMBINATION

Several randomized large-scale trials have been reported on PEG-IFNα-2a and LAM combination therapy. In hepatitis B e antigen (HBeAg)-negative cases, 48-wk PEG-IFNα-2a monotherapy was compared with 48-wk PEG-IFNα-2a and LAM combination therapy, demonstrating that the combination therapy showed relatively stronger antiviral effects, although no difference was noted at 24 wk after the therapy in terms of viral response (VR rates: 43% *vs* 44%, respectively)[15]. In HBeAg-positive patients, 48-wk PEG-IFNα-2b monotherapy was also compared with 48-wk PEG-IFNα-2b and LAM combination therapy, again demonstrating no difference in the therapeutic effects (VR rates: 32% *vs* 27%, respectively)[16]. Therefore, PEG-IFN monotherapy is recommended as the firs-line treatment because there was no advantage of 48-wk PEG-IFNα-2a and LAM combination in therapeutic effects of CHB[17].

We have conducted another clinical trial wherein we combined ETV that exerted a stronger antiviral effect than LAM, with PEG-IFN for 48 wk[18]. Seventeen CHB patients with genotype C received ETV and PEG-IFNα-2b combination for 48 wk, and were observed for additional 24 wk to know the virological and biochemical response. Our results showed that serum HBV-DNA levels continued to reduce after the 48-wk administration. At 24 wk after the administration, low viral loads were sustained at < 4 log copies/mL in 12 cases (71%). Of 11 HBeAg-positive cases, four (36%) and eight (73%) showed HBeAg seroconversion at the end of the treatment and at 24 wk after the administration, respectively. Hepatitis B s antigen (HBsAg) disappeared in one case. Since only a small number of patients were analyzed in this study, the good antiviral effect of this combination should be confirmed with a larger sample size. However, the virological and biochemical effects observed in this study were superior to those reported in the previous study of HBeAg-positive CHB patients using PEG-IFNα-2a monotherapy or combination therapy with LAM[18]. This is partially explained by the use of ETV that has more potent antiviral effects than LAM. On the other hand, our cohort of CHB consisted of the patients who developed flare-up hepatitis before the treatment with low baseline HBsAg levels. Therefore background condition of hepatitis with active immune response should be an important factor to achieve a good antiviral effect.

A recent report showed randomized control trial of monotherapy *vs* combination therapy: Comparison of the antiviral response between HBeAg-positive patients who received ETV alone for 24 wk *vs* patients treated with PEG-IFN for 24 wk after 24-wk ETV administration[19]. HBeAg loss with an HBV DNA < 200 IU/mL (18% *vs* 32%, respectively, $P = 0.032$) rates were significantly higher in the 24-wk PEG-IFN combination group, and the relapse rate after ETV discontinuation was also lower in the 24-wk PEG-IFN combination group than ETV-monotherapy group. Thus, ETV and PEG-IFN combination may provide favorable outcomes. It is also known that tenofovir (TDF) also exerts a good antiviral effect to HBV. Therefore, TDF and PEG-IFN combination should be conducted in a large-scale study.

SEQUENTIAL THERAPY

Administration of NAs followed by IFN therapy is known as sequential therapy. The results of the treatment with the 20-wk LMV administration, followed by the 4-wk combination of LMV with IFN and subsequent 24-wk IFN monotherapy were reported[20]. Fourteen CHB patients received the therapy, resulting in HBeAg seroconversion in 45% and negative for HBV-DNA in 57%. Thus, this sequential method could be a promising antiviral therapy. However, the majority of the patients examined in this trial had a HBV-genotype A. Therefore, antiviral effect for other genotypes is still unknown.

Other trials were performed using different protocols at many facilities. Sequential therapy was conducted in 36 HBeAg-negative patients using 6-mo LMV monotherapy, followed by 6-mo combination of LAM with IFN, and additional 12-mo IFN monotherapy[21]. At 12 mo after the therapy, biological effects and HBV-DNA-negative rates were not significantly different from those of the age- and sex-matched IFN-alone control group. The antiviral response reported in this study were markedly different from those reported in the previous report[20]. Sequential therapy was also conducted in 24 HBeAg-positive patients using 16 to 32-wk LMV therapy, followed by 4-wk combination with IFN-β and additional 20-wk IFN monotherapy[22]. Virological effects were noted in 29% of the patients, which is also much lower than those reported by Serfaty *et al*[20]. In this trial, the majority of the CHB patients carried genotype C virus that was known as a resistant genotype to IFN therapy. Therefore, these controversial results among the studies may be explained by the difference of the HBV genotype in addition to the differences of sex, and ethnic groups analyzed. In this report, background factor that associated with therapeutic effects were also analyzed: IFN therapy is markedly effective in young patients with low HBV-DNA levels before the therapy[23]. Sequential therapy with ETV and IFNα was also reported, demonstrating no additional therapeutic advantage as compared to those with LMV[24]. However, this therapy is more likely to be effective in patients who achieved clearance of HBeAg during ETV administration. Therefore, the effects of sequential therapy should be enhanced through the appropriate patient selection.

IMMUNE RESPONSE AND THERAPEUTIC EFFECTS

In the early stage of HBV infection, viruses are controlled by natural immunity, mainly consisting of NKT cells, and activated NKT cells activate NK, T, and B cells to ameliorate the HBV infection and eliminate infected hepatocytes[25,26]. Thus, activated NKT cells are essential for viral clearance in acute hepatitis B. On the other hand, attempts have been made to treat chronic hepatitis B by activating NKT cells. In a clinical trial on chronic hepatitis B treatment with α-GalCer, a ligand for type 1 NKT cells, no marked antiviral effects were noted[27]. This should be further investigated in the future.

The expression levels of activation markers in NK cells from the peripheral blood and liver were higher in chronic hepatitis B patients with high alanine transaminase (ALT) levels than in those with low ALT levels, with the degree of activation being correlated with the severity of hepatocyte damage[28]. As described above, IFN exerts antiviral effects through the activation of NK cells[25,26]. Hence, in patients with high ALT levels, antiviral effects may be increased by IFN intervention through the increased activation of NK cells. The marked therapeutic effects of the combination therapy with PEG-IFN and ETV can be explained by the selection of patients with high ALT levels (157 ± 143 IU/L) before the intervention[18]. A high viral load has been reported as an IFN-resistance factor. The combination with ETV may have increased the IFN effects by reducing viruses early. The activities of interleukin 15 and 6 and CD8 were increased by the combination with tenofovir and PEG-IFN as compared to PEG-IFN monotherapy, suggesting that tenofovir improves the immune response to IFN[29]. This should be further examined in the future.

The numbers of NKT cells in chronic hepatitis B patients were lower than those in healthy subjects. However, the numbers of NKT cells were restored in patients successfully treated with telbivudine[30]. In addition, the IFN-γ production capacity of NK cells was improved in patients successfully treated with ETV[31]. Before analog treatment, sequential therapy is conducted to reduce viruses, followed by IFN treatment. IFN intervention may exert effects in patients with the numbers and functions of NKT cells restored by analog treatment.

In both combination therapy with PEG-IFN and analog and sequential therapy, immunocompetent cells, mainly NKT cells, may be associated with the therapeutic effects. From this viewpoint, therapeutic indications and effectiveness should be examined.

CONCLUSION

The concurrent administration of IFN and NAs is intended to enhance the effect of IFN. Baseline viral loads are considered to be associated with IFN resistance, while flare up of hepatitis lead to susceptibility to IFN treatment because active immune response should accelerate the antiviral action of IFN. Thus, IFN exerts maximum effects in a conflicting situation with low HBV-DNA level accompanied by active hepatitis. From this point of view, IFN should be started during an initial decreasing phase of HBV-DNA under the administration of NAs in patients with active hepatitis. To achieve this condition, simultaneous initiation of NAs and IFN should be ideal. Indeed, in addition to the types of combinations of NAs and IFN, various factors, such as ALT, HBV-DNA, and HBsAg levels before the treatment may affect the therapeutic effects.

Sequential therapy is aimed at enhancing thera-

peutic effects and safety discontinuing of the NAs administration. However, it should also induce inactive hepatitis after a long-term administration of NAs, which could affect the effect of IFN. On the other hand, many studies reported the effectiveness of sequential therapy specifically in patients with low serum HBV-DNA levels and negative HBeAg after administration of NA. The factors that predict the effectiveness of sequential therapy should be investigated.

Combination therapy and sequential therapy are based on the different treatment concepts. However, both are basically aimed at drug-free treatment. We should take the advantages as well as the risk of treatment failure into reconsideration for the treatment of CHB.

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P- Reviewer: Gehring AJ, Ohkoshi S, Parvez MK, Pandey VN
S- Editor: Yu J **L- Editor**: A **E- Editor**: Liu SQ

World Journal of Hepatology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4254/wjh.v7.i23.2432

World J Hepatol 2015 October 18; 7(23): 2432-2448
ISSN 1948-5182 (online)

TOPIC HIGHLIGHT

2015 Advances in Liver Transplantation

Transoesophageal echocardiography during liver transplantation

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Author contributions: De Pietri L conceived, designed and performed the review; Mocchegiani F, Montalti R, Vivarelli M and Agnoletti V revised the manuscript; Leuzzi C performed the research.

Conflict-of-interest statement: All authors of the work submitted for consideration of publication have no conflicting interests (including but not limited to commercial, personal, political, intellectual, or religious interests) to declare.

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Received: April 26, 2015
Peer-review started: April 28, 2015
First decision: June 2, 2015
Revised: June 22, 2015
Accepted: September 2, 2015
Article in press: September 9, 2015
Published online: October 18, 2015

Abstract

Liver transplantation (LT) has become the standard of care for patients with end stage liver disease. The allocation of organs, which prioritizes the sickest patients, has made the management of liver transplant candidates more complex both as regards their comorbidities and their higher risk of perioperative complications. Patients undergoing LT frequently display considerable physiological changes during the procedures as a result of both the disease process and the surgery. Transoesophageal echocardiography (TEE), which visualizes dynamic cardiac function and overall contractility, has become essential for perioperative LT management and can optimize the anaesthetic management of these highly complex patients. Moreover, TEE can provide useful information on volume status and the adequacy of therapeutic interventions and can diagnose early intraoperative complications, such as the embolization of large vessels or development of pulmonary hypertension. In this review, directed at clinicians who manage TEE during LT, we show why the procedure merits a place in challenging anaesthetic environment and how it can provide essential information in the perioperative management of compromised patients undergoing this very complex surgical procedure.

Key words: Liver transplantation; Transoesophageal echocardiography; Cirrhotic cardiomyopathy; Liver cirrhosis; Perioperative anaesthesia management

Core tip: The allocation of organs to the sickest liver transplant candidates has made their management more complex and anaesthesia for perioperative liver transplantation (LT) more challenging. Transoesophageal echocardiography, which can visualize dynamic cardiac function and overall contractility and provide real-time feedback on the adequacy of therapeutic interventions, has gained an irreplaceable role in the perioperative management of LT. We believe that echocardiography can play a key role in the care of and decision making for compromised liver transplant candidates undergoing this complex surgical procedure.

De Pietri L, Mocchegiani F, Leuzzi C, Montalti R, Vivarelli M, Agnoletti V. Transoesophageal echocardiography during liver transplantation. *World J Hepatol* 2015; 7(23): 2432-2448 Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i23/2432.htm DOI: http://dx.doi.org/10.4254/wjh.v7.i23.2432

INTRODUCTION

Liver transplantation (LT) is a life-saving procedure for patients with end-stage liver disease not responsive to other medical treatment. Unfortunately, many transplant candidates will die on the waiting list because of the marked shortage of donor organs[1]. In an effort to reduce waiting list mortality, organ allocation is based on the Model of End-Stage Liver Disease (MELD). This score prioritizes allocation to the sickest patients, and for this reason, patients undergoing LT today have more severe end stage liver disease (ESLD), are older (> 60 years), and are more complex regard both their comorbidity burden and their risk for perioperative complications[2]. The patient's first examination will confirm his status as a LT candidate, and intraoperative transoesophageal echocardiography (TEE) will help optimize the anaesthetic management of these highly complex patients.

Patients with cirrhosis requiring LT have an increased cardiac output and a decreased peripheral vascular resistance and arterial pressure but a compromised ventricular response to stressors, such as haemorrhage, vasoactive drugs, vascular clamping, volume overload, and reperfusion. This condition is defined as cirrhotic cardiomyopathy[3,4] and is associated with increased left ventricular wall thickness and cardiac chamber enlargement. Assessing the optimal volaemia and excluding left or right ventricular dysfunction in the perioperative course of LT is a challenge for the anaesthesiologist. TEE, by providing a rapid visualization of dynamic cardiac function, volume status, overall contractility, regional wall motion, embolization of large vessels, and pericardial effusion, can provide irreplaceable help[5].

PREOPERATIVE TEE ASSESSMENT IN LIVER TRANSPLANT CANDIDATES

Liver transplant candidates usually have cardiac changes associated with their advanced age and the presence of several comorbidities that increase the potential for cardiovascular complications, particularly during the haemodynamic stresses that characterize the perioperative period. The preoperative cardiac evaluation usually includes a trans thoracic echocardiography which is essential to assess cardiac function and to look for the effects on the heart of the two main pulmonary syndromes caused by the end-stage liver disease: Hepatopulmonary syndrome and portopulmonary hypertension (POPH).

Echocardiography with agitated saline contrast is commonly used to detect intrapulmonary arteriovenous shunts, which are common in patients with ESLD. Microbubbles that appear late (after a time delay of 4 to 8 cardiac cycles) in the left side of the heart after agitated saline injection into the venous system are consistent with the diagnosis of hepatopulmonary syndrome. Immediate or early shunting (1-2 cardiac cycles) is more consistent with an atrial septal defect or patent foramen ovale[6].

If diagnostic questions remain after a transthoracic study, a TEE can provide increased sensitivity and can directly visualize bubbles entering the left atrium from the pulmonary veins rather than crossing the interatrial septum[7]. TEE is believed to be the test of choice for the diagnosis of patent foramen ovale or interatrial shunts[8].

For a preoperative patent foramen ovale (PFO) diagnosis, it is important to assess the severity of the problem and decide whether preoperative correction is needed to avoid significant amounts of venous air entering the systemic circulation at the time of reperfusion.

PFO

PFO, usually a benign and silent lesion (present in approximately 25% of adults in the general population), can cause hypoxemia and paradoxical embolic phenomena under circumstances when right atrial pressure exceeds left atrial pressure[9]. These circumstances may occur perioperatively as a result of mechanical ventilation, changes in intra-abdominal pressure, hypotension, and/or severe reperfusion syndromes. Numerous case reports implicate PFO as a cause of perioperative hypoxemia and systemic thromboembolism in LT[10]. Air from the right atrium can embolize the coronary arteries, particularly the right coronary artery, resulting in acute myocardial ischaemia, ventricular fibrillation, and severe right ventricular hypokinesis. Even in the absence of systematic study, screening for PFO before surgery has been suggested for high-risk patients to reduce the risk of paradoxical embolization[10,11].

Other authors, in contrast, state that LT can be performed safely in patients with a PFO and other types of intra-cardiac shunts because the overall incidence of this complication appears to be quite low (isolated case reports)[12]. They argue that although TEE offers the best sensitivity and specificity for PFO diagnosis, it is semi-invasive and expensive and that most patients

with portal hypertension and oesophageal varices are at higher risk of oesophageal bleeding provoked by the TEE probe. Therefore, these authors argue that a TEE should be reserved for LT patients with specific indications[13]. Although a PFO is not a contraindication to LT, extra care should be taken to prevent thrombus formation and air entry into the venous system during surgery. Further studies are needed to determine impact of a PFO on LT morbidity and the potential role, if any, for percutaneous PFO closure in liver transplant candidates.

Contraindication to TEE

The value of a TEE must of course be balanced against the risk of performing the procedure. The insertion and manipulation of a transoesophageal echocardiographic probe may, even if infrequently, cause arrhythmias, respiratory distress, hemodynamic effects, provoke dental injuries, pharyngeal and/or laryngeal, esophageal and/or gastric trauma, and of course bleeding, that can be more severe and dangerous in cirrhotic patients with gastric varices or coagulopathy. Gastro-oesophageal varices are very common in patients listed for LT, and their presence is indicated by worse laboratory parameters and MELD scores. Varices are present in 73% of patients with ESLD awaiting LT[14,15], and 5% will develop new varices; moreover, up to 28% of patients will have oesophageal varices in the three years following a diagnosis of cirrhosis[16]. Preoperative endoscopy to evaluate the grade of the varices, oropharyngeal examination, limited probe manipulation and exam performance by an experienced operator is, recommended for patients with ESLD prior to TEE[17,18].

Patients with oesophageal stricture, cancer, diverticulum, and recent oesophageal surgery are generally considered to have near absolute contraindications for TEE.

Gastro-oesophageal varices are considered a relative contraindication to TEE, and the transgastric view is sometimes avoided to prevent damaging gastro-oesophageal varices in the distal oesophagus[19]; however, concerns over damage to these varices have been shown to be largely unfounded[20]. On the other hand, the right message should be that TEE is not completely safe in patients with oesophageal varices because their presence still remains a relative contraindication for TEE.

There is a paucity of data related to the manipulation of the TEE probe in patients with gastro-oesophageal varices, but a recent retrospective analysis by Spier *et al*[17] specifically analysed this cohort and found no major bleeding complications, even in higher-risk patients. No bleeding episodes were reported in a small study of 23 patients with oesophageal varices undergoing intraoperative TEE during LT[21].

An editorial by Spencer[22] accompanying Spier's study provides some recommendations to aid decision-making regarding the use of TEE in patients with gastro-oesophageal varices and concludes by saying that "if a patient has an important indication for TEE, which cannot be answered first with TTE or any other noninvasive technique, then TEE should not be contraindicated by the presence of oesophageal varices".

Physicians who perform TEE need to make individual decisions on the risk/benefit ratio of performing TEE in patients with known or suspected varices. Burger-Klepp *et al*[14] agreed, suggesting that TEE is a relatively safe method for monitoring cardiac performance of LT patients with a moderate MELD score and documented gastro-oesophageal varices but a low risk of major haemorrhagic complications. Markin *et al*[23], based on their retrospective study, state that intraoperative TEE is a relatively safe method for monitoring cardiac performance in liver transplant patients, with a major complication rate of 0.86%. Although not without risk, TEE provided valuable information that would not otherwise be detected, such as pulmonary thromboembolism occurring at the time of graft reperfusion, unidentified PFO and the presence of ventricular dysfunction after graft reperfusion[23].

INTRAOPERATIVE TEE ASSESSMENT IN LIVER TRANSPLANT PATIENTS

Intraoperative TEE use during LT has been increasing because of its unique ability to rapidly visualize the dimensions and function of the heart chambers; to detect intra-cardiac air or thrombus, myocardial ischaemia, or pulmonary thromboembolism[24]; and for its invaluable role in intraoperative haemodynamic management[25]. It is an invasive medical procedure that is focused on intraoperative monitoring rather than specific diagnosis. It carries rare but potentially life threatening complications and therefore must be performed by only qualified physicians.

Training and certification

Although TEE is reported to be routine in 40%-72% of high-volume liver transplant centres[13,26], formal TEE certification is the exception. A thorough understanding of anatomy, physiology, and the surgical procedure is critical to its proper use. The intraoperative use of TEE is limited by the need for advanced training and the lack of credentialed anaesthesiologists. Most anaesthesiologists learn to use TEE in LT in an informal manner after completing training[26]. A significantly smaller proportion of anaesthesiologists who work in low volume liver transplant centres do not use perioperative TEE because they are unfamiliar with the procedure and the data it provides[27]. The American Society of Anesthesiology, in cooperation with the National Board of Echocardiography (NBE), defined the components of basic perioperative TEE training as including independent clinical experience, supervision, and continuing education requirements. The NBE's Basic perioperative TEE training pathways require an extensive training, at least 150 basic intraoperative procedures, and a written exam to obtain certification[28,29].

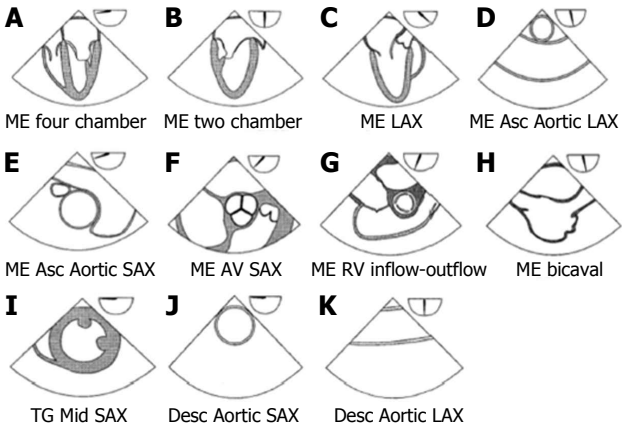

Figure 1 List of the 11 views suggested by the American Society of Echocardiography and Society of Cardiovascular Anesthesiologists guidelines on basic perioperative transoesophageal echocardiography. Modified from Reeves *et al*[28]. ME: Mid esophageal; LAX: Long axis view.

The American Society of Echocardiography (ASE) and the Society of Cardiovascular Anesthesiologists (SCA) state that because of the risks, technical complexity, and potential impact of TEE on perioperative management, the basic TEE echocardiographer must be a licensed physician[28].

Although a basic perioperative TEE echocardiographer should be familiar with the 20 classical views necessary to obtain a comprehensive intraoperative transoesophageal echocardiogram[30], recent ASE and SCA guidelines state that it is more realistic to expect an anaesthesiologist to be familiar with the 11 most relevant TEE views (Figure 1), which can provide him with the necessary information for an aetiologic diagnosis of haemodynamic instability during surgery[28].

The basic perioperative TEE examination should be performed using three primary positions of the probe within the gastrointestinal tract: The mid-oesophageal level, the transgastric level, and the upper oesophageal level.

TEE assessment of haemodynamic alterations during LT

The haemodynamic instability typical of LT can result from heart failure, real hypovolaemia, or reduced peripheral vascular resistance[31].

Hemodynamic instability and rapid changes in volume status, mainly due to acute blood loss or vascular clamping, are the most serious complications and challenges that the anaesthetist has to manage during LT.

TEE allows the intraoperative causes of hypotension to be identified and can help optimize volaemia and avoid impaired organ perfusion and ischaemia. Identifying the cause of hypotension is the key to treatment. It is the inability of the cirrhotic patient to respond to cardiac stress together with their hypovolaemia and the massive fluid shifts that accompany clamping of the inferior vena cava and subsequent reperfusion of the liver graft that make intraoperative fluid management so difficult[32,33]. Unique haemodynamic changes occur in the transition from the anhepatic to the reperfusion phases of LT, and rapid fluid assessments and adjustments are needed to optimize the outcome. TEE analysis based on the close approximation of the papillary muscles in the transgastric mid papillary view (TG Mid SAX) allows for a rapid qualitative assessment of ventricular filling so that fluids can be adjusted for the desired preload.

Cirrhotic patients have an increased risk for right ventricular failure, so aggressive fluid repletion or blood transfusion or the increased blood flow to the right heart at reperfusion can cause volume overload and precipitate pulmonary oedema due to occult cardiac disease[3]. In these circumstances, TEE allows the right heart chambers to be visualized, allowing for a real-time diagnosis of right ventricle failure (due to pulmonary embolism or overload), pulmonary hypertension, or even a reduced preload. Right and left ventricular dysfunction can be exacerbated during the transplant by the clamping and unclamping of major vessels, such as the inferior vena cava or portal vein, and the sudden fluid shifts associated with such manoeuvres can be assessed during surgery by TEE.

TEE can be useful not only in assessing the patient's volume status and intraoperative fluid management but also when evaluating any left ventricular hypertrophy or hyperdynamic systolic function typical of ESLD, which may result in haemodynamically significant left ventricular outflow tract obstruction (LVOTO) during LT. TEE makes the diagnosis of an haemodynamically significant LVOTO possible intraoperatively together with the recognition and management of refractory hypotension through inotropic agents and careful volume administration[34].

Monitoring cardiac output (CO) during LT is particularly important because it is considered, despite its limitations, one of the main determinants of oxygen transport and wrongly considered to be a surrogate for left ventricular function. TEE seems to be a valid alternative to standard methods for measuring CO, providing both a numerical value for CO and separate qualitative determinations of right and left ventricular function and ejection fraction.

Because a normal CO value does not always imply an adequate peripheral perfusion, it may be more useful to monitor CO variations over time, especially under conditions of haemodynamic instability or after therapeutic interventions rather than considering a single numerical value.

Comparison of cardiac output measurements by TEE or pulmonary artery catheter

The thermodilution method is the most common technique used at the bedside to monitor CO during LT; this method uses a pulmonary artery catheter (PAC), which is considered a gold standard due to its extensive past use[35]. PAC measurements are based on changes in the temperature of the blood surrounding the catheter, but the large core body temperature shifts that are frequently witnessed before and after revascularization

of the new graft can affect the reliability of both continuous and bolus determination of CO with PAC[36,37]. Massive peripheral or central venous infusion of fluids or unheated blood from a veno-venous by-pass (VVB) may affect the accuracy of thermodilution by increasing thermal noise and lead to erroneous measures of CO as well[38]. Using right-heart catheterization for volumetric left ventricular preload assessment highlights some limitations. Right ventricular function differs considerably from left ventricular one. The major determinant of left ventricular function is myocardial wall tension, whereas for the right it is ventricular afterload. Therefore, the relationship between right ventricular preload assessment and cardiac output readings may be weak[39]. Another underlined PAC limitation comes from a possible delayed reactivity to rapid changes in cardiac output and intravascular volume detection. CO monitored continuously by the thermodilution technique yields values averaged over a period of time (3-6 min or longer)[36], so changes in the left ventricular stroke volume (SV) or CO cannot be assessed with a high time resolution[36]. TEE cardiac output monitoring instead seems to detect changes in output during LT, as can occur during acute haemodynamic changes, more rapidly than thermodilution[40]. Other authors argue instead that a sudden change in filling pressures or SvO2, as indirect indicator of cardiac output, is an extremely valuable information provided by PAC, that allows the proper detection and identification of certain intraoperative events that TEE cannot detect[41]. TEE application does not guarantee a continuous monitoring and it's not good at trending information (especially preload): No quantitative online evaluation of right ventricular function is available, and only sporadic right ventricular ejection fraction values can be obtained[42]. On the other hand TEE, unlike PAC, is not affected by blood temperature changes and provides a calculated numerical value for left ventricular volume and cardiac output (*e.g.*, by Simpson's rule) as well as a qualitative determination of right and left ventricular filling and ejection fraction.

In experienced hands, the correlation between echocardiographic and thermodilution measurements of cardiac output is generally acceptable[43,44] even if there is no general agreement on this subject. Other authors have declared that CO measurements by TEE are not interchangeable with PAC thermodilution because of limited agreement and a large percentage of errors[45]. Unlike PAC, TEE provides volumetric rather than pressure data, which can be misleading in the setting of pulmonary hypertension, valvular dysfunction or ventricular failure. The unique shape and function of the right ventricle may delay changes in PAC readings until the right ventricle is significantly dilated as central venous pressure readings do not necessarily correlate with right ventricular preload or ejection[21].

Despite PAC limits, transoesophageal echocardiography for monitoring left ventricular preload has some limitations which should be emphasized as well.

Determination of the left ventricular end diastolic area (LVEDA) index provides a measure of left ventricular filling that has been shown to correlate with changes in SV during volume therapy[46] only if, the compliance and contractility of the left ventricle remain unchanged[47]. Quantitative assessment of LVEDA may be altered by dislocation of the probe from the midpapillary level as well[47]. The technical complexity of TEE performance can be increased by the difficulty in obtaining short-axis visualization of the left ventricle which is limited due to the common posterior retraction of the stomach during LT[48]. The apparent reduced invasiveness of TEE compared to PAC, is however offset by a greater complexity of the technique. TEE users should be qualified in displaying standardized cross-sections, and skilled to interpret findings in order to avoid potential serious misinterpretation of the images[49]. Beside this technical difficulty this method is either not practicable in a perioperative setting or cannot be routinely performed for logistic and economic reasons.

In summary PAC allows for the nearly continuous measurement of CO, right ventricle ejection fraction, and right ventricle end diastolic volume showing some great advantages like its continuous nature and the relative lack of user input. TEE, besides aiding in the estimation of preload, is very valuable in the overall assessment of cardiac function, detection of air embolism or intracardiac clot formation, diagnosis of hepatopulmonary syndrome, and management of pulmonary hypertension[50]. Both methods have several limitations which make the actual available literature inconclusive about how to best monitor the hemodynamics during LT and whether a single monitoring device is absolutely superior to the others in terms of accuracy, validity and reproducibility of data. Nowadays a complete, accurate, non-invasive device, suitable for instantaneously detecting hemodynamic alterations typical of LT is still unavailable, and the integration of various data from different monitoring systems probably remains the only way to properly manage haemodynamic instability.

How to measure CO with TEE

In the clinical setting, SV is an important parameter of cardiac performance. Assessment of CO is an important measure of responses to medical and surgical therapies, such as administration of inotropic agents to treat right and left heart failure[51]. SV and CO are most reliably and easily measured at the left ventricular outflow tract (LVOT) or at the level of the aortic valve. SV and CO can also be measured at the level of the mitral valve or the pulmonary artery, but this is less commonly done because, unlike the mitral or pulmonary valves, the cross sectional area of the LVOT and ascending aorta (because they are circular structures) change very little throughout the cardiac cycle.

SV and CO measurement at LVOT: The TEE-derived CO can be calculated as the product of SV and heart rate, where left ventricular SV is calculated by multiplying the time-velocity integral at the left ventricular outflow

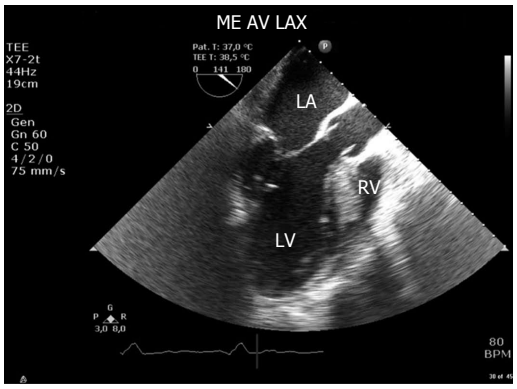

Figure 2 Transoesophageal echocardiography view (mid esophageal long axis view) used to measure left ventricular outflow tract diameter usually best imaged at a multiplane angle of 110°-140°. AV: Aortic valve; LA: Left atrium; LV: Left ventricle; RV: Right ventricle; ME AV LAX: Mid esophageal aortic valve long axis view. Modified by Møller-Sørensen *et al*[45].

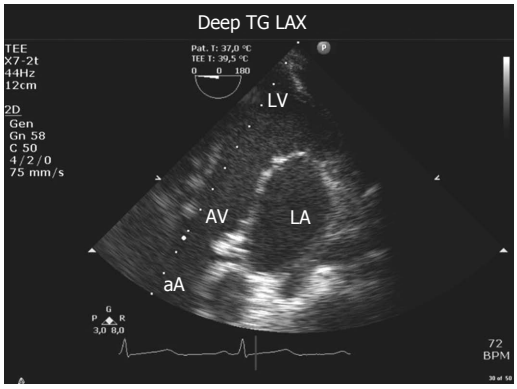

Figure 3 Transoesophageal echocardiography view (deep transgastric long axis view), used to measure velocity time integral. AV: Aortic valve; LA: Left atrium; LV: Left ventricle; aA: Ascending aorta; TG LAX: Transgastric long axis view. Modified by Møller-Sørensen *et al*[45].

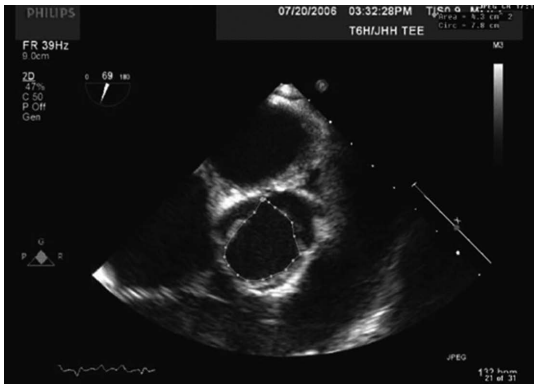

Figure 4 Transesophageal measurement of aortic valve planimetry from mid esophageal aortic valve SAX, usually best imaged at a multiplane angle of 40°-60°.

tract by the LVOT area. It is important to remember that area and flow measurements must be made at the same anatomical site. This calculation assumes that flow is laminar (*i.e.*, not turbulent) and that the conduit being measured is an unchanging circular orifice such that it has the area of πr^2.

Stroke volume measured at LVOT level is simplified by the following equations:

$$SV = VTI \times CSA_{LVOT}$$

CSA: Cross-sectional area; VTI: Velocity-time integral.

The CSA (LVOT) is calculated from the LVOT diameter as follows:

$$CSA_{LVOT} = 0.785 \times diameter^2$$

The CSA of the LVOT is usually obtained from the mid-oesophageal long axis (ME LAX) view at 110°-140° (Figure 2). Errors in diameter measurements are quadrupled because the formula requires squaring the diameter. Therefore, very small errors in measurement make a dramatic difference to the calculation.

The diameter should be measured multiple (usually three) times at mid-systole in the mid oesophageal aortic valve long axis (ME AV LAX) view, using the inner edge to inner edge technique, and then averaged. This measurement assumes that the annular size does not vary much throughout the cardiac cycle, so the timing of this measurement is not crucial.

VTI measured at the level of the LVOT using pulse wave Doppler requires the sample volume to be positioned in the LVOT just proximal to the aortic valve. Because the blood flow is nearly parallel to the ultrasound beam, the best transoesophageal views for this measurement are the transgastric long axis (TG LAX) and the deep transgastric long axis (deep TG LAX) views with PW Doppler sample volume placed in the LVOT (Figure 3)[45,52].

When aortic stenosis is present, the CW Doppler signal shows a characteristic flow image with two densities[53]. The most intense part of the time velocity integral is the SV, whereas the outer contour shows the speed of the peak that allows the pressure gradient at the level of aortic valve to be calculated using the modified Bernoulli equation. This technique cannot be used when there is a significant aortic regurgitation.

SV and CO measurement at aortic valve: If measuring at the level of the aortic valve, the CSA of the valve can be measured using planimetry of a short axis view of the aortic valve in mid systole (Figure 4). VTI is measured using continuous wave Doppler with the Doppler beam directed through the valve orifice; the TG LAX view or the deep TG LAX view are the most helpful views for this purpose.

The stroke volume measured at aortic valve is simplified by the following equation:

$$SV = VTI \times \text{aortic valve area}$$

It is important to remember that if significant aortic stenosis is present, flow distal to the valve is not laminar and SV measurements will therefore be inaccurate.

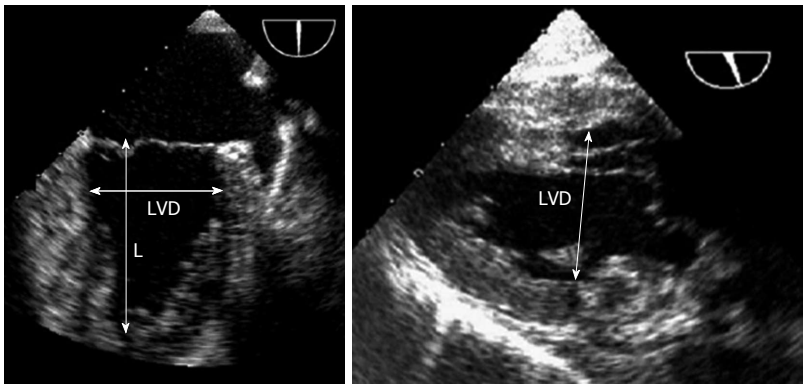

Figure 5 Transesophageal measurements of left ventricular length and minor left ventricular diameter from the ME 2C, usually best imaged at a multiplane angle of approximately 60°-90° and from the trans-gastric two-chamber view of the left ventricle, usually best imaged at an angle of approximately 90°-110°. L: Length; LVD: Left ventricular diameter.

Volume status assessment and volume therapy

Volume status assessments are a challenge in peri-operative haemodynamic management of LT and the most common haemodynamic changes are secondary to changes in volume status and cardiac function.

The filling pressures (*i.e.*, central venous pressure and pulmonary artery occlusion pressure), as indirect indicators of filling volumes, have been the "standard" methods for decades but, following significant criticism, volumetric measurements are now preferred[5,54]. The most commonly used parameters for left ventricular preload assessment are the left ventricular end-diastolic diameter (LVEDD) and the LVEDA, both obtained in the TG Mid SAX view[28]. It is important to remember that retractor placement during LT may, unfortunately, obstruct the transgastric view required to obtain these parameters[20,55]. Additionally, some authors recommend avoiding this view so as not to disrupt gastro-oesophageal varices in the distal oesophagus[19].

Left ventricular internal end diastolic diameter: Volume status or trends can be rapidly estimated by measuring LVEDD. A small left ventricular (LV) internal diameter at end-diastole can be indicative of hypovolemia, whereas the LV internal diameter at end systole (LVESD) is a less specific indicator of hypovolemia because a low value may be caused by a decreased systemic vascular resistance, an increased inotropic state, or by decreased ventricular filling. Both LVEDD and LVESD are decreased in hypovolemia, whereas LVEDD is normal and LVESD is decreased when systemic vascular resistance is decreased[52]. Serial measurements of both diameters can be useful to monitor a patient's response to administered fluids. The LV diameters can be measured by the TEE mid oesophageal two chamber (ME 2C) view at the mitral valve leaflet tips and by the TG Mid SAX view using M-mode imaging at the mid-papillary level[52]; however, the TG LAX view has been recommended because it is easier to align. LV diameters are measured from the endocardium of the anterior wall to the endocardium of the inferior wall in a line perpendicular to the long-axis of the ventricle at the junction of the basal and middle thirds of the long-axis (Figure 5).

Reference ranges for un-indexed LVEDD are 3.9 to 5.3 cm in women and 4.2 to 5.9 cm in men[56].

LVEDA: Compared with baseline imaging, measurements of LVEDA can be used as an indirect measurement of LV preload[57] and can be used to monitor the response to fluid therapy[58].

Variations of LVEDA, measured by definition at end-diastole at the mid-papillary muscle level, closely reflect changes in left ventricular end diastolic volume (LVEDV)[59]. The LVEDA index provides a measure of LV filling, which correlates with changes in the stroke volume index during volume replacement[60].

LVEDA can be calculated most easily in the TG Mid SAX view. The endocardium should be traced at the mid-papillary muscle level in end-diastole where the LV area is maximal (Figure 6)[61]. By convention, the papillary muscles are excluded from the tracing. Once traced, ultrasound software can calculate the LVEDA, which normally ranges from 8 to 14 cm^2[56].

TEE provides a better index of LV preload in patients with normal LV function than filling pressure values obtained by the more invasive PA catheterization approach[62].

Intraoperative TEE monitoring offers an indirect assessment of preload, but the validity of LVEDA as a preload index is still under discussion because it only correlates with changes in stroke volume index during volume therapy if the compliance and contractility of the left ventricle remain unchanged[47]. An index LVEDA < 5.5 cm^2/m^2 is very suggestive of low filling of the left ventricle[63].

Left and right ventricular function assessment

Although PAC study parameters provide an index of global myocardial function, they do not provide information on specific areas of myocardial performance. Assessments of left and right ventricular performance during surgery are advantages that TEE offers. TEE can detect areas of regional wall dysfunction that would otherwise remain

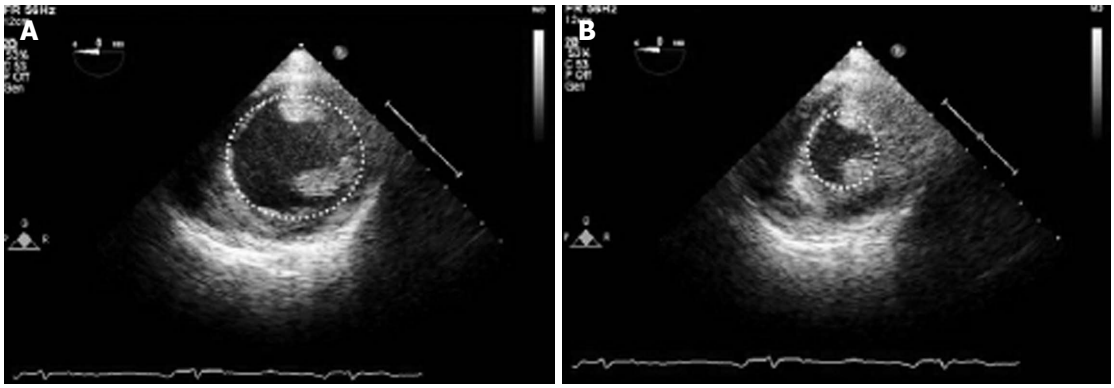

Figure 6 Transesophageal measurements of fractional area change. Transgastric Mid SAX view of the left ventricle showing measurement of left ventricular end diastolic area (A) and left ventricular end-systolic area (B). The bidimensional image is usually best imaged at a multiplane angle of 0°. Modified by Guarracino Fabio *et al*[61].

undiagnosed. Clearly, time consuming methods requiring multiple equations or measurements are not helpful in an acute setting, such as with the hemodynamic instability of LT. So, despite many published quantitative measures of global left ventricular function[28,64], most basic echocardiographers rely on qualitative, visual estimations of systolic function. This approach is far from precise but allows a basic echocardiographer to differentiate those patients who might benefit from inotropic therapies from those who simply need more fluid volume.

Left ventricular function: The American Society of Echocardiography and by the Society of Cardiovascular Anaesthesiologists[28] has recommended that ventricular function is assessed by a regional wall motion analysis based on a 17-segment wall motion score, as described in the ASE guidelines[64]. This approach suggests that a physician trained in basic transoesophageal echocardiography obtains mid oesophageal four-chamber (ME 4C), ME 2C and ME LAX views for a more comprehensive evaluation and to monitor global and regional LV function. However, visualization of 6 mid-papillary segments from the TG Mid SAX view may suffice and is important for prognosis[65].

The TG Mid SAX view provides significant diagnostic information regarding regional and global ventricular function to allow for efficient patient care and to minimize any distraction under intraoperative conditions while the patient is haemodynamically unstable.

Quantitative measurements of left ventricular function can also be obtained by measuring the Fractional Shortening, fractional area change and Ejection Fraction.

Fractional shortening: Fractional shortening (FS) expresses the percentage change between the LVEDD and the LVESD according to the following formula:

$$FS\ (\%) = (LVEDD - LVESD)/LVEDD \times 100$$

The LV internal diameters are measured at the ends of diastole and systole on an M-mode tracing of a TG Mid SAX view taken just above the papillary muscles and sometimes from an M-mode tracing of a TG LAX view from the inner edge to the inner edge of the endocardial borders (Figure 7).

Fractional shortening can be used to determine ventricular function, with normal values ranging from 25% to 45%[56].

Although FS gives a rapid and simple estimate of LV systolic function, it is not representative of global ventricular function if there are ventricular regional wall abnormalities or aneurysmal deformities[56]. It assesses only the selected cross section of the left ventricle, so there should be no alterations in regional LV contractility either at the apex or at the base if FS is to reflect global LV function accurately.

Fractional area change: Fractional area change (FAC) is a two-dimensional measurement that is easily obtained from the TG Mid SAX view. It expresses the percentage change between the LVEDA and the left ventricular end-systolic area (LVESA) according to the following formula:

$$FAC\ (\%) = (LVEDA - LVESA)/LVEDA \times 100$$

Normal values range from approximately 55% to 65%[56].

This approach requires both the LVEDA and LVESA measurements, tracing the left ventricular endocardium during the end of diastole and systole. The measurements are usually made in the TG Mid SAX view, but when this view is suboptimal, long axis views can also be used. The endocardium can be traced manually traced around the LV cavity, ignoring the papillary muscles, or the endocardial borders can be detected automatically (Figure 6)[61].

FAC can provide a reasonable global estimate of LV function but, like the FS, it has its limitations. It may represent global LV function poorly in cases of myocardial infarction or aneurysmal dilatation in areas of the ventricle other than the mid papillary level, where FAC is evaluated. Changes in loading condition may also influence the FAC.

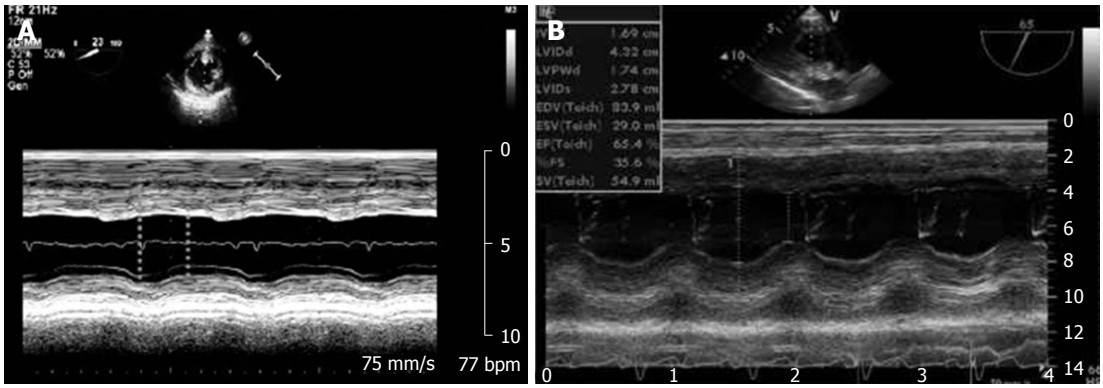

Figure 7 Transesophageal measurements of fractional shortening. A: TG Mid SAX view of the left ventricle showing M-mode measurement of LVEDD and LVESD normalized for LVEDD. The bidimensional image is usually best imaged at a multiplane angle of 0°; B: TG LAX view of left ventricle showing M-Mode measurement of LVEDD and LVESD normalized for LVEDD usually best imaged at an angle of approximately 80°-110°. LAX: Long axis view; TG: Transgastric; LVEDD: Left ventricle end diastolic diameter; LVESD: Left ventricle end systolic diameter. Modified by Guarracino Fabio *et al*[61].

Ejection fraction: This measure requires the application of algorithms that can approximate the left ventricle to a conventional solid. The ejection fraction (EF) is the most widely used index in clinical practice to describe left ventricular function, even if it measures ejection ability rather than the contractility of the left ventricle. The B-mode evaluation is most widely used to study systolic function because it is reliable even in the presence of geometric distortion or wall motion abnormalities and because it correlates well with radionuclide ventriculography and scintigraphy measurements[66]. In contrast to fractional shortening, which depends on a single cavity dimension in systole and diastole, the ejection fraction examines the entirety of myocardial contraction by expressing stroke volume as a percentage of LV end-diastolic volume:

$$LVEF\% = (LVEDV - LVESV)/LVEDV \times 100$$

Where LVEF is the left ventricle ejection fraction, LVEDV is the LV end-diastolic volume, and LV end-systolic volume (LVESV).

The LVEF represents a composite of cardiac performance involving preload, contractility, and afterload. It is widely regarded to be a predictor of outcome and survival. Normal EF values range from 55% to 70%.

The area-length and the Simpson's methods are among the commonest used to calculate the EF. The previously used Teichholz method of calculating LV ejection fraction from LV linear dimensions is not recommended for clinic practice because inaccuracies can arise from the geometric assumptions required to convert a linear measurement to a 3-D volume[67].

The area-length method is an alternative method to calculate LV volumes when the apical endocardial definition precludes an accurate tracing. This method assumes that the LV is bullet shaped[68], and the volumes are obtained by measuring LV areas and lengths at both end-diastole and end-systole. The measurements require a long-axis view of the chamber without foreshortening of the LV. This view may be difficult to obtain from the standard ME 4C view, and some retroflexion of the probe may be required to visualize the true apex and prevent any foreshortening of the LV.

The Simpson method is the most widespread EF calculation method and consists of 2-D measurements of volume with the biplane method of discs (modified Simpson's rule). The EF is calculated from the summation of a series (20) of overlapping slices from apex to base, each of which is assumed to be an elliptical disc. This is the currently recommended method of choice of the American Society of Echocardiography and the European Association of Echocardiography[56]. Modern ultrasound is equipped with software to calculate the EF by using the area of the LV and its diameter measured from the apex to the mitral floor in the two phases of the cardiac cycle. The EF is calculated from projections of the ME 4C and 2C views (Figure 8).

Right ventricular function

A normal right ventricle (RV) has an oblong shape and a complex architecture and, in the ME 4C and TG Mid SAX views, it is approximately two-thirds the size of the LV. In the ME 4C view, the RV extends more than half way to but does not normally share the LV apex.

The RV cavity on the short axis has a half-moon shape with the concave side of the interventricular septum towards the VS. A first echocardiographic sight provides information regarding RV function because, at the onset of right ventricular dysfunction, the ventricular chamber enlarges and the usually convex septal wall of the RV, facing the crescent shaped RV cavity, loses its classical shape and anatomical relationships with VS. An increase in RV pressure or volume overload can cause flattening or leftward deviation of the septal wall, producing an elliptical or circular short-axis shape of the RV cavity. The normal right ventricle is accustomed to a low pulmonary resistance and hence low afterload; thus, normal RV pressure is low and right ventricular compliance is high. Elevations in RV afterload result acutely in RV dilatation, whereas chronic elevations cause RV hypertrophy. Right ventricular size is best

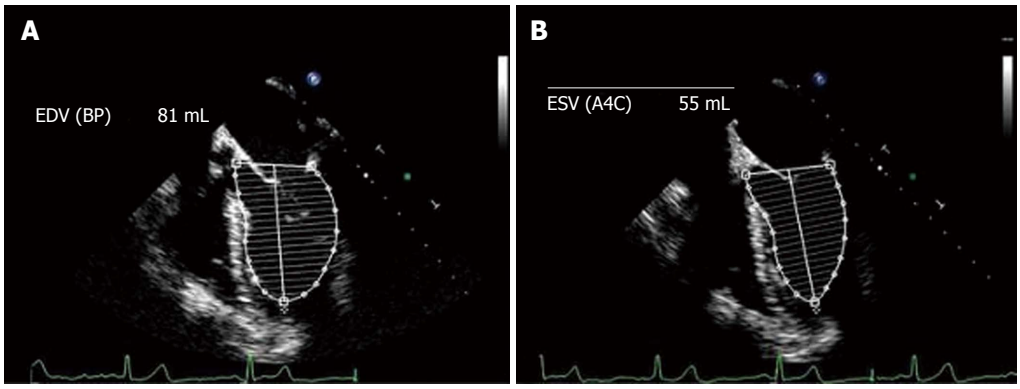

Figure 8 Calculation of ejection fraction using the disc method (Simpson's rule). Transoesophageal echocardiography ME 4C view in diastole (A) and systole (B). The bidimensional image is usually best imaged at a multiplane angle of 0°. Modified by Guarracino Fabio *et al*[61]. EDV: End diastolic volume; ESV: End-systolic volume.

estimated by TEE from a right ventricle focused ME 4C view, with the multiplane angle adjusted to maximize the tricuspid annulus diameter, usually between 10° and 20°[56]. Care should be taken to obtain the image of the maximal right ventricular diameter without foreshortening[69].

Many techniques have been described to obtain quantitative measurements of the overall function of the right ventricle, but in most cases, the physician relies on qualitative measures, and on visual estimates of systolic right ventricular function.

Assessments of right ventricular function are required whenever a patient presents with unexplained or refractory hypotension. Patients undergoing LT, for example, can present with hypotension secondary to right ventricular failure[70]. If their RV dysfunction is related to acute changes in pulmonary pressures associated with lung volume shifts or acid-base changes during the transplant, they can also be at risk for pulmonary hypertension[21].

TEE has the significant advantage that right ventricular failure can be identified by dynamic rather than the pressure changes, which can be easily missed in PAC measurements. Because right ventricular failure is an important complication during the reperfusion phase of LT, TEE monitoring confers very significant advantages at this stage.

When the evaluation is based on a quantitative assessment, a number of echocardiographic techniques may be used to assess RV function.

Tricuspidal annular plane systolic excursion: In systole, the tricuspid annulus will normally descend towards the apex 1.6-2.0 cm. A tricuspidal annular plane systolic excursion (TAPSE) of less than 1.6 cm has been associated with a poor prognosis in a variety of cardiovascular diseases; it is highly specific for RV dysfunction and can be used to monitor RV systolic function serially. It is important to place the cursor at the annulus side and to apply the M-Mode. Once the image is frozen, the TAPSE is the difference between the lowest and highest excursion points of the tricuspidal annulus. It has the advantage of easy reproducibility, speed of measurement and less dependence on optimal-quality images. There are some disadvantages: The measurement is angle dependent, and the displacement in the ME 4C view is representative of the function of the entire right ventricle only if there are no regional RV wall motion abnormalities. The TAPSE may also be load-dependent, even if less preload-dependent than other markers of RV function[69].

Right ventricular fractional area change: The FAC provides an estimate of the systolic function of the right ventricle. Usually measured in the ME 4C view, it is a simple method to assess RV function that correlates with RV ejection fractions measured by magnetic resonance imaging and has been related to outcome in a number of diseases[56,71]. It is important to verify that the entire right ventricle is in the view, including the apex and the lateral wall, in both systole and diastole. Care must be taken to exclude trabeculations while tracing the RV area. The normal range is 35%-60% and a two-dimensional FAC < 35% indicates RV systolic dysfunction[69].

UTILITY OF TEE EXAMINATION AND SURGICAL PHASES OF LT

In addition to all the pathophysiological processes that characterize ESLD, the three major stages of LT (pre-anhepatic, anhepatic and reperfusion phases) pose particular challenges in terms of anaesthetic management. TEE permits accurate monitoring of these clinical phases with rapid anaesthetic management during complicated procedures, maximizing the opportunities for a successful outcome.

Pre-anhepatic phase

The pre-anhepatic phase begins with surgical incision and concludes with cross clamping of the vascular inflow to the liver. The conventional technique involves clamping of the portal vein, the suprahepatic inferior

vena cava, the infrahepatic inferior vena cava, and the hepatic artery. The piggyback technique requires temporary clamping of portal flow only and tangential clamping of the retrohepatic inferior vena cava, which allows venous return to the heart.

Surgical bleeding, when present, could be the main issue during the pre-anhepatic phase. Dissection may be complicated by steady and sometimes rapid haemorrhage from varices in the abdominal wall or adhesions within the abdominal cavity. Fluid shifts and third space fluid losses may result from the drainage of ascitic fluid and from venous congestion. Bleeding during this phase of surgery is related to the degree of pre-existing coagulopathy, the presence and severity of portal hypertension, and the duration and complexity of the surgical procedure[72,73].

Vascular clamping and manipulation of the liver, together with an inadequate volume resuscitation, result in decreased venous return and reduced cardiac output, resulting in critical organ hypoperfusion[74].

Sudden haemodynamic variations and a reduced preload are the most serious complications and challenges that the anaesthetist has to manage during LT.

TEE allows circulatory volumes to be optimized, avoiding impaired organ perfusion, ischaemia and, at the same time, volume overload. Identifying the cause of any hypotension is critical for successful treatment and aggressive fluid repletion leading to overload must be avoided to prevent pulmonary oedema or right ventricular failure due to unrecognized preoperative cardiac disease[3,75].

Close approximation of the papillary muscles and decreases in LVEDA and LVEDD in the TG Mid SAX view signal a reduced preload due to haemorrhage or vascular clamping: After a fluid challenge; TEE allows the anaesthetist to assess the adequacy the interventions rapidly. Signs of RV and right atrial dilation together with RV hypokinesis[76] or atypical regional wall motion abnormalities of the RV free wall[77] can draw attention to right ventricular failure.

If the conventional surgical technique with inferior vena cava and portal vein clamping is poorly tolerated, the VVB might be warranted to guarantee venous return to right atrium and to decompress the portal venous system, reducing bleeding, vascular congestion to the intestines, and injury to the bowel capillary bed[78].

In this case, TEE can assist in the placement of transcutaneous VVB lines, confirm the correct location of guide wires in the venous system, and detect the tip of the cannula in the superior vena cava[79]. Air embolism, thromboembolism, and inadvertent decannulation are among the reported and feared complications of VVB and can increase its morbidity[80].

Anhepatic phase

The anhepatic phase starts after complete occlusion of vascular inflow to the liver; includes the removal of the native liver and the completion of the vascular anastomoses; and ends with the reperfusion of the newly grafted liver. This phase is mainly characterized by hemodynamic changes induced by the cross-clamping of the inferior vena cava and portal vein, which reduce venous return, cardiac output and renal perfusion pressure while increasing the splanchnic and lower caval pressures[25]. VVB is used routinely in some centres to facilitate return of blood from the portal system and lower body to the heart *via* a centrifugal pump to the axillary vein[78]. The TG Mid SAX view or ME 4C view can be ideal for monitoring ventricular function and volume status continuously during this phase. If preload is diminished or systemic vascular resistance is reduced, the left ventricular papillary muscles will approximate each other during systole and LVEDA will be reduced.

Reperfusion phase

LT represents a special case of acute right ventricular stress. Cardiac output increases acutely at the time of reperfusion (up to 3-fold in 15 min) and this increased blood flow to the right heart can result in volume overload and pulmonary oedema due to occult cardiac disease[3].

This phase extends from the period immediately after the reperfusion of the graft to the end of surgery and includes the arterial anastomosis and biliary tract reconstruction. Cardiovascular instability is greatest during this phase of the operation, and it is as accompanied by a decrease in mean arterial pressure of 30% or more from baseline for at least 1 min's duration, and occurring within 5 min of reperfusion[81,82].

The reperfusion syndrome is typically characterized by severe hypotension, decreased heart rate, a significant reduction in systemic vascular resistance, and increases in pulmonary arterial pressure and wedge pressure. All these changes are thought to result from the sudden release of cold, acidotic and hyperkalaemic preservation fluid into the circulation while myocardial dysfunction, often observed after reperfusion, is caused by several vasoactive mediators released into the circulation by the re-perfused graft[83].

The characteristic echocardiographic features of the reperfusion phase may include acute right ventricular systolic dysfunction, left ventricular systolic dysfunction or both; new global or focal wall motion abnormalities; decreased FAC; and because of rapid cardiac influx after vascular unclamping, increases in ventricular end-diastolic volume and LVEDA are common. Selecting and maintaining TEE, typically either in the ME 4C or TG Mid SAX views, allows for real time monitoring of all the effects of reperfusion on the heart. During transition from the anhepatic to the reperfusion phase, TEE views can also be useful for detecting intracardiac air, thrombosis, mitral or tricuspid valve regurgitation; severe diastolic dysfunction; or a previously undiagnosed outflow obstruction that can occur during reperfusion.

Following reperfusion TEE may be useful to detect the temporary opening of a foramen ovale[84] or elevation of pulmonary arterial pressures, facilitating prompt

management of these complications.

Unique haemodynamic changes occur from the anhepatic to the reperfusion phases, and a real time TEE allows for a rapid assessment and adjustment of fluid shifts and an optimal outcome.

USEFULNESS OF TEE IN THE DIFFERENTIAL DIAGNOSIS OF RARE CARDIOVASCULAR CONDITIONS OFTEN ASSOCIATED WITH HAEMODYNAMIC INSTABILITY

Pulmonary embolism

Although patients with liver disease were long assumed to have a natural bleeding tendency and to be protected from thrombosis, the real coagulation state of the cirrhotic patients combines changes in both pro- and anti-haemostatic pathways in a new haemostatic balance[85]. However, the occurrence of both bleeding and thrombotic complications in a significant proportion of patients shows that this haemostatic balance is relatively unstable[86]. Although LT is associated with increased bleeding and altered coagulation, a prothrombotic state may also occur. Intravascular thrombus formation and subsequent embolization is a potentially fatal complication that most often occurs after reperfusion. Both surgery (vascular clamping) and trauma pose an increased risk for pulmonary embolism (PE) and an incidental cardiac thrombosis, in particular, may lead to serious complications in LT, including intraoperative death[87]. Thus, anaesthesiologists may be responsible for both PE diagnosis and treatment, even if the diagnostic sensitivity of TEE for PE by direct visualization of a thrombus in the pulmonary artery is actually quite low[88]. Although TEE is not the gold standard for PE diagnosis, it can compare with the sensitivity of a TC scan when the PE is acute, central and characterized by severe haemodynamic instability[89,90]. Notably, only 30% obstruction is needed for RV dysfunction to be recognized on TEE[91] but the echocardiographic diagnosis of a PE using direct evidence often requires advanced TEE skills.

The Consensus Statement of the American Society of Echocardiography and the Society of Cardiovascular Anesthesiologists recommends that "a physician trained in basic perioperative TEE at least should be able to use ME 4C, ME AV SAX, and ME RV inflow-outflow views to identify indirect echocardiographic findings consistent with a PE"[28]. Before initiating any specific treatment, the presence of thrombus and/or signs of RV dysfunction, typically due to elevated right-sided pressures, should be identified. The direct visualization of intracardiac thrombus or emboli in the main pulmonary artery or its right or left branches by TEE allows for diagnosis, though it is important to remember that the left pulmonary artery is not completely visualized with TEE due to the interposition of the left bronchus.

Echocardiographic findings consistent with acute PE include: Signs of RV dilation, RV hypokinesis[76], atypical regional wall motion abnormalities in the RV free wall[77], and decreased TAPSE.

Another typical sign of elevated right-sided pressures is the leftward shift of the interatrial septum or interventricular septum, which is easily seen in the ME 4C view. Although the RV is dysfunctional, the LV may appear hyperkinetic and underfilled, due to a leftward shift of the interventricular septum[92].

LVOTO

Cirrhosis is associated with increased left ventricular wall thickness, cardiac chamber enlargement, and a significantly impaired systolic and diastolic response to stress, especially in the setting of volume overload. The hypertrophic cardiomyopathy, which characterizes the cirrhotic patient, makes these patients particularly susceptible to LVOTO especially when hypovolemia, tachycardia, and increased ventricular contraction can cause apposition of the mitral valve anterior leaflet and the septal wall during systole[93]. The pre-anhepatic and anhepatic phases of LT are usually associated with decreases in the left ventricular preload secondary to intraoperative surgical or medical bleeding and to vascular clamping, whereas the post-reperfusion phase is usually associated with a marked decrease in systemic vascular resistance. Therefore, the patients undergoing LT often have several risk factors for dynamic LVOTO[94]. TEE, by virtue of its unique features, guarantees a continuous monitoring of cardiac function and structures, especially during the anhepatic and reperfusion phases[34] when the occurrence of dynamic LVOTO is more common, and can facilitate appropriate management and therapeutic interventions[95].

Dynamic LVOTO can be assessed readily in the ME LAX view at an approximately 120° angle. The characteristic features to look for are turbulence through the left ventricular outflow tract, hypercontractility of the left ventricle, systolic anterior leaflet motion of the mitral valve, and some degree of late mitral regurgitation[96]. A severe mitral regurgitation due to the systolic anterior leaflet motion represents a rapid qualitative method to quantify the degree of LVOTO. The degree of mitral regurgitation is a qualitative method to determine the severity of LVOTO because it usually correlates with the degree of outflow tract obstruction. Another rapid qualitative measure of LVOTO can be obtained by Colour-Doppler, where a mosaic pattern indicates turbulence associated with an elevated LVOT gradient. A quantitative assessment of the obstruction is otherwise obtained by a Doppler quantification of blood flow velocities through the LVOT, using the TG LAX view or the deep TG LAX view. Blood flow velocity in the LVOT is measured by positioning the continuous wave Doppler sample volume in the centre of the LVOT just proximal to the AV. Normal LVOT and AV flow velocities

are less than 1.5 m/s[97]. Colour flow Doppler imaging of the LVOT and AV is useful in directing the Doppler beam through the area of maximum flow when these velocity measurements are made.

Portopulmonary hypertension

Liver disease and portal hypertension can be associated with pulmonary vascular complications, such as POPH, which is characterized by the presence of portal hypertension, a mean pulmonary artery pressure > 25 mmHg at rest, a mean pulmonary capillary wedge pressure < 15 mmHg, and a pulmonary vascular resistance > 240 dynxsxcm^{-5}[98]. The prevalence of POPH in liver transplant candidates is reported to be 6.3%[99] and 8.5%[100]

Severe pulmonary hypertension and elevated right ventricular systolic pressure predicted a high risk of morbidity and mortality from fulminant right ventricular failure among patients undergoing LT[101]. Patients presenting for liver transplantation with pulmonary hypertension have an additional risk for RV dysfunction secondary to acute changes in pulmonary pressures associated with the volume shifts and acid base disturbances that characterize LT.

For this reason, most transplant centres consider severe POPH an absolute contraindication to transplantation[102]. On the other hand, a number of reports have confirmed that LT can be performed safely if the patient haemodynamic state is suitably controlled[102].

The most important test to screen for POPH is the two dimensional TTE, which is a routine part of an LT evaluation[103].

Unfortunately, the TTE cannot fully discriminate between increased PVR due to true vaso-occlusive arteriopathy, a hyperdynamic state, or fluid overload, with normal/low PVR. Therefore, right heart catheterization is the gold standard for the diagnosis of POPH[104].

For this reason, POPH may be missed by preoperative echocardiography and may be recognized only during right heart catheterization in the intraoperative phase.

The diagnosis of unexpected POPH on the operating table may still be best handled through TEE. Continuous intraoperative transoesophageal echocardiography has been recommended for following right heart function, and in the event of a pulmonary hypertensive crisis, the anaesthetist has to be ready to address acute pulmonary hypertension with effective agents such as inhaled or intravenous vasodilators[102,105].

The echocardiographic intraoperative findings of severe pulmonary hypertension include right ventricular hypertrophy, dilatation and dysfunction, as well as right atrial enlargement. A paradoxical septal movement can be seen as well.

The best views to visualize the right heart chambers are ME 4C and ME RV inflow-outflow.

CONCLUSION

TEE has evolved as an important diagnostic tool outside the field of cardiac anaesthesia and it has gained increasing importance as a monitoring tool in liver anaesthesia. One of the most advantageous features of the TEE over PAC is the direct visualization of the heart in real time, which allows for instantaneous assessment of the state of the cardiovascular system, changes in global and regional contractility, and the rapid diagnosis of ventricular dilatation and failure. TEE can overcome the limitations of PAC measurements arising from the large core body temperature shifts typical of massive fluid infusion or revascularization of the new graft during LT.

TEE allows the intraoperative causes of hypotensive episodes during LT to be identified and can help rapidly optimize volaemia, avoiding organ perfusion impairment and ischaemia. TEE also allows for the intraoperative diagnosis and management of specific cardiovascular conditions that like portopulmonary hypertension, air embolism and thromboembolism, can complicate LT and the management of patients with hypertrophic cardiomyopathy, who may experience LVOTO.

Notwithstanding the published guidelines that define the basic and advanced competency requirements for TEE users, transoesophageal echocardiography is being used to monitor haemodynamics and for direct therapy in liver transplant patients, and clinically useful interpretations are possible even during the skill-acquisition phase of TEE training.

The value of TEE must of course be balanced against the risk of performing the procedure. Gastro-oesophageal varices are very common in patients listed for LT; thus, patients with ESLD should have a preoperative endoscopic surveillance and oropharyngeal examination, and probe manipulation should be limited to experienced operators.

Despite these limitations, the intraoperative TEE is a relatively safe method for monitoring cardiac performance in liver transplant patients and should not be contraindicated by the presence of oesophageal varices if the indications for the exam are important.

Although the interpretation of TEE remains mostly subjective, TEE has been helpful for assessing haemodynamic alterations, guiding fluid replacement and inotropic therapy, and identifying potential complications during LT, allowing for better management of patients.

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P- Reviewer: Niu ZS **S- Editor**: Ma YJ
L- Editor: A **E- Editor**: Liu SQ

World Journal of Hepatology

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DOI: 10.4254/wjh.v7.i23.2449

World J Hepatol 2015 October 18; 7(23): 2449-2458
ISSN 1948-5182 (online)

REVIEW

Programmed death-1/programmed death-L1 signaling pathway and its blockade in hepatitis C virus immunotherapy

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Author contributions: Salem ML and El-Badawy A contributed equally to this work.

Supported by Science and Technology Development Fund (STDF; grants No. 1469 and No. 5245); and Tanta University Fund, Egypt to Mohamed L Salem, the Principal investigator of these projects.

Conflict-of-interest statement: We declare that authors have no conflict of interest.

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Received: April 11, 2015
Peer-review started: April 15, 2015
First decision: June 2, 2015
Revised: June 29, 2015
Accepted: September 2, 2015
Article in press: September 7, 2015
Published online: October 18, 2015

Abstract

Chronic hepatitis C virus (HCV) infection is a public health issue that often progresses to life-threatening complications, including liver cirrhosis, fibrosis, and hepatocellular carcinoma. Impaired immune responses to HCV are key features of chronic HCV infection. Therefore, intervention strategies usually involve enhancing the immune responses against HCV. Cytotoxic $CD8^+$ T lymphocytes (CTLs) play a critical role in the control of HCV infection. However, their cytolytic function can be impaired by the expression of co-inhibitory molecules. Programmed death-1 (PD-1) receptor and its ligand PD-L1 function in a T cell co-inhibitory pathway, which either blocks the function of CTLs or the differentiation of $CD8^+$ T cells. During chronic HCV infection, the immune inhibitory receptor PD-1 is upregulated on dysfunctional HCV-specific $CD8^+$ T cells. As such, blockade of the PD-1/PD-L1 pathway in these $CD8^+$ T cells might restore their functional capabilities. Indeed, clinical trials using therapies to block this pathway have shown promise in the fostering of anti-HCV immunity. Understanding how chronic HCV infection induces upregulation of PD-1 on HCV specific T cells and how the PD-1/PD-L1 interaction develops HCV specific T cell dysfunction will accelerate the development of an efficacious prophylactic and therapeutic vaccination against chronic HCV infections, which will significantly improve HCV treatments and patient survival. In this review, we discuss the relationship between PD-1 expression and clinical responses and the potential use of PD-1 blockade for anti-HCV therapy.

Key words: Hepatitis C virus; Programmed death-1; Hepatitis C virus immunotherapy; Exhausted T cells; Hepatitis C virus immune escape

Core tip: The programmed death-1 (PD-1)/PD-L1

pathway is an attractive target for anti-hepatitis C virus (HCV) immunotherapy because it restores the functional capacities of HCV-specific T cells. This is an extremely promising development in anti-HCV vaccines research since restoration of exhausted anti-HCV T cells is a major challenge when developing either prophylactic or therapeutic vaccines. This review will discuss the correlation between PD-1 expression and the clinical outcome in HCV patients and how this information can be potentially applied to block PD-1/PD-L1 pathway for HCV immunotherapy.

Salem ML, El-Badawy A. Programmed death-1/programmed death-L1 signaling pathway and its blockade in hepatitis C virus immunotherapy. *World J Hepatol* 2015; 7(23): 2449-2458 Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i23/2449.htm DOI: http://dx.doi.org/10.4254/wjh.v7.i23.2449

INTRODUCTION

Chronic viral infections, including hepatitis C virus (HCV), hepatitis B virus (HBV), and human immunodeficiency virus (HIV), are among the main causes of death worldwide[1]. While most viral infections prompt successful T cell responses that remove the infections, HCV, HBV, and HIV have acquired mechanisms to avoid immune elimination, permitting them to persist in many, if not all, infected individuals. These escape mechanisms lower the responsiveness of patients to anti-viral therapy.

HCV is found in nearly every region of the world, affecting an estimated 170 million patients and 1%-2% of the overall population in most infected countries[2]. HCV not only causes hepatitis C, but it also provides the perfect infection setting to study viral evasion mechanisms, since the infection persists in most infected individuals while 25% of infected patients effectively clear the virus. This allows for the comparison of immune responses between responders and non-responders. How these immune responses determine whether a patient eliminates infection or develops a chronic infection is not completely understood. Accordingly, viral escape from immune cells has been suggested as a contributing factor to HCV as well as HIV and HBV infection.

In the acute stage of HCV infection, 20%-40% of patients improve spontaneously[3], and this recovery is associated with a robust, HCV-specific T cell responses[4-6]. The discriminating role of the HCV specific $CD8^+$ cells responses in the unprompted recovery of acute HCV infection was demonstrated in chimpanzees[5,6], the only animal model for the study of HCV. Even in chimpanzees with chronically developing, acute HCV infection, intrahepatic infusion of $CD8^+$ T cells promoted a partial decline in the HCV load[7,8]. In chimpanzees, vaccination with an experimental prophylactic vaccine induced HCV-specific $CD8^+$ cell responses and suppression of acute HCV infection[9]. These studies led to the hypothesis that recovery from HCV may be due to induction of HCV-specific T cell responses. Hence, research efforts for the development of novel treatments for chronic HCV infection have focused on T cell responses.

Dysfunction of virus-specific $CD8^+$ cells is a fundamental property of persistent viral infections like HCV; consequently, restoration of T cell capacity is a major aim in the generation of immune-based therapies for persistent infection of viruses[10]. Many factors are known to contribute to T cell dysfunction, including inhibitory cytokines, regulatory T cells, and inhibitory receptors expressed on T cells[10]. Accordingly, removal or blockade of these inhibitory factors may be a promising approach for the treatment of persistent viral infection.

Overall, the mechanisms that have been proposed to date to explain impaired immunity in chronic HCV infection are summarized as follows: (1) HCV escapes immune responses by developing mutations; (2) primary T cell exhaustion after an extensive response; (3) impaired antigen presentation of dendritic cells (DCs); (4) impaired natural killer (NK) cell activities; (5) skewing the Th1 type cytokine to a Th2 type; (6) suppression by HCV proteins; (7) impaired T cell maturation; (8) suppression by regulatory T cells; (9) the nature of the tolerogenic environment in the liver; and (10) the expression of co-inhibitory molecules on immune cells[11].

An important inhibitory receptor that downregulates T cell function is programmed death-1 (PD-1)[12]. PD-1, with its two known ligands B7-H1/PD-L1 and B7-DC/PD-L2, has recently been shown to be upregulated on HCV- and HIV-specific $CD8^+$ cells, indicating that PD-1 upregulation may be an essential mechanism for viral immune escape in chronic HCV and HIV infections[13-17].

Here, we provide up to date review on the role of PD-1 in HCV immune evasion and the potential use of PD-1 blockade for anti-HCV therapy. Understanding the relationship between PD-1/PD-L1 and T cell dysfunction and its role in HCV persistence will accelerate the development of an efficacious prophylactic and therapeutic vaccination against chronic HCV infection.

HCV TREATMENT AND FAILURE

Combination therapy with pegylated interferon (PEG-IFN) and ribavirin (RBV) is the current standard therapy for individuals with chronic HCV infection[18-21]. Treatment duration is 48 wk for HCV genotypes 1 and 4, and 24 wk for genotypes 2 and 3. The dominant majority of treated patients, especially those with HCV genotypes 2 or 3, show a significant virologic response. Almost 66% of patients with HCV genotype 2 or 3 accomplish rapid virologic response (RVR), characterized by untraceable HCV RNA within 4 wk of starting treatment, and 97% have undetectable HCV RNA within 12-24 wk of starting treatment. Seventy-six percent attain sustained virologic response (SVR)[21,22]. Unfortunately, only approximately half of all patients accomplish SVR with 24-48 wk of therapy with PEG-IFN and RBV[18,20].

Factors that contribute to non-responsiveness, other than genotype, are high baseline HCV viral-load, high fibrosis stage in the liver, male gender, old age, race, obesity, alcohol intake, insulin resistance, liver steatosis, and alterations in the host immune response, such as high interleukin (IL)-8 and IL-10 serum levels[23-25].

Current IFN-based therapy does not work in many patients, possibly due to a combination of viral and host factors. Innate immunity to HCV is activated by cellular sensors that identify the presence of pathogen-associated molecular patterns (PAMPs). Key fundamental cellular sensors for HCV infection are toll-like receptor 3 (TLR3), which recognizes double-stranded RNA (dsRNA), and the RNA helicase retinoic acid-inducible gene 1 (*RIG-1*). The HCV PAMP sensors TLR3 and RIG-1 signal through the adaptor proteins TIR-domain-containing adapter-inducing interferon-β (TRIF) and Cardif, respectively. Remarkably, the HCV NS3-4A serine protease relieves both Cardif[26,27] and TRIF[28] to disable signals initiated by RIG-1 and TLR3. In addition to blockade of the upstream events of IFN-β transcription by NS3-4A, there is evidence that other HCV proteins block IFN signaling downstream of the IFN-α/β receptor. Overexpression of HCV core protein causes activation of suppressors of cytokine signaling (SOCS) 3 protein[29], which in turn hinders signal transducer and activator of transcription 1 (STAT1) phosphorylation by janus kinase 1 (Jak1). These mechanisms support viral persistence even in the face of IFN-based therapies. Further understanding of the molecular mechanisms underlying HCV resistance to the host immune response will lead to generation of novel therapeutic strategies. Moreover, host factors, such as insulin resistance and race, have considerable effects on treatment responsiveness. Adjustment of adverse host factors, whenever possible, may be a feasible alternative for the optimization of HCV therapy.

PEG-IFN is contraindicated in decompensated cirrhosis[30] and is associated with constitutional, autoimmune, neuropsychiatric, and hematological side effects[31], whereas RBV is contraindicated in renal failure[32] and is associated with rash, cough, hemolysis, and teratogenesis[31]. Therefore, many patients are ineligible for or intolerant to PEG-IFN and RBV therapy. However, the approval of sofosbuvir (Sovaldi®, Gilead Sciences), which is a direct acting pyrimidine nucleotide analog that represents the first NS5B HCV polymerase inhibitor, is considered a key step towards a new era in chronic hepatitis C therapy. It was among the first approved antiviral agents with strong activity and high genetic barrier against all HCV genotypes. Additionally, its safety profile is highly favorable, even when it is prescribed to patients with very advanced liver disease and high risk of complications (*e.g.*, cirrhosis with portal hypertension and liver transplant recipients).

ANTI-HCV IMMUNITY

Impaired immune responses to HCV are hallmarks of chronic HCV infection. Therefore, intervention approaches commonly include those that can boost the immune responses against HCV. These immunotherapies for chronic HCV infections include anti-HCV neutralizing antibodies, antagonists of T cell inhibitory factors, therapeutic vaccines, agonists for TLRs, and cytokines[33]. These therapies can be utilized alone or in combination with other antiviral drugs for chronic HCV therapy.

To date, immune-based therapies have not demonstrated satisfactory efficacy. In general, a virologic response was shown only in a small group of patients, and in these cases, the effect was marginal and transient. A critical reason for the poor efficacy is the inadequate activation and stimulation of immune responses. It should be mentioned, however, that the virologic responders showed the strongest T cell responses in a late study that tested the peptide vaccine IC41[34]. This observation demonstrated that a sufficient virologic response might be accomplished by sufficient activation and stimulation of the immune system. Thus, enhancement of the protocol/regimen is needed to improve the efficacy of immune-based therapies.

One possible critical mechanism underlying the inability of HCV patients to resolve the infection is the imbalance between the stimulatory and regulatory immune cells[35]. The poor adequacy of immune-based therapies may be due to various factors. First, many individuals with chronic HCV infection have delayed impairment of the anti-HCV immune response, and it seems unlikely that longstanding immune dysfunction can be repaired by immune-based therapies. Second, HCV advances quickly, and persistent HCV infection brings about the specific survival of viruses that are most proficient at evading host immune responses. Accordingly, these viruses may have the capacity to resist clearance despite the improvement of immune responses by immunotherapies. Finally, the poor adequacy may be credited, in part, to the selection of patients.

Combination therapy might be an efficient strategy for improving the efficiency of immunotherapies For instance, the impact of therapeutic vaccines could be enhanced by combining them with antagonists of T cell inhibitory factors and/or agonists of TLRs. Interestingly, combining antagonists of IL-10R or PD-1 with a therapeutic vaccine strengthened the effects in a murine model of persistent lymphocytic choriomeningitis virus (LCMV) infection[36]. In general, immunotherapies have been well-endured and have not been associated with severe adverse effects. However, improvements in immunotherapies aiming to prompt stronger immune responses may aggravate liver injury and cause severe hepatitis in extreme situations. In this regard, it would be useful to demonstrate the differences between cytotoxic virus-clearing and tissue-damaging T cell responses. A better understanding of the cellular and molecular mechanisms implicated in T cell dysfunction will pave the way for highly efficacious immunotherapies for chronic HCV.

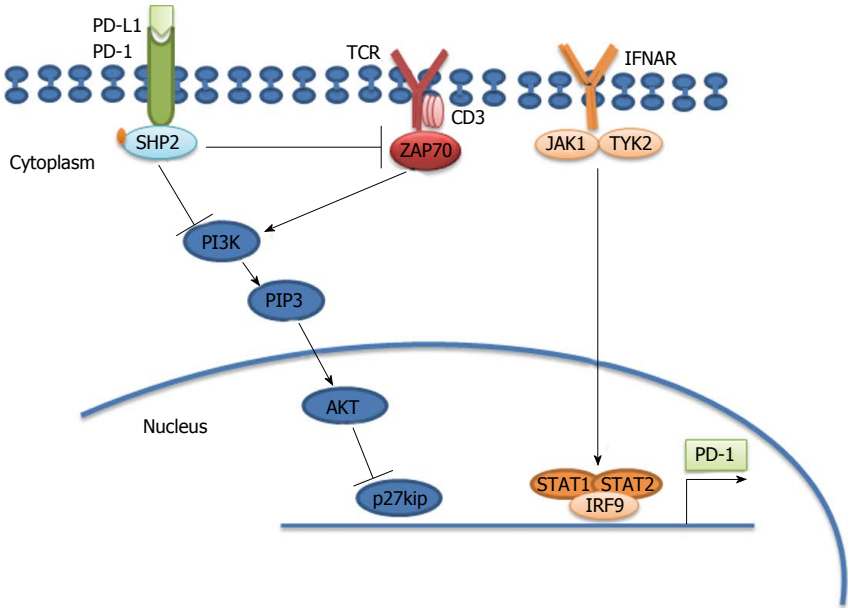

Figure 1 Programmed death-1 causes T cell exhaustion. Programmed death-1 (PD-1) inhibits the T cell receptor (TCR) signaling pathway through src homology 2-containing protein-tyrosine phosphatase 2 (SHP2). PD-1 is located in the immune synapse at the T cell-antigen presenting cell (APC) interface. When its physiological ligand (PD-L1 or PD-L2) binds, PD-1 suppresses the activation and function of T cells through the recruitment of SHP-2, which dephosphorylates and inactivates ZAP70, a major integrator of TCR-mediated signaling. In chronically activated ("exhausted") T cells, interferon (IFN)-α causes overexpression of PD-1 through the binding of the transcription factor IRF9 to the signal transducer and activator of transcription (STAT)1 and STAT2 promoters. PD-1 also results in accumulation of p27kip1, which is an inhibitor of cyclin dependent kinases to block cell cycle and proliferation[84]. ZAP70: Zeta-chain (TCR) associated protein kinase 70 kDa; IRF9: Interferon regulatory factor 9; JAK1: Janus kinase 1.

PD-1 EXPRESSION ON IMMUNE CELLS IN HCV PATIENTS

PD-1 and its ligands play a critical role in the inhibition of the immune system by banning the activation of T-cells, which subsequently decreases autoimmunity and advances self-tolerance. The inhibitory effect of PD-1 is achieved through a dual mechanism of inducing apoptosis in antigen specific T-cells in lymph nodes and decreasing apoptosis regulatory T cells (Tregs) (Figure 1). New classes of drugs that block PD-1, such as Nivolumab, Pembrolizumab, Pidilizumab, and BMS-936559, activate the immune system to attack cancers and are used to treat tumors.

PD-1 has two ligands-PD-L1 (B7-H1)[37,38], which is largely expressed on both hematopoietic and parenchymal cells, and PD-L2 (B7-DC)[39,40], which is mainly expressed on macrophages and DCs. Barber *et al*[12] found that PD-L1 was expressed at very high levels in splenocytes from persistently infected mice, particularly on virally infected cells. Consequently, not only did the exhausted cytotoxic T cells express high levels of PD-1, but its ligand was upregulated on infected cells (Figure 1). PD ligands are differentially regulated, where IFN-γ primarily stimulates PD-L1 expression and IL-4 stimulates PD-L2 expression[41,42]. Recent studies showed that antibody-mediated interference with PD-1 caused regression of several tumor types, including melanoma, renal-cell cancer, and non-small-cell lung cancer, in some patients[43,44]. The inhibitory effect of PD-1 is achieved through a dual mechanism that involves simultaneous induction of apoptosis in antigen specific T-cells in lymph nodes and decreasing apoptosis in regulatory T cells[45,46] (Figure 1).

In the acute stage of HCV infection, HCV specific T cells have been shown to be inadequately functional regardless of the final outcome of the disease[47-51]. A possible mechanism directing this behavior of the HCV-specific T response is exhaustion. When T cells are chronically exposed to high antigen loads, the PD-1/PD-L1 ligand pathway may play a role in T-cell exhaustion. Blocking the PD-1/PD-L1 interaction can permit restoration of exhausted T cells[12,52-55]. These studies indicated that high expression of the inhibitory PD-1 receptor appears to be a signature of functional T cell exhaustion.

Kasprowicz *et al*[17] have demonstrated elevated PD-1 expression on almost all HCV specific $CD8^+$ and $CD4^+$ T cells through the early phase of acute infection, irrespective of clinical outcome or viral load. They also showed that PD-1 expression is reliant on the tissue microenvironment, where the T cells execute their antiviral functions. Interestingly, the overall PD-1 expression levels of infiltrating $CD8^+$ and $CD4^+$ T lymphocytes in the liver were significantly higher compared to peripheral blood[17]. The mean PD-1 expression level on most of $CD8^+$ T liver-residing lymphocytes was 71%, while the median expression on peripheral blood $CD8^+$ T cells from the same subjects was 33%. For liver-derived $CD4^+$ T lymphocytes, the median PD-1 expression level was 53%, while PD-1 expression for cells in the peripheral blood was only 25%[17].

It has been shown that chronic HCV infection has a wide effect on PD-1 expression. For example, PD-1 is

Table 1 Differences between the studies made on the correlation between programmed death-1 expression and clinical outcome

Ref.	No. of patients	Mode of transfection	Symptoms	PD-1 expression levels in acute infection	PD-1 expression levels in resolved patients
Urbani *et al*[16]	19	Sexually transmission	Symptomatic	High	Decreased
Kasprowicz *et al*[17]	37	Sexually transmission	Symptomatic	High	Irrespective
Rutebemberwa *et al*[62]	20	Injection drug use	Asymptomatic	High	Decreased

PD-1: Programmed death-1.

highly expressed on peripheral B cells and monocytes, since it is induced upon activation[56]. In patients with chronic HCV, $CD56^{high}$ NK cells expressed greater levels of PD-1, convenient considering their greater functional deficiency and less mature $CD56^{low}$ differentiation state[57]. In addition, PD-1 is expressed on Kupffer cells in the liver, other monocyte-derived cells, as well as epithelial, endothelial, and tumor cells[52,58,59].

Although HCV-specific $CD8^+$ T-cells are generally dysfunctional in HCV persistence, their level of impairment varied considerably among patients depending on PD-1 expression. Within an individual patient, the function and PD-1 expression of HCV-specific $CD8^+$ T-cells varied between the liver and peripheral blood[60]. Additional studies on the expression patterns of diverse splice variants of PD-1, PD-L1, and receptor-ligand interactions in diseased tissue will be important in determining a more comprehensive estimation of the level of the inhibitory signal and its effect on the outcome of human infection. Such studies will not only provide a superior mechanistic understanding of the PD-1 pathway in controlling T cell responses but will also encourage specific manipulation of this pathway therapeutically.

CORRELATION OF PD-1 EXPRESSION AND CLINICAL RESPONSES

The identification of cellular and molecular factors predicting clinical response to immunotherapy is strongly desirable, not only to aid in the design of therapies that overcome and enhance the inhibitory and stimulatory mechanisms, but also to preselect patients most likely to benefit from therapy and spare others from unnecessary exposure to possible side effects. Similar to its inhibitory role in anti-cancer immunity, the PD-1 signaling pathway has also been found to shape the overall immunity in HCV infection. For instance, PD-1 expression in acute HCV infection was found to be a signature of functional HCV-specific CD8 T cell exhaustion[16]. In this study, and as reported for the acute infection of HBV[61], the expression of PD-1 by HCV specific cytotoxic T cells was decreased in self-limited infections after the acute stage of infection in conjunction with $CD8^+$ T cell differentiation towards a memory CD127 phenotype[61]. In contrast, HCV specific $CD8^+$ T cells maintained high levels of PD-1 in patients with chronic advancement of infection and remained functionally impeded with no change from an effector to a memory phenotype[16]. Other studies, however, reported high levels of PD-1 expression (60%-100%) on all HCV specific $CD8^+$ and $CD4^+$ cells during the early stage of acute infection, irrespective of the clinical outcome or viral load[17]. Taken together, these results suggested that a role for the PD-1/PD-L1 interaction in regulating $CD8^+$ T cell function may exist under conditions of continuous high levels of HCV antigen stimulation.

Consistent with this suggestion, another study showed that the level of PD-1 expression in early phases of HCV infection was significantly more on HCV-specific T cells from patients who advanced to chronic HCV infection than from those who eliminated infection; and this correlation was independent of HCV RNA titer levels[62]. The reason for this difference is unclear, but it may be due to differences between the routes of infection between the two studies. This suggests that some of the biological differences that cause the development of symptoms likewise affect PD-1 expression. Additionally, the duration of infection in patients defined as acutely infected could be different between studies. Table 1 provides a list of studies involving PD-1 expression and the role of PD-1 in HCV infection.

PD-1 expression was investigated in 72 treatment-naïve patients with persistent HCV[57]. In this study, PD-1 expression was upregulated significantly not only on $CD4^+$ and $CD8^+$ T cells but also on NK cells, connecting with failed early and persistent virologic response to therapy. In contrast, patients with SVR demonstrated decreases in PD-1 after therapy completion, demonstrating that PD-1 expressed by NK cells is critical in persistent HCV[57].

$PD\text{-}1^+$ HCV-specific $CD8^+$ T cells in chronic HCV infection have a tendency to co-express Tim-3[63], 2B4, CD160 and other inhibitory molecules[64], particularly in the liver[65] since intrahepatic T cells showed a more exhausted phenotype than in the blood. In addition, the level of TIM-3 from patients with persistent HCV infection was greater than those who resolved the infection.

The results from other studies, however, are inconsistent regarding the differences in levels of PD-1/PD-L1 on HCV-specific $CD8^+$ cells contrasts between those who clear HCV infection and those with persistent infection[16,17,62]. Most of these studies though concluded that PD-1 expression levels are elevated on HCV-specific T cells *vs* naïve $CD8^+$ T cells or on T cells specific for some control antigens in the acute stage of infection, regardless of the outcome.

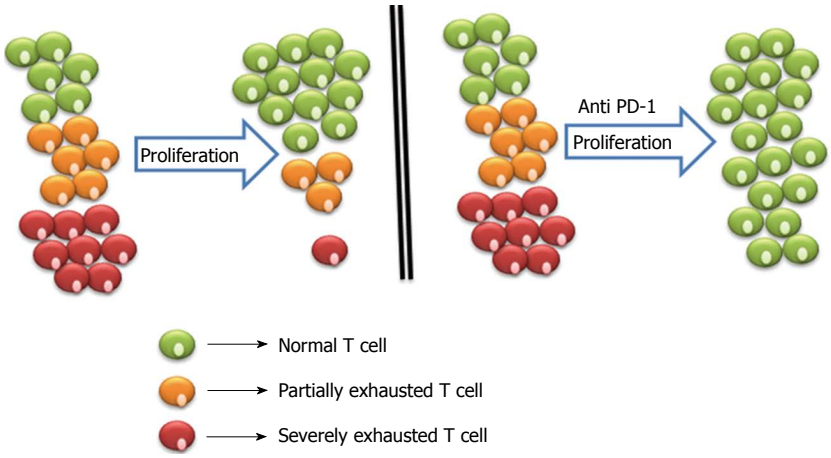

Figure 2 Proliferation of exhausted T cells and blockage strategies to reverse exhaustion. Severely exhausted T cells (red cells) proliferate poorly in comparison to partially exhausted and normal T cells (yellow and green cells). Antibody blockade of the pathway with PD-1 and its ligand reverses exhaustion and restores the functional capacities of exhausted T cells. PD-1: Programmed death-1.

RESTORATION OF ANTI-HCV RESPONSES *IN VITRO* BY BLOCKING PD-1

During the acute phase of HCV infection, HCV specific T cells have been characterized as poorly functional, regardless of the outcome of the disease[47-51]. A possible explanation for this behavior of the HCV-specific $CD8^+$ T response is exhaustion, which is supported by the initial rapid kinetics of HCV spread, replication after infection, and later on, by the continuous exposure of $CD8^+$ T cells to high antigen load. Several studies demonstrated that the PD-1/PD-L1 pathway plays a role in T-cell exhaustion when $CD8^+$ cells are chronically exposed to high level antigen loads. Blockade of this pathway can permit restoration of exhausted $CD8^+$ T cells[12,52-55] and results in expansion of HCV specific T cell proliferation[15,16,66] (Figure 2).

For instance, PD-L1 blockade improved HCV-specific T cell proliferation in a dose-dependent manner. However, proliferation of cytomegalovirus (CMV)-specific $CD8^+$ T cells was not affected by PD-1/PD-L1 blockade, consistent with their low expression levels of PD-1. Similar to its effects on $CD8^+$ T cells in HCV, blocking the PD-1/PD-L1 interaction *in vitro* restored effector function and enhanced the proliferative ability of exhausted $CD8^+$ T cells in many chronic infections, including HIV, HBV, simian immunodeficiency virus (SIV), LCMV, and Epstein-Barr virus (EBV)[13,15,67-71].

This functional T cell restoration by blocking the PD-1/PD-L1 pathway is a hierarchical phenomenon that appears to reflect the different sensitivities to exhaustion of the diverse T cell functions[72]. Restoration of proliferation capacity is relatively faster than the restoration of IFN-γ and IL-2 production but with no effect on cytotoxicity. In line with this, treatment with anti-PD-L1 antibodies does not always predict the expected positive effect of PD-1 blockade[73], suggesting that PD-L1 binds at least one additional receptor other than PD-1 to mediate its costimulatory function. Moreover, exhaustion was more effectively overcomed in HCV, than in HIV[68] by PD-1/PD-L1 blockade, as demonstrated by the increased capability of HCV-specific $CD8^+$ T cells to expand and to produce IFN and IL-2 after incubation with anti PD-L1 antibodies[15].

Collectively, these *in vitro* data suggest that PD-1 signaling on T cells is a significant inhibitory pathway during chronic HCV infection. Consequently, the possibility of partially restoring $CD8^+$ T cell function by blocking PD-1/PD-L1 interaction may provide a valuable tool for the enhancement of available therapies to cure chronic hepatitis C.

POTENTIAL USE OF *IN VIVO* PD-1 BLOCKING FOR ANTI-HCV THERAPY

As demonstrated above, *in vitro* blockade of PD-1 can restore the functionality of HCV-specific T cells. Blockade of PD-1 signaling was tested *in vivo* in both chimpanzees[74] and in patients with chronic HCV infection[75]. In the chimpanzee study, an increase in HCV specific $CD8^+$ cell responses and a considerable, although transient, reduction in HCV viremia was only seen in one of three chimpanzees. This chimpanzee had the strongest and broadest $CD4^+$ and $CD8^+$ T cell response before the development of chronic infection, which suggested that PD-1 blockade alone is not sufficient to attain viral clearance[74]. In the patient study, a single dose (10 mg/kg) of the PD-1 blocking antibody BMS-936558 was followed by a greater than 0.5 log10 IU/mL decrease in HCV RNA titer in five of 45 (11%) patients. At the highest dose given (10 mg/kg), a > 4 log10 IU/mL decrease in HCV RNA titer was seen in three of 20 (15%) patients. This decrease of HCV replication continued for more than 8 wk in most patients[75].

Interestingly, *in vivo* PD-L1 blockade does not appear to affect IL-10 level during chronic infection, although

it downregulated IL-10 and upregulated IL-2 and IFN-γ during *in vitro* stimulation[76,77]. Another *ex vivo* study showed that intrahepatic T cells were significantly dysfunctional and insusceptible to PD-1 blockade[60]. Therefore, the effect of PD-1 blockade is characterized by T cell compartmentalization. Additionally, PD-1 expression on HCV specific $CD8^+$ cells is affected by viral immune-evasion in chronic HCV infections[62].

Indeed, an efficient helper T cell function is compulsory for the development of virus specific CD8 cells[78]. Thus, the synergistic effect of CD8- and CD4-mediated T cell functions enhanced by anti-PD-L1 may represent a strategy to enhance the effect of available anti-HCV drugs. Consequently, it is conceivable that utilizing PD-1/PD-L1 blockade can enhance the antiviral effect of IFN/RBV therapy. Additional studies are required, however, to survey whether the enhancement of the T cell function induced by PD-1/PD-L1 blockade and favored by IFN-α therapy in patients with a recent HCV infection can be also accomplished in chronic infections of longer duration, where the effect of long lasting exhaustion may be more difficult to succeed. Therefore, the potential of partially restoring $CD8^+$ cells function by blocking PD-1/PD-L1 interaction could provide an additional tool to enhance available therapies to cure chronic HCV.

CONCLUSION

In spite of much progress in our understanding of T cell responses against HCV over the past decade, many critical research questions remain to be answered. It will be important to determine whether there is a causal correlation between the outcome of HCV infection and PD-1 expression. Functionally deficient exhausted HCV specific T cells are a significant cause and outcome of chronic viral infection. This is a great challenge to vaccine designs that either aim to eliminate or prevent such infections by interceding the T cell response. Whether developing a therapeutic vaccine to eliminate disease in infected patients or a prophylactic vaccine to prevent HCV in healthy individuals, it is imperative to keep this considerable obstacle in mind.

The possibility of restoring the function of HCV specific T cells by blocking the PD-1/PDL-1 pathway and reverting T-cell dysfunction in chronic HCV seems a worthy direction for anti-HCV immunotherapy. While the therapeutic application of this strategy in HCV infection is constrained by the recent, ongoing development of highly efficacious new treatments, it is promising that further investigation of PD-1 pathway blockade during antiviral therapies is warranted.

However, the systemic administration of PD-L1/PD-1 blocking antibodies carries the high risk of violating peripheral tolerance. Most tissues depend on PD-L1 expression to decrease T-cell effector activities that might cause autoimmune attack. To demonstrate this, knocking out PD-1 or PD-L1 pathways in mouse models causes severe, deadly autoimmunity[79,80]. In humans, single-nucleotide polymorphisms (SNP) of the *PDCD1* gene (encoding PD-1) are connected with systemic lupus erythematosus[81]. Therefore, efforts have to be made to enhance this promising strategy to maximize anti-HCV therapeutic activities while minimizing toxicity. We propose that one possible attractive alternative to the systemic blockade of PD-1 for HCV immunotherapy is to target the suppression of PD-1/PD-L1 co-stimulation during antigen presentation. This was demonstrated in mouse antigen presenting cells (APCs)[82,83]. This local and transient blockade may provide the positive effects needed to adequately boost anti-HCV immunity while restricting possible side effects to a minimum.

Overall, continuing work to understand better how the PD-1/PD-L1 pathway functions is imperative and will encourage development of new vaccination approaches that can overcome HCV specific T cell exhaustion.

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P- Reviewer: Jin B **S- Editor**: Ma YJ
L- Editor: A **E- Editor**: Liu SQ

World Journal of Hepatology

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DOI: 10.4254/wjh.v7.i23.2459

World J Hepatol 2015 October 18; 7(23): 2459-2469
ISSN 1948-5182 (online)

REVIEW

Contribution of the toxic advanced glycation end-products-receptor axis in nonalcoholic steatohepatitis-related hepatocellular carcinoma

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Author contributions: All authors contributed to this study.

Supported by The Japan Society for the Promotion of Science (JSPS) KAKENHI, Grant No. 22300264 and No. 25282029 (to Takeuchi M); the Ministry of Education, Culture, Sports, Science; Technology (MEXT), Regional Innovation Strategy Support Program (to Takeuchi M); and Kanazawa Medical University, No. SR2012-04.

Conflict-of-interest statement: The authors declare no conflict of interest associated with this manuscript.

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Received: March 18, 2015
Peer-review started: March 18, 2015
First decision: April 23, 2015
Revised: May 7, 2015
Accepted: August 29, 2015
Article in press: September 7, 2015
Published online: October 18, 2015

Abstract

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. The main etiologies of HCC are hepatitis B virus and hepatitis C virus (HCV), and non-hepatitis B/non-hepatitis C HCC (NBNC-HCC) has also been identified as an etiological factor. Although the incidence of HCV-related HCC in Japan has decreased slightly in recent years, that of NBNC-HCC has increased. The onset mechanism of NBNC-HCC, which has various etiologies, remains unclear; however, nonalcoholic steatohepatitis (NASH), a severe form of nonalcoholic fatty liver disease, is known to be an important risk factor for NBNC-HCC. Among the different advanced glycation end-products (AGEs) formed by the Maillard reaction, glyceraldehyde-derived AGEs, the predominant components of toxic AGEs (TAGE), have been associated with NASH and NBNC-HCC, including NASH-related HCC. Furthermore, the expression of the receptor for AGEs (RAGE) has been correlated with the malignant progression of HCC. Therefore, TAGE induce oxidative stress by binding with RAGE may, in turn, lead to adverse effects, such as fibrosis and malignant transformation, in hepatic stellate cells and tumor cells during NASH or NASH-related HCC progression. The aim of this review was to examine the contribution of the TAGE-RAGE axis in NASH-related HCC.

Key words: Hepatocellular carcinoma; Nonalcoholic steatohepatitis; Advanced glycation end-products; Toxic advanced glycation end-products; Receptor for advanced glycation end-products; Hepatic stellate cells

Core tip: Expression of the receptor for advanced glycation end-products (RAGE), which is a multi-ligand cell surface receptor, is correlated with the poor therapeutic outcomes and malignancy of hepatocellular

carcinoma (HCC). The synthesis of toxic advanced glycation end-products (TAGE), ligands of RAGE, is increased in nonalcoholic steatohepatitis (NASH) as well as in NASH-related HCC. Interactions between TAGE and RAGE induce oxidative stress, which may, in turn, lead to adverse effects in tumor cells and hepatic stellate cells during NASH or NASH-related HCC progression. Therefore, these findings prompted us to suggest that the TAGE-RAGE axis may be a treatment target in NASH-related HCC.

Takino J, Nagamine K, Hori T, Sakasai-Sakai A, Takeuchi M. Contribution of the toxic advanced glycation end-products-receptor axis in nonalcoholic steatohepatitis-related hepatocellular carcinoma. *World J Hepatol* 2015; 7(23): 2459-2469 Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i23/2459.htm DOI: http://dx.doi.org/10.4254/wjh.v7.i23.2459

INTRODUCTION

Hepatocellular carcinoma (HCC), which accounts for approximately 90% of all primary liver cancers, is one of the most common malignancies in men and women, and is the third leading cause of cancer-related mortality worldwide[1-3]. Hepatitis B virus (HBV) and hepatitis C virus (HCV) are known to be the main risk factors for HCC, accounting for over 75% of HCC worldwide[4]. The incidence of HCC is particularly high in Asia[3,4], and HBV infection is endemic in eastern/south-eastern Asia, while HCV infection is prevalent in Japan[5-7]. Among diagnosed HCC patients in Japan in 2006-2009, 84.1% had virus-related HCC (HCV: 66.3%, HBV: 14.1%, HCV + HBV: 3.7%) and 15.9% had non-hepatitis B/non-hepatitis C HCC (NBNC-HCC) [alcoholic: 7.2%, etiology unknown: 5.1%, nonalcoholic fatty liver disease (NAFLD): 2.0%, Others: 1.6%][7,8]. Although the incidence of HCV-related HCC has decreased slightly in recent years, that of NBNC-HCC has increased[9,10]. Nonalcoholic steatohepatitis (NASH), a severe form of NAFLD, has been identified as an important risk factor among the etiological factors of NBNC-HCC. The incidence of NASH-related HCC is expected to increase in the future as the number of patients with NAFLD is increasing worldwide.

Advanced glycation end-products (AGEs) formed by the Maillard reaction, a nonenzymatic reaction between the ketone or aldehyde groups of sugars and the amino groups of proteins, have been implicated in aging and diabetes-related pathological complications[11,12]. This reaction begins with the conversion of reversible Schiff base adducts to more stable covalently bound Amadori rearrangement products. Over the course of days to weeks, these Amadori products undergo further rearrangement reactions to form irreversibly bound moieties known as AGEs[13]. Recent studies have suggested that AGEs are formed not only from sugars, but also from carbonyl compounds produced as a result of the autoxidation of sugars and from other metabolic pathways[14,15]. There is evidence to suggest that glyceraldehyde-derived AGEs (Glycer-AGEs), the predominant components of toxic AGEs (TAGE), are closely associated with insulin resistance, obesity, hypertension, diabetes complications, cardiovascular diseases, dementia, NASH, and cancer[16-24]. We recently demonstrated that TAGE were present at significantly higher concentrations in the sera of patients with NASH than in those with simple steatosis or healthy controls, and that TAGE accumulated in the livers of patients with NASH[25]. Extracellular TAGE induce oxidative stress by binding with the receptor for AGEs (RAGE), which, in turn, causes adverse effects in various types of cells[26-31]. The TAGE-RAGE axis has been shown to increase the malignancy of various types of cancer cells[18,32-35].

These findings suggest that TAGE play an important role in the development and progression of NASH and NASH-related HCC. In this review, we discuss the contribution of the TAGE-RAGE axis in NASH-related HCC.

BACKGROUND OF NASH AND NBNC-HCC

NAFLD, which is the most common liver disease worldwide, is a disease that ranges from simple steatosis to NASH[36-41]. Approximately 20%-30% of the population has evidence of fatty liver disease attributed to NAFLD, and approximately 10% of patients with NAFLD progress to NASH[42]. NASH, which is a disease that has the typical histopathological findings of alcoholic liver disease in patients without a history of significant alcohol abuse, is recognized as a component of the metabolic syndrome and has been closely associated with insulin resistance as well as glucose and lipid metabolic disorders[43-46]. Although simple steatosis appears to be a benign and non-progressive condition, NASH is a potentially progressive disease that can lead to fibrosis, cirrhosis, and HCC[47,48]. Approximately 8%-26% of patients with NASH progress to cirrhosis, and approximately 10% of patients with cirrhotic NASH transform to HCC after 5 years[47,49]. Several case series have recently been published on NASH-related HCC[50,51]. Furthermore, NASH was shown to increase the risk of HCC without the development of cirrhosis[52]. While NASH is a risk factor for HCC, the cirrhosis caused by NASH is also considered to be an important risk factor.

The etiology of NBNC-HCC is often cryptogenic cirrhosis (CC). Most cases of CC are considered to be end-stage NASH because the prevalence of obesity and diabetes among patients with CC is similar to that of patients with NASH[53]. In addition, patients who undergo orthotopic liver transplantation for CC often develop NAFLD and NASH after transplant[54]. However, the histopathological features of NASH often disappear when cirrhosis is established[55]. Marrero *et al*[56] reported that HCV (51%) and CC (29%) were the first and second most common etiologies among 105

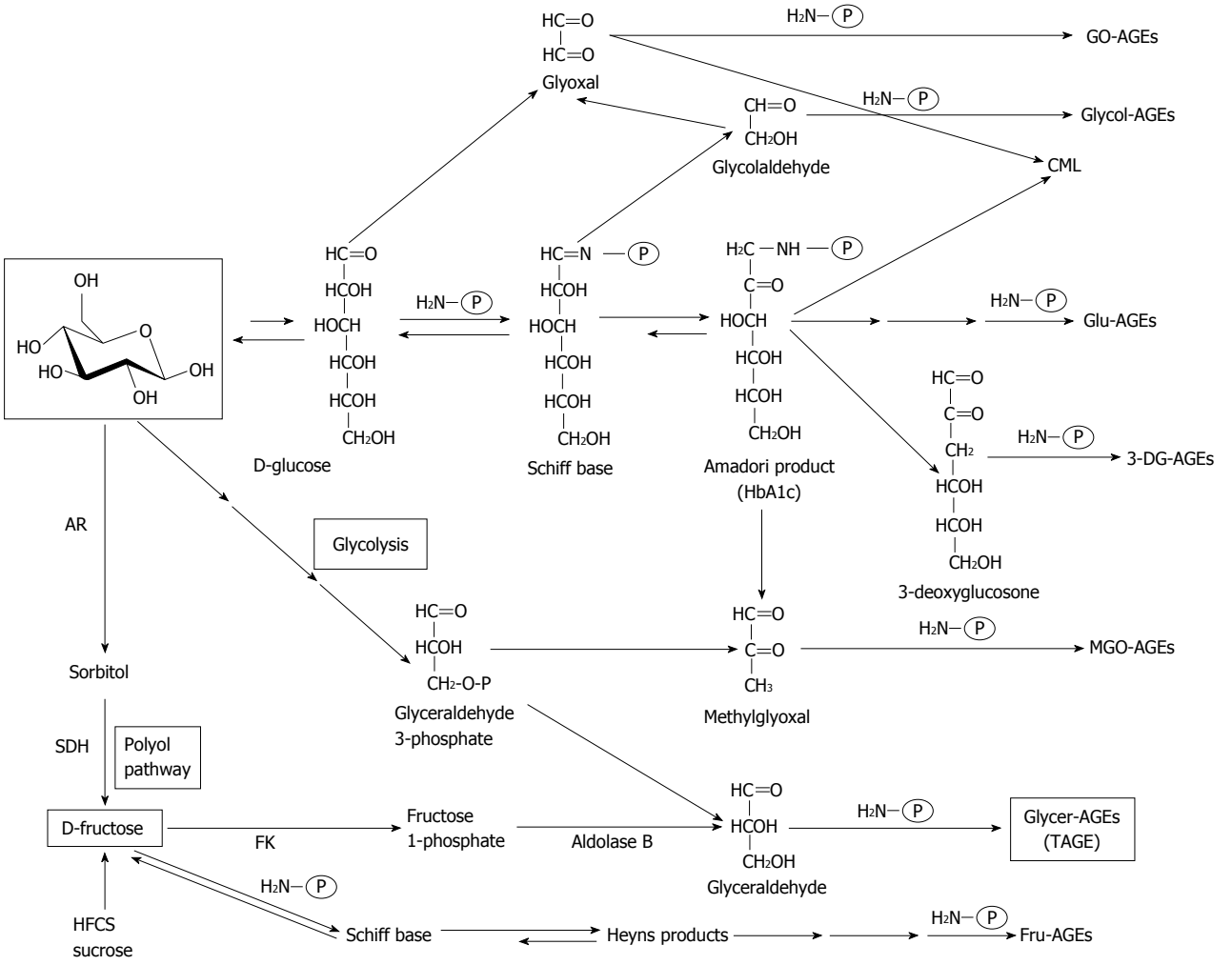

Figure 1 Alternative routes for the formation of advanced glycation end-products *in vivo*. Reducing sugars, such as glucose, fructose, and glyceraldehyde are known to react non-enzymatically with the amino groups of proteins to form reversible Schiff bases and Amadori product/Heyns products. These early glycation products undergo further complex reactions such as rearrangement, dehydration, and condensation to become irreversibly cross-linked, heterogeneous fluorescent derivatives, termed advanced glycation end-products (AGEs). Glu-AGEs: Glucose-derived AGEs; Fru-AGEs: Fructose-derived AGEs; Glycer-AGEs: Glyceraldehyde-derived AGEs; Glycol-AGEs: Glycolaldehyde-derived AGEs; MGO-AGEs: Methylglyoxal-derived AGEs; GO-AGEs: Glyoxal-derived AGEs; 3-DG-AGEs: 3-deoxyglucosone-derived AGEs; CML: N_{ε}-(carboxymethyl)lysine; P-NH_2: Free amino residue of a protein; AR: Aldose reductase; SDH: Sorbitol dehydrogenase; FK: Fructokinase; HFCS: High-fructose corn syrup; HbA1c: Hemoglobin A1c; TAGE: Toxic advanced glycation end-products.

patients with HCC in the United States, respectively, that 50% of patients with CC had a prior histological diagnosis of NASH or clinical features associated with NAFLD, and that NAFLD-related CC accounted for 13% of patients with HCC. These findings suggested the existence of NASH-related HCC.

ALTERNATIVE ROUTES FOR THE FORMATION OF AGES *IN VIVO*

The formation of AGEs, which occurs through a non-enzymatic glycation reaction, is known to result from not only glucose, but also the actions of various metabolites that are primarily located intracellularly[13,15,57].

We previously reported the contribution of fructose, α-hydroxyaldehydes (glyceraldehyde and glycolaldehyde), and dicarbonyl compounds (methylglyoxal, glyoxal, and 3-deoxyglucosone) as well as glucose in the glycation of proteins. Seven immunochemically distinct classes of AGEs (Glu-AGEs, glucose-derived AGEs; Fru-AGEs, fructose-derived AGEs; Glycer-AGEs, glyceraldehyde-derived AGEs; Glycol-AGEs, glycolaldehyde-derived AGEs; MGO-AGEs, methylglyoxal-derived AGEs; GO-AGEs, glyoxal-derived AGEs; and 3-DG-AGEs, 3-deoxyglucosone-derived AGEs) were detected in the sera of type 2 diabetic subjects undergoing hemodialysis[13,58-61]. These findings suggested that all seven forms of AGEs were synthesized *in vivo* (Figure 1).

PATHWAY FOR THE *IN VIVO* FORMATION OF TAGE

Glyceraldehyde, a precursor of TAGE, is produced by two pathways (the glycolytic pathway and fructose metabolic pathway)[17,21,22,24,62]. In the glycolytic pathway (glycolysis), the intermediate glyceraldehyde-3-phosphate (G-3-P) is metabolized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH), G-3-P accu-

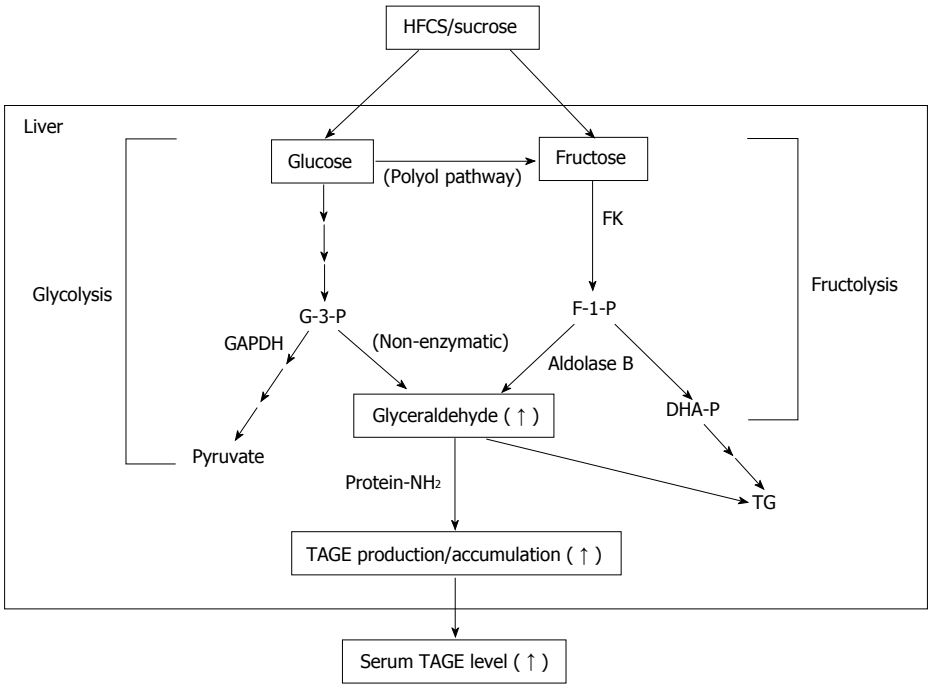

Figure 2 ***In vivo*** **production routes of glycer-advanced glycation end-products (toxic advanced glycation end-products).** The chronic and excessive ingestion of sugar-sweetened beverages (HFCS/sucrose) increases the levels of the sugar metabolite, glyceraldehyde in the liver. The glycolytic intermediate glyceraldehyde-3-phosphate (G-3-P) is normally catabolized (glycolysis) by the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). G-3-P accumulates intracellularly with a decline in GAPDH activity. The metabolism of G-3-P then shifts to another route, resulting in an increase in the amount of glyceraldehyde, which promotes the formation of glycer-advanced glycation end-products (AGEs) (TAGE). Fructose from the daily diet and polyol pathway is phosphorylated to fructose-1-phosphate (F-1-P) by fructokinase and is then catabolized to glyceraldehyde and dihydroxyacetone phosphate by aldolase B (fructolysis). The newly synthesized glyceraldehyde is then transported or leaks passively across the plasma membrane. Glyceraldehyde promotes the formation of TAGE both intracellularly and extracellularly. DHA-P: Dihydroxyacetone-phosphate; FK: Fructokinase; HFCS: High-fructose corn syrup; TAGE: Toxic advanced glycation end-products; TG: Triglyceride; Protein-NH2: Free amino residue of a protein.

mulates intracellularly due to a decrease in GAPDH enzyme activity. Accumulated G-3-P then shifts to another metabolic route, and the amount of glyceraldehyde is increased. In the fructose metabolic pathway (fructolysis), fructose is mainly metabolized in the liver. Fructose is phosphorylated to fructose-1-phosphate (F-1-P) by a fructokinase, and liver aldolase B cleaves F-1-P to produce dihydroxyacetone phosphate and glyceraldehyde. The accumulated glyceraldehyde due to a metabolic disorder is then transported or leaks passively across the plasma membrane, thereby promoting the intracellular and extracellular formation of TAGE (Figure 2).

SERUM TAGE LEVELS IN NASH AND NBNC-HCC

AGEs were originally characterized by their ability to form cross-links with and between amino groups and have a yellow brown fluorescent color. However, this term is now used for a broad range of advanced products of the glycation process, including Nε-(carboxymethyl)lysine (CML), Nε-(carboxyethyl)lysine, and pyrraline, which are not cross-linked proteins and do not have color or fluorescence[13,63-67]. CML was previously shown to be formed from precursors such as glycolaldehyde and glyoxal *via* the intra-molecular Cannizzaro reaction, a process that is largely independent of glucose autoxidation[14]. CML may also be formed independently of the presence of fructose-lysine during the metal-catalyzed oxidation of low-density lipoproteins and peroxidation of polyunsaturated fatty acids[68]. A recently supported concept is that CML is a marker of oxidation rather than glycation.

Sebeková *et al*[69] initially suggested that the catabolism and clearance of circulating CML was impaired by various liver diseases. In their study, plasma CML levels were measured in 51 patients with liver cirrhosis (five of whom were followed for 36 mo after liver transplantation) and 19 healthy controls. The main findings obtained were that: (1) plasma CML levels were markedly elevated in patients with liver cirrhosis and positively correlated with severity of the disease; (2) plasma CML levels were inversely associated with residual liver function in patients, as estimated by serum albumin and plasma bilirubin levels; and (3) plasma CML levels were markedly decreased (to approximately 50% of those before treatment) within 3 mo of liver transplantation. These findings suggested that the liver may play an important role in the removal of circulating CML and that the hepatic clearance of circulating CML may be impaired due to liver cirrhosis. Yagmur *et al*[70] also reported that serum CML levels were significantly higher in patients with liver cirrhosis than in patients without cirrhosis, and were positively associated with the severity of cirrhosis defined by the Child-Pugh score.

These findings suggested that circulating CML levels may be a useful biomarker for evaluating residual liver function. However, Moy *et al*[71] recently reported that serum CML levels were inversely correlated with the risk of HCC. They measured serum CML levels in 145 patients with HCC and 340 control patients, who were male Finnish smokers, and found that high serum CML levels correlated with a lower risk of HCC. Furthermore, this relationship did not change in the case of NBNC-HCC. Therefore, the relationship between circulating CML levels and liver disease needs to be examined in more detail in future studies.

Our clinical data indicated that TAGE played a role in the etiology of NASH and that serum TAGE levels were a useful clinical tool for discriminating between NASH-related HCC, NASH, and simple steatosis[25,72,73]. We measured serum AGE levels (CML, Glu-AGEs, and TAGE) in 66 NASH patients without cirrhosis, 10 patients with simple steatosis, and 30 control patients. We found that serum TAGE levels were significantly higher in NASH patients than in those with simple steatosis and the controls; however, no significant difference was observed in CML and Glu-AGE levels between the groups[25]. We measured serum TAGE levels in 43 NASH patients with dyslipidemia in order to determine whether they played a role in the treatment of NASH. Serum TAGE levels were measured and clinical laboratory tests were performed periodically during the administration of atorvastatin (10 mg daily), a hydroxymethylglutaryl-CoA reductase inhibitor, for 12 mo. This treatment significantly decreased serum TAGE levels, and significantly improved biochemical and histological findings[72]. We also measured serum TAGE levels in 90 patients with NBNC-HCC, 56 NASH patients without HCC, and 27 control patients, and found that serum TAGE levels were significantly higher in NBNC-HCC patients than in those with NASH without HCC and controls. Among the patients with NBNC-HCC, 10 had NASH-related HCC, 49 alcoholic-related HCC, and 31 etiology unknown HCC, and no significant differences were observed in serum TAGE levels between these groups[73]. These findings suggested that the formation of TAGE, but not CML, was enhanced by the development and progression of NASH, and that enhanced TAGE may influence the development and progression of NBNC-HCC. Brenner *et al*[74] previously reported that increases in AGE-RAGE-mediated inflammation in patients with end-stage liver diseases including HCC following liver transplantation were dependent on reactive carbonyl species-derived AGEs, but not CML. Therefore, TAGE may play a more important role in the development and progression of NASH-related HCC than CML[24,75].

RAGE EXPRESSION IN HCC

RAGE, a multi-ligand cell surface receptor, interacts with distinct molecules that have been implicated in homeostasis, development, and inflammation. RAGE binding by ligands such as AGEs, high mobility group box 1, and S100/calgranulins has been shown to trigger the activation of key cell signaling pathways, thereby reprogramming cellular properties[76]. Previous studies suggested that the expression of RAGE was associated with the malignant progression of cancer[77-80].

In the liver, RAGE is expressed in hepatocytes and hepatic stellate cells (HSCs)[81,82]. Liver damage caused by various factors such as inflammation, drugs, and hepatic ischemia/reperfusion (I/R) is known to increase the expression of RAGE, which induces further liver failure[83-89]. For example, Kuhla *et al*[89] reported that exposure to galactosamine/lipopolysaccharides induced RAGE expression, leading to inflammation, and a pre- or post-treatment with an anti-RAGE antibody attenuated enhanced inflammation, apoptosis, and necrosis. Therefore, RAGE is crucially involved in the exacerbation of liver disease and may also play a role in HCC, which occurs following liver failure. A previous study reported that the expression of RAGE mRNA was higher in the liver cells of hepatitis and HCC patients than in normal liver cells[81], and RAGE expression correlated with the poor therapeutic outcomes and malignancy of HCC[90,91]. Ito *et al*[90] investigated the relationship between RAGE expression and clinical outcomes in 65 patients who underwent initial hepatectomy for HCC. The number of patients that expressed RAGE was significantly higher among those with well and poorly differentiated HCC than those with moderately differentiated HCC, and the 5-year survival rate was significantly lower in the RAGE-positive group than in the RAGE-negative group. Yang *et al*[91] investigated the relationship between RAGE expression and clinicopathological features in 75 patients with HCC. HCC tissues expressed significantly higher levels of RAGE than non-cancerous tissues, and the expression of RAGE was closely associated with pathological staging and lymph-vascular space invasion. Furthermore, recent studies suggested that RAGE expression increased under hypoxic conditions in HCC cell lines[79,81]. The survival of RAGE-transfected cells was significantly prolonged under hypoxic conditions, and an anti-RAGE siRNA treatment eliminated this influence[81]. Therefore, inhibitors of RAGE expression may be effective as new HCC therapeutic drugs. Koh *et al*[88] reported that losartan, a peroxisome proliferator-activated receptor-γ (PPAR-γ) activator, attenuated the enhanced expression of RAGE in I/R and attenuated liver damage-inducing factors such as aspartate or alanine aminotransferase, tumor necrosis factor-α, and interleukin-6. Yang *et al*[91] demonstrated that pioglitazone, a PPAR-γ agonist, decreased the expression of RAGE in HCC cell lines, suppressed cell proliferation and cell invasion, and induced apoptosis and cell cycle arrest. We also previously reported that telmisartan may down-regulate the expression of RAGE through its PPAR-γ-modulating activity in the human HCC cell line, Hep3B. Telmisartan, but not candesartan, decreased RAGE mRNA and protein expression levels, and GW9662, an inhibitor of PPAR-γ, blocked the inhibitory effects of telmisartan on RAGE mRNA and protein expression.

Troglitazone and ciglitazone, which are full agonists of PPAR-γ, mimicked the effects of telmisartan[92]. Therefore, PPAR-γ activators may become important targets as inhibitors of RAGE expression in treatment strategies for HCC.

Full-length RAGE, which is generally referred to as RAGE, induces adverse effects, whereas the soluble form of RAGE (sRAGE) attenuates these effects. sRAGE, a circulating isoform of RAGE, has been detected in plasma and consists of an endogenous secretory RAGE (esRAGE), which is a splice variant, as well as a proteolytically cleaved isoform of cell surface RAGE. sRAGE, including esRAGE, is known to act as a decoy receptor of RAGE by binding with AGEs and other ligands competitively[93,94]. Zeng *et al*[83] reported that the survival of mice treated with sRAGE after hepatic I/R injury was better than that of mice treated with PBS, and that the blockade of RAGE signaling by sRAGE attenuated hepatic I/R injury. In order to elucidate the relationship between serum sRAGE levels and NASH, Yilmaz *et al*[95] measured serum sRAGE levels in 48 patients with NASH (definite NASH, n = 40, and borderline NASH, n = 8) and 14 control patients, and found that serum sRAGE levels were significantly lower in patients with NASH than in controls. Furthermore, regarding the relationship between serum sRAGE levels and HCC, Moy *et al*[71] reported that serum sRAGE levels inversely correlated with the risk of HCC or NBNC-HCC, in addition to serum CML levels, as discussed above. Kohles *et al*[96] also recently demonstrated that serum sRAGE levels were significantly lower in patients with progressive HCC than in patients without progressive HCC. On the other hand, we, along with others, recently found that serum sRAGE levels were positively, rather than inversely, associated with serum TAGE levels in non-diabetic and diabetic subjects[97,98]. These findings suggested that since TAGE up-regulates the expression of RAGE, increases in sRAGE levels may reflect the expression of full-length RAGE[99]. However, the relationship between sRAGE and TAGE in NASH or NASH-related HCC has not yet been elucidated in detail; therefore, further analyses will be necessary in the future.

THE TAGE-RAGE AXIS IN HCC AND HSCS

We previously described the effects of the TAGE-RAGE axis in HCC[100]. TAGE induced the expression of C-reactive protein (CRP), an inflammatory marker, *via* the activation of Rac-1 in Hep3B, and its induction was attenuated by a pretreatment with anti-RAGE anti-serum. Signal transducer and activator of transcription 3 - and nuclear factor-kappa B-dependent pathways and a reactive oxygen species (ROS)-dependent pathway exist in this signaling pathway, and have been suggested to participate with each other in the early and late stages of CRP induction[100]. A previous study reported a relationship between increases in the expression of CRP and the malignancy of HCC. Kinoshita *et al*[101] analyzed the relationship between serum CRP levels and poor prognoses in 186 patients with HCC, and demonstrated that serum CRP levels correlated with a poor prognosis in HCC patients. Furthermore, Kim *et al*[102] examined serum CRP levels in 83 HCC patients with malignant portal vein invasion and 1056 HCC patients without portal vein invasion who underwent liver resection. They found that CRP levels were significantly higher in HCC patients with malignant portal vein invasion than in HCC patients without portal vein invasion, and that CRP levels correlated with the risk of tumor recurrence in HCC patients with malignant portal vein invasion. TAGE significantly enhanced cell proliferation in the human HCC cell line HuH7, which expressed RAGE on the cell surface, but not in the human HCC cell line HepG2, which does not express RAGE. Flow cytometry with anti-RAGE antibody staining demonstrated the expression of membrane-bound RAGE in both HuH7 and HepG2 cells at 24.3% and 6.2%, respectively. Furthermore, MK615, an extract of the Japanese apricot, was shown to suppress TAGE-induced cell proliferation by decreasing the expression of RAGE on the cell surface[34]. The expression of vascular endothelial growth factor mRNA and protein was significantly greater in Hep3B cells treated with TAGE than with the control non-glycated bovine serum albumin (BSA). Furthermore, the proliferation and migration of as well as tube formation by human umbilical vein endothelial cells was significantly greater with the conditioned medium of TAGE-treated Hep3B cells than with the conditioned medium of control non-glycated BSA-treated Hep3B cells. On the other hand, TAGE did not influence HepG2 cells[34]. This may have been due to differences in the expression of RAGE on cell surfaces. Glu-AGEs were also found to have no influence on Hep3B or HepG2 cells because they are known to have lower binding affinity with RAGE than TAGE. The findings suggested that the TAGE-RAGE axis played an important role in the malignant transformation of HCC (Figure 3).

We previously reported the effects of the TAGE-RAGE axis in HSCs. TAGE induced the expression of transforming growth factor-β1 and collagen type Iα2, which are fibrogenic factors, as well as that of monocyte chemoattractant protein-1, an inflammatory factor, *via* the generation of NADPH oxidase-derived ROS in the human stellate cell line LI90[103]. The activation of HSCs, which mainly produce the extracellular matrix, has been shown to play a pivotal role in liver fibrogenesis[104], and promotes the onset and progression of HCC[105,106]. Amann *et al*[107] found that activated HSCs increased the malignancy of HCC. The main findings of their study were: (1) the conditioned medium of activated HSCs significantly increased the proliferation and migration of human HCC cell lines (HepG2, Hep3B, and PLC); (2) activated HSCs significantly increased the volumes of spheroids formed in the three-dimensional coculture of HSCs and HCC, and these spheroids showed smaller

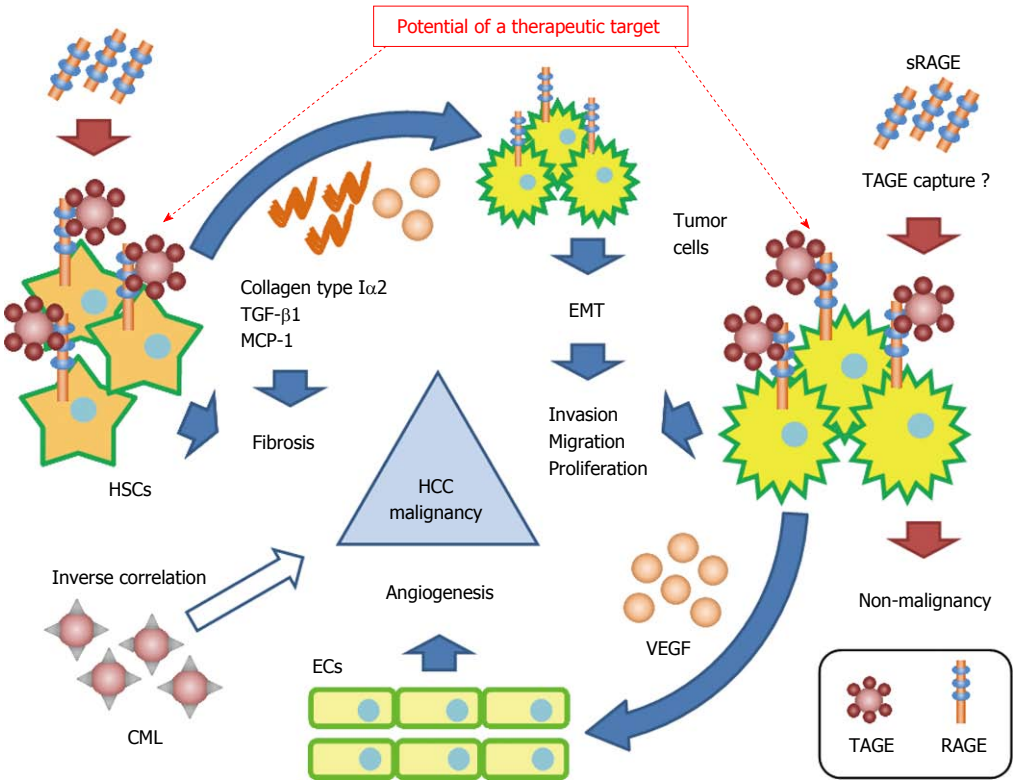

Figure 3 Proposed model for the contribution of the toxic advanced glycation end-products-receptor axis in nonalcoholic steatohepatitis-related hepatocellular carcinoma. The interaction between TAGE and RAGE alters intracellular signaling in tumor cells and hepatic stellate cells, and induces angiogenesis, invasion, migration, proliferation, and fibrosis. This cooperation by the TAGE-RAGE axis may lead to the malignant progression of nonalcoholic steatohepatitis (NASH)-related hepatocellular carcinoma (HCC). CML and sRAGE inversely correlate with the risk of HCC, and sRAGE, which plays the role of a decoy receptor of RAGE, prevents the malignant progression of HCC. The TAGE-RAGE axis may become a treatment target in NASH and NASH-related HCC. CML: Nε-(carboxymethyl)lysine; ECs: Endothelial cells; EMT: Epithelial mesenchymal transition; HSCs: Hepatic stellate cells; MCP-1: Monocyte chemoattractant protein-1; RAGE: Receptor for advanced glycation end-products; sRAGE: Soluble receptor for advanced glycation end; TAGE: Toxic advanced glycation end-products; TGF-β1: Transforming growth factor-β1; VEGF: Vascular endothelial growth factor.

central necrotic areas than those of spheroids formed only with HCC; and (3) tumor size and invasion ability were significantly greater following the co-implantation of HSCs and HepG2 into nude mice than the implantation of HepG2 alone *in vivo*. Furthermore, Yang *et al*[108] reported that collagen type Ⅰ, which is secreted by HSCs, enhanced the metastatic ability of HCC *via* epithelial mesenchymal transition. These findings suggested that the TAGE-RAGE axis in HSCs indirectly caused the malignant transformation of HCC (Figure 3).

CONCLUSION

Slight increases in the incidence of NBNC-HCC in recent years have changed the etiology of HCC. The onset mechanism of NBNC-HCC, the etiology of which is varied, currently remains unclear, and, as a consequence, has led to its later diagnosis and larger NBNC-HCC tumors than virus-related HCC tumors. However, the survival rate of early stage NBNC-HCC patients was previously reported to be higher than that of virus-related HCC patients[109-111]. The clinical data suggest that NBNC-HCC can be resolved by early pathogenesis-based treatment, because most of patients diagnosed with NBNC-HCC may have NASH, an important etiological factor of NBNC-HCC. We herein indicated that TAGE, enhanced by NASH, contributes to the malignancy of NASH-related HCC *via* RAGE. Therefore, the TAGE-RAGE axis may become an important treatment target in NASH-related HCC, and warrants further study.

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P- Reviewer: Ampuero J, Gallego-Duran R, Lukacs-Kornek V
S- Editor: Yu J **L- Editor**: Webster JR **E- Editor**: Liu SQ

World Journal of Hepatology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4254/wjh.v7.i23.2470

World J Hepatol 2015 October 18; 7(23): 2470-2473
ISSN 1948-5182 (online)

MINIREVIEWS

Training *vs* practice: A tale of opposition in acute cholecystitis

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Conflict-of-interest statement: The authors disclose no conflict of interests.

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Received: April 27, 2015
Peer-review started: May 1, 2015
First decision: July 6, 2015
Revised: September 1, 2015
Accepted: September 7, 2015
Article in press: September 8, 2015
Published online: October 18, 2015

Abstract

Acute cholecystitis is one of the most common surgical diagnoses encountered by general surgeons. Despite its high incidence there remains a range of treatment of approaches. Current practices in biliary surgery vary as to timing, intraoperative utilization of biliary imaging, and management of bile duct stones despite growing evidence in the literature defining best practice. Management of patients with acute cholecystitis with early laparoscopic cholecystectomy (LC) results in better patient outcomes when compared with delayed surgical management techniques including antibiotic therapy or percutaneous cholecystostomy. Regardless of this data, many surgeons still prefer to utilize antibiotic therapy and complete an interval LC to manage acute cholecystitis. The use of intraoperative biliary imaging by cholangiogram or laparoscopic ultrasound has been demonstrated to facilitate the safe completion of cholecystectomy, minimizing the risk for inadvertent injury to surrounding structures, and lowering conversion rates, however it is rarely utilized. Choledocholithiasis used to be a diagnosis managed exclusively by surgeons but current practice favors referral to gastroenterologists for performance of preoperative endoscopic removal. Yet, there is evidence that intraoperative laparoscopic stone extraction is safe, feasible and may have added advantages. This review aims to highlight the differences between existing management of acute cholecystitis and evidence supported in the literature regarding best practice with the goal to change surgical practice to adopt these current recommendations.

Key words: Cholangiography; Acute cholecystitis; Ultrasound; Laparoscopy; Cholecystectomy; Evidence based

Core tip: General surgeons commonly perform laparoscopic cholecystectomy for acute cholecystitis; however, current practices in biliary surgery often vary regarding timing, intraoperative biliary imaging, and management of bile duct stones. In spite of growing evidence in the literature defining best practice and societal guidelines supporting early cholecystectomy, intraoperative cholangiogram and ultrasound, and laparoscopic bile

duct exploration utilizing laparoscopic ultrasound and performing common bile duct exploration, an overwhelming number of surgeons still perform delayed operations, rarely perform intraoperative imaging and defer treatment of common bile duct stones. Efforts should be made to adopt the evidence-based data supported in the literature.

Patel PP, Daly SC, Velasco JM. Training *vs* practice: A tale of opposition in acute cholecystitis. *World J Hepatol* 2015; 7(23): 2470-2473 Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i23/2470.htm DOI: http://dx.doi.org/10.4254/wjh.v7.i23.2470

THE DICHOTOMY OF LAPAROSCOPIC CHOLECYSTECTOMY TIMING

The timing of cholecystectomy in the management of patients presenting with acute cholecystitis is variable and controversial in spite of well-published guidelines from surgical societies: SAGES and SSAT advocating early cholecystectomy during same hospital admission[1]. Two current approaches exist in the treatment of acute cholecystitis: (1) the traditional, conservative approach consisting of initial antibiotic therapy or percutaneous cholecystostomy followed by delayed cholecystectomy (DC) once inflammation has resolved; and (2) the preferred approach of early surgical intervention, ideally utilizing laparoscopic cholecystectomy (EC) within three to seven days of admission. Many surgeons continue to practice the former due to a number of reasons, such as a belief that an operation will be technically easier after a two to six week delay. Patient comorbidities, and the thought that laparoscopic cholecystectomy may be relatively contraindicated in acute cholecystitis for it may lead to high conversion rates have led to a resurgence of percutaneous cholecystostomy beyond the accepted indications in medically compromised patients. However, a wide range of practicing surgeons in the United States, ranging from 20% to 65% favor performing a laparoscopic cholecystectomy early during the initial presentation of acute cholecystitis[2-4]. Current surgical training has shifted this paradigm more towards early surgical intervention based on strong evidence of its benefit. However, practicing surgeons have been slow to embrace the change for a variety of reasons[3,5,6].

The concern for increased morbidity by operating in a surgical field of acute inflammation still persists. While significantly decreased morbidity rates for patients undergoing EC (12.0%) compared to DC (33.3%) was demonstrated in the ACDC trial, these results were not replicated on a recent meta-analysis where similar morbidity rates between EC patients and DC patients (0.12% *vs* 0.27%) were observed[7,8]. In the elderly population, where the number of comorbidities is often higher, similar morbidity rates were shown between EC patients and DC patients[9]. However, decreased rates of cholangitis and persistent cholecystitis (1.3% *vs* 10.3%) and a decreased rate of septic shock (0.0% *vs* 1.3%) have been demonstrated in EC patients[7]. Most studies with the highest level of evidence demonstrated similar morbidity rates between EC and DC patients while some even show improved outcomes for EC patients[4,7-9]. A recent study analyzing the management of acute cholecystitis in the elderly did not show any significant differences in outcomes between the early and late cholecystostomy groups. Furthermore, percutaneous cholecystostomy did not confer any significant benefit as a bridge to cholecystectomy except in medically compromised patients[1].

The concern for an increased risk of bile duct injuries when operating in acute inflammation may deter some surgeons from early cholecystectomy. However, a meta-analysis, which included seven trials and over 1106 patients, demonstrated a similar common bile duct injury rate between patients undergoing an EC *vs* a DC (0.002% *vs* 0.004%)[8]. In addition to the meta-analysis, a database review by Zafar *et al*[4], which included over 95000 patients, failed to show a difference in observed bile duct injury rates. The literature supports equal rates of bile duct injury irrespective of laparoscopic cholecystectomy timing.

Many surgeons may choose to delay laparoscopic cholecystectomy in times of acute inflammation to lessen the chance of converting to an open procedure. The current literature does not support this concern. There exists no difference in conversion to open operations in patients undergoing an EC, with rates as low as 0.14% in a large meta-analysis to ranges of 5.0%-9.9% in elsewhere in the literature[4,7,8]. These rates compare to patients undergoing DC with rates as lows as 0.16% in meta-analysis and ranges of 1.7%-11.9% elsewhere in the literature[4,7,8]. Results were similar in the elderly population[9]. An increase in conversion rates after 5 d of symptoms to open cholecystectomy was shown by Zafar *et al*[4], further supports EC.

Some surgeons may be hesitant to perform a laparoscopic cholecystectomy because such an operation may be thought to lead to longer hospital stays, higher costs and decreased patient satisfaction. Significantly shorter hospital stays in EC patients, when compared to DC patients, have been demonstrated and includes the elderly population[4,9,10]. Zafar *et al*[4] demonstrated an increased postoperative stay of two days in patients whose laparoscopic cholecystectomy was performed after 5 d. Decreased hospital stay in one factor that has led to decreased costs in EC. A cost savings of nearly 31% has been observed in the literature[7]. In addition, Johner *et al*[11] demonstrated not only a $2028 (2009 Canadian dollar) cost savings but a gain of 0.03 quality-adjusted life year (QALY) gain in patients undergoing EC. Compared to EC, delayed cholecystectomy led to a significantly increased rate of persistent abdominal pain, 10.0% *vs* 2.3% in EC patients, and increased rates of persistent fever, 3.3% *vs* 0.3% in the EC group[7]. When mean patients satisfaction scores were determined, EC patients had significantly higher satisfaction (92.7)

compared to DC patients (75.3)[10]. This difference was attributable to persistent and recurrent biliary attacks in patients undergoing DC[10].

THE DICHOTOMY OF BILIARY DUCT IMAGING IN ACUTE CHOLECYSTITIS

Two current approaches exist regarding intraoperative evaluation of the biliary tree: One that routinely evaluates biliary ductal anatomy during a laparoscopic cholecystectomy and the alternative, a selective approach, that completes intraoperative imaging based on individual clinical factors. To date, no randomized controlled study has been appropriately powered to endorse routine biliary imaging, however many studies have demonstrated a trend towards decreased biliary ductal injuries with routine evaluation[12,13]. Despite this trend in data and despite a bile duct injury being one of the most dreaded complications of biliary surgeons, few training programs endorse a curriculum of routine imaging and few surgeons have adopted it as part of their practice.

When a surgeon undertakes biliary imaging, the next decision to be made is which method of imaging should be employed. Many techniques have been described in the literature but the two most popular are intraoperative transcystic cholangiography (IOC) and laparoscopic ultrasound (LUS). Intraoperative cholangiogram is completed by cannulating the transected cystic duct with a small lumen catheter and is the most common method utilized. Alternatively, laparoscopic ultrasound uses a flexible probe dressed in a sterile sheath to evaluate both ductal anatomy and the hepatic vasculature. In an attempt to try and demonstrate one method superior to the other, Aziz *et al*[14] performed a meta-analysis including 11 studies whose results demonstrated no significant difference in either sensitivity or specificity between each method. However, more recent studies have been able to demonstrate a higher specificity, in some cases nearly 100%, using laparoscopic ultrasound[15]. Additional advantages to LUS include that it is efficient, does not require cannulating the biliary system, and it can be accomplished prior to a complete and sometimes tedious dissection as it is easily repeated as needed during the course of an operation. Furthermore, LUS does not require fluoroscopy, and it has a lower failure rate than IOC, being extremely useful in defining the hepaticoduodenal anatomy[15-17]. In spite of these many benefits, LUS is rarely taught and rarely utilized in practice because of the technique's large learning curve and the traditional acceptance of IOC as best care.

THE DICHOTOMY OF TREATING COMMON BILE DUCT STONES IN ACUTE CHOLECYSTITIS

The rate of common bile duct stones in acute cholecystitis ranges from 3%-18%[18]. Many algorithms have been established to manage choledocholithiasis but controversy exists to which is the best method. When open cholecystectomy was standard of care, the majority of common bile duct stones were removed at the time of surgery by means of a common bile duct exploration *via* a choledochotomy or an indirect transcystic method. Early in the laparoscopic era, there were limited capabilities of laparoscopic instruments and surgeons' lacked the expertise to complete a common bile duct exploration. As such, endoscopic retrograde cholangiopancreatography (ERCP) became standard practice. With improved laparoscopic instruments and advanced training, laparoscopic common bile duct exploration (LCBDE) rates are on the rise. In conjunction with intraoperative biliary ductal imaging, LCBDE allows for the management of common bile duct stones during one procedure. This technique has been shown to have a statistically significant reduction in total costs, length of hospital stay from 98 h with ERCP to 55 h, and number of procedures performed[19,20]. In addition, a trend towards better ductal clearance has been demonstrated[19]. Although utilization of LCBDE is gaining popularity, use is largely limited to fellowship trained minimally invasive or hepatobiliary surgeons and has not yet been readily adopted by most general surgeons despite improved outcomes.

THE FUTURE OF BILIARY SURGERY

Even though laparoscopic cholecystectomy for acute cholecystitis is one of the most commonly performed operations by general surgeons, current practices in biliary surgery remain varied despite growing evidence in the literature defining best practice. Despite improved outcomes by performing an early cholecystectomy in acute cholecystitis and current training mirror such recommendations, an overwhelming number of surgeons still perform delayed operations. Despite superior outcomes when LUS and IOC are utilized, a very limited number of residents will be proficient at these techniques upon completion of training and thus will not incorporate them into clinical practice. Due to this lack of expertise, many surgeons have come to rely on other tools including MRCP for preoperative definition of anatomy and ERCP to diagnose and remove common bile duct stones. Although these alternative methods have utility in a majority of cases, they come with additional potential morbidity and costs. Efforts should be made for surgical practice to catch up to surgical training and the evidence supported in the current literature.

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P- Reviewer: Garg P **S- Editor**: Song XX
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World Journal of Hepatology

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DOI: 10.4254/wjh.v7.i23.2474

World J Hepatol 2015 October 18; 7(23): 2474-2481
ISSN 1948-5182 (online)

MINIREVIEWS

Role of biomarkers in the prediction and diagnosis of hepatocellular carcinoma

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Author contributions: All authors equally contributed to acquiring the data, reviewing the literature, drafting the article, making critical revisions to the manuscript, and approving of the final version of the article to be published.

Conflict-of-interest statement: All authors declare that they have no conflict of interest to be declared.

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Received: December 1, 2014
Peer-review started: December 2, 2014
First decision: January 12, 2015
Revised: August 24, 2015
Accepted: September 10, 2015
Article in press: September 16, 2015
Published online: October 18, 2015

Abstract

The prevalence of hepatocellular carcinoma (HCC) has progressively increased in recent years and is now the fifth and the second most common cancer in the World and in Egypt, respectively. Much work has focused in the development of assays for detecting hepatic carcinogensis before the observance of hepatic focal lesions. Particular attention has been directed towards HCC-specific biomarkers for use in the early diagnosis of HCC and in the confirmation of radiological studies. Although a number of biomarkers have been identified, none have been considered reliable indicators of early HCC lesions. This review presents a few of the most relevant HCC biomarkers and suggests improvements to the accuracy of diagnostic assays through their combined use. Furthermore, we present an algorithm for the biomarker-based diagnosis of HCC and highlight its important role in the early prediction of HCC.

Key words: Hepatocellular carcinoma; Epidemiology; Pathogenesis; Biomarkers; Diagnosis

Core tip: Alpha-fetoprotein (AFP) has been widely used as a reference biomarker to validate the diagnosis of hepatocellular carcinoma (HCC). However, normal physiological-levels of AFP are observed in approximately one third of HCC cases. Furthermore, a number of HCC positive patients have AFP levels less than the threshold value of 400 ng/mL. These factors make an AFP-based diagnosis of HCC far from reliable. However, high diagnostic accuracy indices have been reported when AFP is combined with other biomarkers such as midkine, golgi protein 73, des-γ-carboxyprothrombin, glypican-3, and gamma-glutamyl transferase.

Khattab M, Fouad M, Ahmed E. Role of biomarkers in the prediction and diagnosis of hepatocellular carcinoma. *World J Hepatol* 2015; 7(23): 2474-2481 Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i23/2474.htm DOI: http://dx.doi.org/10.4254/wjh.v7.i23.2474

EPIDEMOLOGY

Hepatocellular carcinoma (HCC) has an annual incidence of 7.9% in men and 6.5% in women (fifth and seventh worldwide respectively)[1]. In regions with a high prevalence of hepatitis C virus (HCV) and hepatitis B virus (HBV) infections, the incidence and prevalence of HCC has progressively increased[2]. In Egypt, due to the high prevalence of HCV and HBV infections, the incidence rate of HCC has doubled in the past ten years[3].

Pathogenesis and risk factors

Important environmental risk factors for HCC include chronic hepatitis virus infections, alcohol abuse, and non-alcoholic steatohepatitis (NASH). These risk factors are also relevant aetiologic factors for cirrhosis[4]. In Eastern Asia and sub-Saharan Africa the main risk factors for HCC are chronic hepatitis B infection and exposure to aflatoxin B1. In North America, Europe, and Japan, the main risk factors are chronic hepatitis C (CHC) infection and alcohol consumption[5]. HCV increases HCC risk by promoting cirrhosis and causing specific genetic lesions to the infected liver cells[6]. Clinically relevant hepatitis viral infections has been shown to predispose individuals to HCC, similarly, occult hepatitis B infection has been associated with the development of HCC[7].

Higher viral loads with prolonged infections have been correlated with the occurrence of HCC and may be due to accumulated risks from chronic oncogenic damage[8]. Daily alcohol consumption and Aflatoxin food contamination have been associated with increased risks of HCC[9,10]. Patients with CHC, increased insulin resistance and high serum adiponectin levels are more likely to develop liver cancer[11]. Metabolic syndrome, a combination of phenomena, including obesity, dyslipidaemia, insulin resistance and type 2 diabetes mellitus (DM), is a known potential risk factor for HCC. Due to obesity or other causes, NASH has been associated with increased risks of HCC[12,13]. DM has been associated with a two to three fold increased risk of HCC. Additionally, DM has been shown to affect the prognosis of HCC after curative therapies[14]. Furthermore, DM type 2 can lead to HCC caused by carcinogenic effects on the liver and other tissues from insulin-like growth factor-1 (IGF-1) due to hyperinsulinaemia and insulin-resistance (Figure 1)[15,16]. The risk for HCC was particularly higher in diabetic patients treated with insulin[17]. However, Yamamoto *et al*[18], 2012, reported a case in which a dramatic regression of HCC was observed after four weeks of treatment with a dipeptidyl peptidase-4 enzyme (DPP-4) inhibitor in a patient with HCV-related chronic hepatitis. $CD8^+$ T-cells were shown to accumulate around the HCC tissue, indicating that a DPP-4 inhibitor may safely exert beneficial effects on HCV-related HCC through immunity modulation[19]. A 1.7-fold increase in the incidence rates of HCC was reported for individuals with hereditary haemochromatosis confirming preliminary observations in smaller studies in related populations[20]. A correlation was also observed for alcoholics presenting with liver iron overload for increased risks of HCC and C282Y mutation in haemochromatosis[21].

MOLECULAR PATHOGENESIS

The pathogenesis of HCC remains undetermined. Evidence exists supporting the notion that DNA damage occurs, resulting in the dysregulation of DNA methylation, chromosomal instability, proto-oncogene activation, and tumour suppressor gene inactivation. The renin angiotensin system signalling pathways have been observed to activate, which leads to cell proliferation (Figure 2)[22,23]. Major risk factors for HCC typically lead to liver cirrhosis and the accumulation of genetic and epigenetic changes, such as the activation of oncogenes and the inactivation of tumour suppressor genes. The signalling pathways (*e.g*., Raf/MEK/ERK, PI3K/AKT, and Wn/β-catenin pathways) are activated through various growth factors. Growth factor receptor signalling typically results in abnormal hepatocyte proliferation and subsequently, tumour angiogenesis (Figure 3)[24,25]. Furthermore, multiple mutations at the chromosomales, genetics and epigenetics levels have been implicated as pathogenetic mechanisms in the development of HCC[26,27]. MicroRNA has been shown to aid in the transcription of HCC oncogenes and (Wnt) signaling, and it plays important roles in the development of HCC through the activation of β catenin, the overexpression of Wnt receptors and the inactivation of E-cadherin[28].

BIOMARKERS IN DIAGNOSIS OF HCC

Alpha-fetoprotein and alpha-fetoprotein-L3

There are three forms of alpha-fetoprotein (AFP) according to electrophoresis lectin-reactivity (AFP-L1, AFP-L2, and AFP-L3). A high percentage of AFP-L3 seems to differentiate HCC from chronic liver diseases and may be an indicator of HCC when the total serum AFP levels are $\geqslant$ 200 ng/mL[29].

AFP has been traditionally used as a reference biomarker to screen and support the diagnoses of HCC. However, approximately one-third of HCC patients have normal, physiological-levels of AFP. AFP levels > 200 ng/mL are specific for HCC, and levels > 500 ng/mL correlate with tumour size. AFP-L3 levels above 10% to 15% threshold level (percentage of AFP-L3 over AFP) have been detected in approximately one-third of HCC patients[30]. The use of AFP levels to diagnose HCC patients relies on a threshold value of 200 ng/mL, a sensitivity of 0.310, a specificity of 0.960 and an area under the curve (AUC) of 0.835[31,32].

Des-γ-carboxyprothrombin

Des-γ-carboxyprothrombin (DCP) is produced in HCC cell lines, and it is found at significantly higher concentrations than normal in 50% to 60% of all HCC patients and in 15% to 30% of early HCC cases. DCP

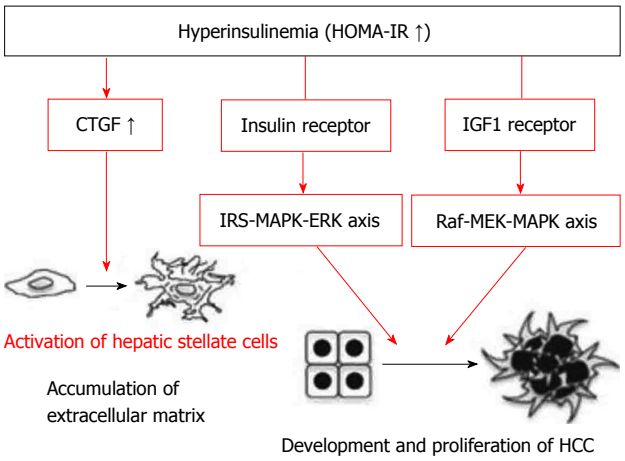


Figure 1 Insulin resistance and the development of hepatocellular carcinoma (cited from Eslam *et al*[16], 2011). HCC: Hepatocellular carcinoma; HOMA-IR: Homeostasis model of insulin resistance; IRS: Insulin receptor; MAPK: Mitogen-activated protein kinase; IGF1: Insulin like growth factor 1; CTGF: Connective tissue growth factor.

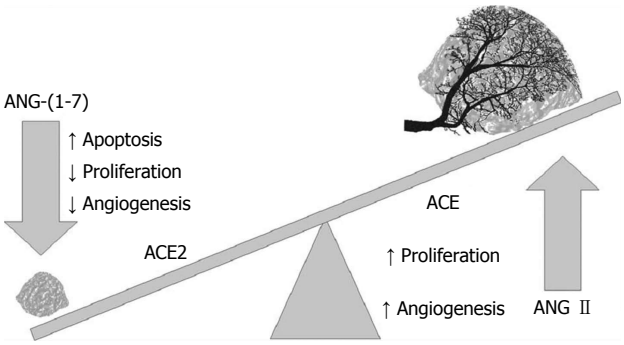


Figure 2 The balancing effects of the renin-angiotensin system on tumourigenesis (cited from Ager *et al*[23], 2008). The renin-angiotensin system can promote or inhibit angiogenesis and cellular proliferation and thereby, support or block tumour neovascularisation, growth and metastasis. ANG: Angiopoietin; ACE: Angiotensin converting enzyme.

can be used together with AFP-L3 to diagnose HCC[33]. At a 125 mAU/mL threshold, DCP has high sensitivity (89%), specificity (95%) and AUC (0.797)[34,35] in the prediction of HCC.

Midkine

AT a 654 ng/mL threshold level, midkine (MDK) serum has higher sensitivity (86.9% *vs* 51.9%), specificity (86.3% *vs* 83.9%) and AUC (0.915 *vs* 0.754) compared with AFP. Therefore, MDK can be used in the diagnosis of AFP-negative HCCs and very early-stages of HCCs. Furthermore, MDK can be used in HCC patients after curative resections to diagnose tumour recurrence[36].

Dickkopf-1

Dickkopf-1 (DKK-1) can be used together with AFP for the diagnosis of HCC and especially for HCC cases with low levels of AFP. DKK-1 can distinguish HCC from non-malignant chronic liver diseases and has sensitivity of 69.1% and a specificity of 90.6%[37].

Golgi protein 73

Golgi protein 73 (GP73) serum levels increase in patients with liver disease and HCC[38]. At a threshold value of RU 10 units, GP73 sensitivity, specificity and AUC are 69%, 75% and 0.914 respectively[39].

The combination of GP73 and AFP (with a 35 ng/mL threshold for AFP and an 8.5 RU threshold for GP73) increased the HCC diagnostic sensitivity to 89.2%, specificity to 85.2%, with an AUC of 0.914[40].

Glypican-3

Glypican-3 (GLP-3) is a 60 kDa cell surface-linked heparin sulfate proteoglycan and is not expressed in adult livers[41]. GLP-3 serum levels are higher in HCC patients than in patients with HCV-induced cirrhosis. Furthermore, GLP-3 is more sensitive than AFP for the detection of smaller HCC. The combined use of GLP-3 and AFP has been shown to provide higher sensitivity and specificity than the individual use of each marker[42].

Gamma-glutamyl transferase

In HCC and liver diseases, significant changes occur in gamma-glutamyl transferase (GGT) serum activity. GGT-Ⅱ is a hepatoma-specific GGT and an early enzyme marker of precancerous and cancerous processes[43]. At threshold levels of 100 U/L and 100 IU/mL for GGT and AFP, respectively, GGT had a higher sensitivity and a lower specificity than AFP (69.5% *vs* 43.5%) and (41.9% *vs* 96.4%), respectively, for the diagnosis of HCC. When AFP and GGT were combined, the sensitivity was 56.5% and specificity was 69.5%[44].

Alpha-l-fucosidase

Alpha-l-fucosidase (AFU) is a lysosomal enzyme. Its serum levels have been shown to increase in patients with cirrhosis and HCC[45]. At a threshold of 2.3005 μmol/L per minute, AFU yielded a sensitivity and specificity of 90% and 97.5%, respectively[46].

Transforming growth factor beta-1

Transforming growth factor beta-1 (TGF-beta-1) is a cytokine with multiple biological functions. It has a role in cell growth and extracellular matrix formation[47]. With a threshold of 64.33 ng/mL, TGF-beta-1 has a sensitivity of 78.3% and a specificity of 29.5% for the diagnosis of HCC. The combined use of AFP and TGF-beta-1 altered the specificity and the sensitivity to 86.6% and 30.4%, respectively[48].

IGF

IGFs Ⅰ and Ⅱ are polypeptides that play important roles in hepatic carcinogenesis. The serum levels of IGF Ⅰ are significantly lower in patients with HCC compared with patients without HCC[49]. At a threshold of 4.1 mg/g IGF Ⅰ has a 63% sensitivity and a 90% specificity for diagnosings of HCC. The combination of IGF Ⅰ and AFP increased the sensitivity to 80% and the specificity to 90%[50].

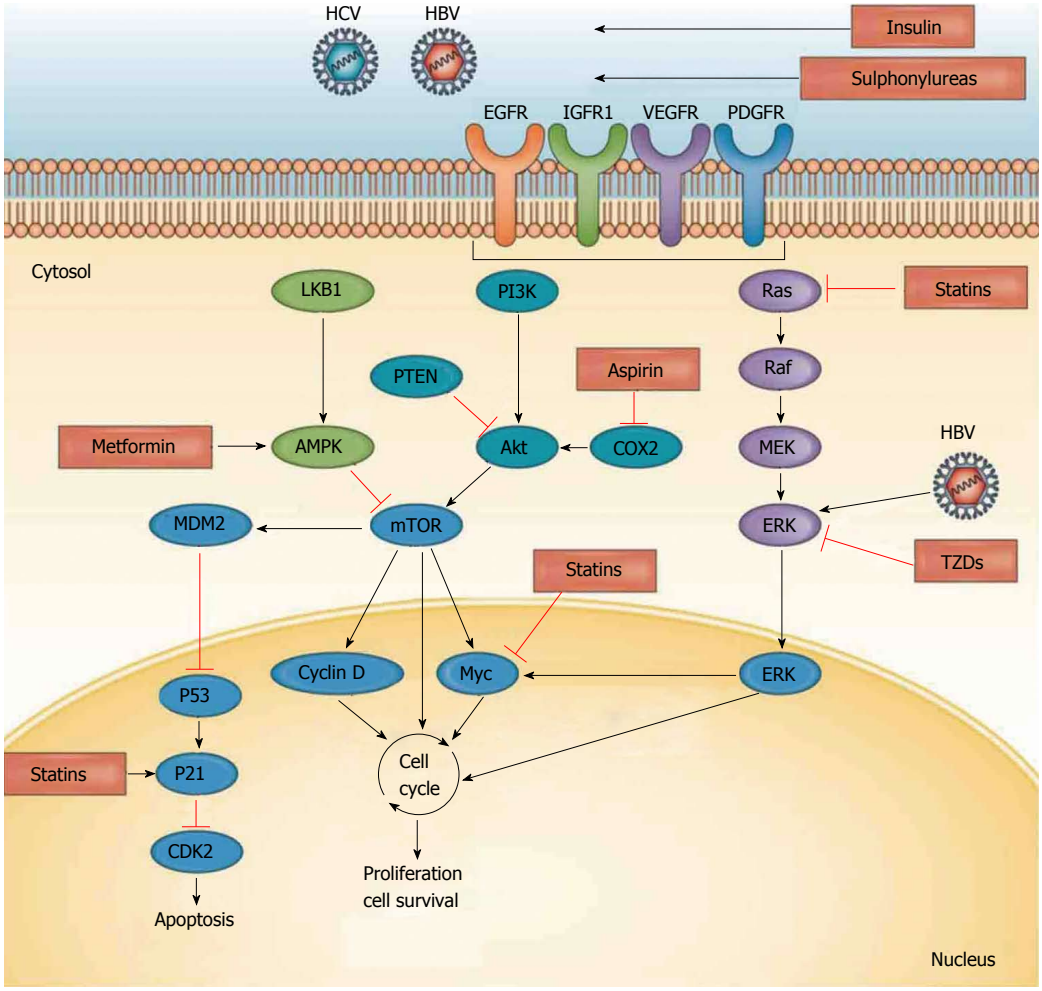


Figure 3 Pathogenesis of hepatocellular carcinoma and targets for chemopreventive agents (cited from Singh *et al*[25], 2014). AMPK: Adenosine monophosphate-activated protein kinase; IGFR1: Insulin-like growth factor receptor 1; MAPK: Ras mitogen-activated protein kinase; mTOR: Mammalian target of rapamycin; PI3K: Phosphatidylinositol 3-kinase; HBV: Hepatitis B virus; HCV: Hepatitis C virus; PTEN: Phosphatase/Tensin homolog deleted on chromosome 10; P53: A tumor suppressor protein; P21: Cyclin-dependent kinase 2 inhibitor; CDK2: Cyclin-dependent kinase 2; ERK: Extracellular signal-regulated kinase protein; MEK: Minase that phosphorylate mitogen activated protein (MAP); PDGFR: Platelet derived growth factor receptor; VEGFR: Vascular endothelial growth factor receptor; IGFR1: Insulin like growth factor receptor 1; EGFR: Epidermal growth factor receptor; Ras: Prototypical member of the RAS superfamily of proteins; Raf: A MAP kinase kinase kinase (a serine/threonine specific kinase); Akt: A protein kinase family of genes involved in regulating cell survival.

Squamous cell carcinoma antigen

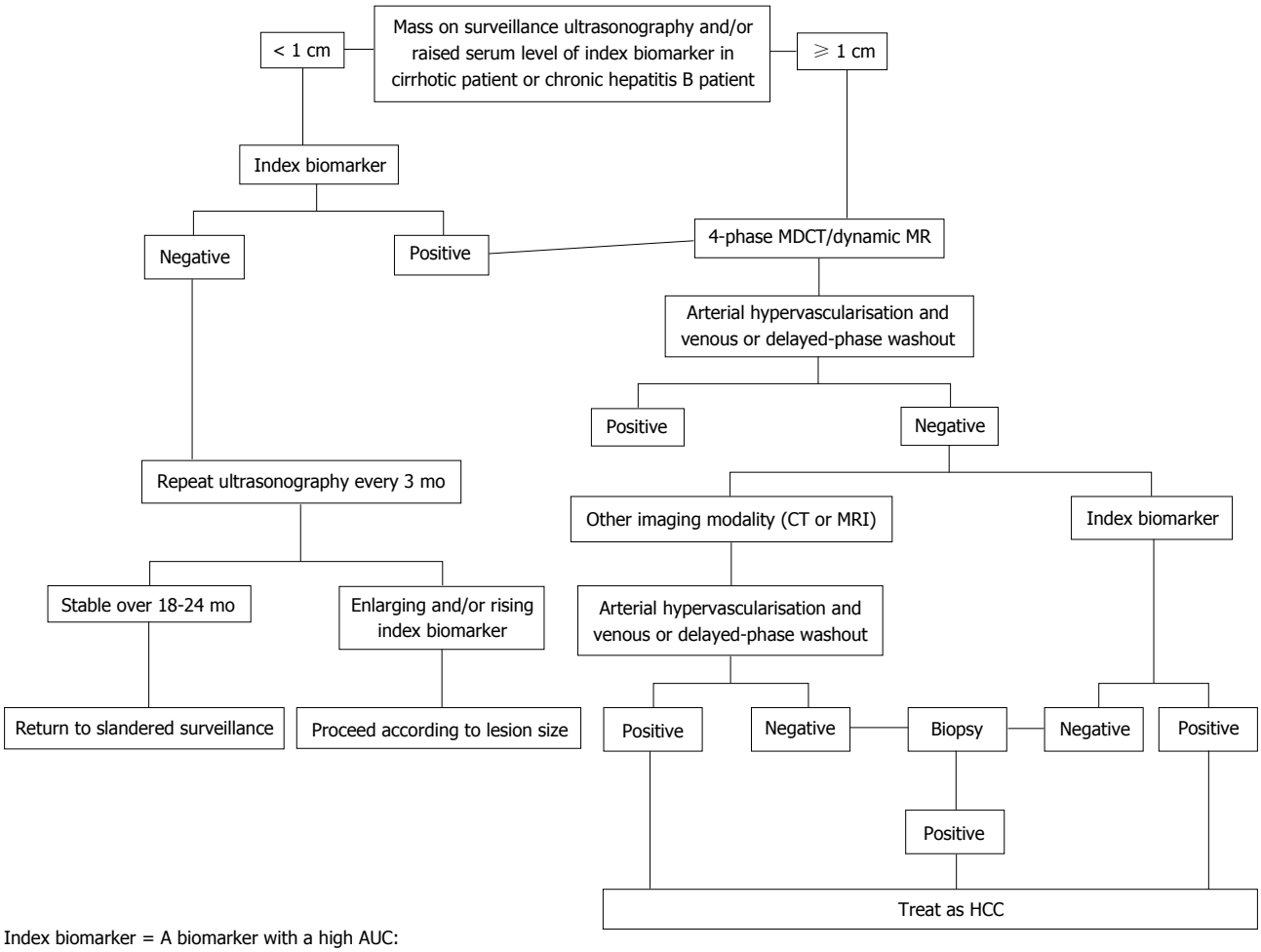
Squamous cell carcinoma antigen (SCCA) levels are persistently elevated in patients with HCC displaying normal, physiological-AFP levels. This property is useful in the early detection and follow-up diagnoses for patients treated for HCC[51]. At a threshold of 0.368 ng/mL, SCCA has an AUC of 0.705, a sensitivity of 84.2% and a specificity of 48.9%[52].

Osteopontin

Osteopontin (OPN) is an integrin-binding glycophosphoprotein involved in many cellular functions, such as regulating the survival, migration, invasion, and metastasis of tumour cells[53]. When compared with cirrhosis, CHC, chronic hepatitis B or healthy controls, OPN plasma levels were significantly elevated in HCC patients[54]. At a serum of 557 ng/mL, OPN has sensitivity, specificity and AUC of 26%, 92.5% and 51%, respectively, for the diagnosis of HCC[55,56]. Furthermore, OPN was more valuable when combined AFP-L3[57].

Heat shock protein

Heat shock proteins (HSP) are stress-induced proteins and belong to the glucose-regulated proteins (GRPs) family. In neoplasms, the expression of HSP has been associated with apoptosis regulation and tumour immune response. The expressions of HSP27, HSP70, HSP90, GRP78, and GRP94 increased in a stepwise pattern as HCC developed from a dysplastic nodules to early HCC and, finally to advanced HCC[58]. In HBV-infected patients, the expressions of GRP78, GRP94, or HSP90 have been significantly correlated with vascular invasion and intrahepatic metastasis. HSP27 has been detected in 90% HCC patients sera and two HBV patients sera, but in none of normal sera[59]. The optimal diagnostic threshold for HSP27 was 456.5 pg/mL. This yielded a sensitivity of 70% and a specificity of 73%, with an AUC



Index biomarker = A biomarker with a high AUC:
GP73 AUC = 0.914
AFP + DCP AUC = 0.874
AFP + GP73 AUC = 0.932
AFP + AFPL3 AUC = 0.748

Figure 4 Diagnostic algorithm for hepatocellular carcinoma. MDCT: Multidetector CT; MR: Magnetic resonance; AFP: Alpha-fetoprotein; DCP: Des-γ-carboxyprothrombin; GP73: Golgi protein 73; AUC: Area under the curve; MRI: MR imaging; CT: Computed tomography; HCC: Hepatocellular carcinoma.

of 0.749[60].

Interleukin-6 and syndecan-1

Syndican: The serum level of syndecan-1 significantly increased in HCC patients when compared with cirrhotic and control groups. Syndecan-1 levels significantly increased with progressive stages of Barcelona-Clinic Liver Cancer Group[61]. The diagnostic value of the test was significantly increased when combined with AFP.

Interleukin-6: At a threshold of 7.9 pg/mL, interleukin-6 (IL-6) has a sensitivity of 0.83, a specificity of 0.83, and an AUC of 0.810[62].

Higher C-reactive protein and IL-6 levels correlated well with larger tumour sizes, poorer Child-Pugh functions, shorter survival times and more predictable outcomes in patients with HCC receiving loco-regional therapy[63].

Soluble CD25

In a study by Cabrera *et al*[64], 2012, sCD25 was found be elevated in HCC patients when compared with those with cirrhosis. At a threshold of 2180 pg/mL, sCD25 based diagnoses of HCC had a sensitivity of 92.3% and a specificity of 37.7%; for the early detection of HCC. sCD25 based diagnoses had a sensitivity of 89.6% and specificity of 39.3%[64].

Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF), especially VEGF-A, was found to be elevated in HCC, particularly in advanced tumour stages and metastasis[65]. High serum levels of VEGF indicate poor HCC prognosis[66].

Glucoregulatory enzymes

In experimental studies, the sera and tissue levels of the alpha feto protein and glucoregulatory enzymes, HK, GAPDH and G6PD of hexose monophosphate shunt were significantly higher in HCC-bearing animals when compared with normal controls[67]. Glucoregulatory enzymes are promising targets as biomarkers for the prediction of HCC in humans (Table 1).

Table 1 Threshold values, sensitivity, specificity and area under the curve of a few biomarkers

Marker	Threshold	Sensitivity	Specificity	AUC	Ref.
AFP	200 ng/mL	0.310	0.960	0.835	[31,32]
DCP	7.5 ng/mL	0.600	0.940	0.797	[34,35]
GP73	10 RU	0.69	0.75	0.914	[39]
AFP-L3	10%	0.410	0.990	0.710	[30]
MDK	654 ng/mL	86.9%	86.3%	0.915	[36]
AFU	2.3005 μmol/L	90%	0.975		[46]
AFP + GP73	7.4 RU	0.770	0.840	0.932	[40]
GGT + AFP	100 U/L + 100 IU/mL	0.57	0.70	-	[44]
SCCA	0.368 ng/mL	0.84	0.49	0.705	[52]
HSP27	456.5 pg/mL	0.70	0.73	0.649	[70]
IL-6	7.9 pg/mL	0.83	0.83	0.810	[62]

AUC: Area under the curve; AFP: Alpha-fetoprotein; DCP: Des-γ-carboxyprothrombin; GP73: Golgi protein 73; MDK: Midkine; AFU: Alpha-l-fucosidase; GGT: Gamma-glutamyl transferase; SCCA: Squamous cell carcinoma antigen; IL-6: Interleukin-6.

Guideline diagnostic work-up for HCC

HCC nodules 1 cm or smaller are difficult to diagnose by imaging and require further tested. Nodules exceeding 1 cm can be diagnosed by imaging computed tomography (CT) or magnetic resonance imaging (MRI) with contrast. The uptake during the arterial phase and contrast washout during the venous or delayed phases provides diagnostic clues for HCC[68]. However, the conventional practices and methods for diagnosing HCC are cumbersome and invasive. Nodular lesions showing an atypical imaging patterns indicative of HCC on one of the dynamic scans (CT or MRI) are validated with the other dynamic scan (CT or MRI). Any liver nodules $\geqslant$ 2 cm showing atypical imaging pattern on both dynamic scans (CT and MRI) subsequently require histological confirmation[69]. However, the use of biomarkers with high HCC diagnoses accuracy indices can preclude the need for these invasive and hazardous liver biopsies (Figure 4).

CONCLUSION

Multiple biological markers are available to aid in the diagnosis of HCC. However, their individual use does not provide sufficient sensitivity and specificity. As presented in this review, the combined use of more than one biomarker may increase the predictive accuracy of HCC diagnoses in cirrhotic patients.

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P- Reviewer: Chen JL, Passadakis PS, Romanelli RG, Wang B
S- Editor: Ji FF **L- Editor**: A **E- Editor**: Liu SQ

World Journal of Hepatology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4254/wjh.v7.i23.2482

World J Hepatol 2015 October 18; 7(23): 2482-2491
ISSN 1948-5182 (online)

Detection of hepatitis B virus infection: A systematic review

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Author contributions: Ghosh M, Nandi S, Dutta S and Saha MK contributed equally to the work; all conceptualized and designed the review, drafted the manuscript, reviewed and approved the final manuscript as submitted.

Conflict-of-interest statement: All the authors declare that they have no competing interest.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at sahamk@yahoo.com. No additional data are available.

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Received: April 28, 2015
Peer-review started: May 6, 2015
First decision: July 29, 2015
Revised: August 18, 2015
Accepted: September 29, 2015
Article in press: September 30, 2015
Published online: October 18, 2015

Abstract

AIM: To review published methods for detection of hepatitis B virus (HBV) infection.

METHODS: A thorough search on Medline database was conducted to find original articles describing different methods or techniques of detection of HBV, which are published in English in last 10 years. Articles outlining methods of detection of mutants or drug resistance were excluded. Full texts and abstracts (if full text not available) were reviewed thoroughly. Manual search of references of retrieved articles were also done. We extracted data on different samples and techniques of detection of HBV, their sensitivity (Sn), specificity (Sp) and applicability.

RESULTS: A total of 72 studies were reviewed. HBV was detected from dried blood/plasma spots, hepatocytes, ovarian tissue, cerumen, saliva, parotid tissue, renal tissue, oocytes and embryos, cholangiocarcinoma tissue, *etc*. Sensitivity of dried blood spot for detecting HBV was > 90% in all the studies. In case of sero-negative patients, HBV DNA or serological markers have been detected from hepatocytes or renal tissue in many instances. Enzyme linked immunosorbent assay and Chemiluminescent immunoassay (CLIA) are most commonly used serological tests for detection. CLIA systems are also used for quantitation. Molecular techniques are used qualitatively as well as for quantitative detection. Among the molecular techniques version 2.0 of the CobasAmpliprep/CobasTaqMan assay and Abbott's real time polymerase chain reaction kit were found to be most sensitive with a lower detection limit of only 6.25 IU/mL and 1.48 IU/mL respectively.

CONCLUSION: Serological and molecular assays are predominant and reliable methods for HBV detection. Automated systems are highly sensitive and quantify HBV DNA and serological markers for monitoring.

Key words: Chemiluminescent immunoassay; Serology; Automated detection; Molecular assay; Hepatitis B virus

Core tip: The article was aimed to review published

methods of detection of hepatitis B virus (HBV) infection. A thorough search on medline database was conducted and 72 studies were included. It was observed that HBV can be detected reliably from dried blood spot (sensitivity > 90%). Serological and Molecular assays are predominant and reliable methods. Chemiluminescent immunoassay is more sensitive than Enzyme linked immunosorbent assay. Rapid tests are useful for screening. Real time polymerase chain reaction (PCR), branched DNA probe assays are principal methods for quantitation. Automated systems are more sensitive compared to in house assays. Abbott real time PCR was found to be most sensitive with a lower detection limit of only 1.48 IU/mL.

Ghosh M, Nandi S, Dutta S, Saha MK. Detection of hepatitis B virus infection: A systematic review. *World J Hepatol* 2015; 7(23): 2482-2491 Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i23/2482.htm DOI: http://dx.doi.org/10.4254/wjh.v7.i23.2482

INTRODUCTION

The enigma of hepatitis started long back in 3rd millennium B.C. in Sumeria with the first description of jaundice. Epidemic icterus was reported initially by Hippocrates (460 to 375 B.C.) followed by various vague descriptions by Greeks and Romans. But the perception of transmissibility came into acceptance with the spread of syphilis by Columbus and crew in 1494[1]. Further innumerable epidemics occurred in recipients of vaccines containing human serum or lymph. The largest was in 1942 among United States Army personnel, who received yellow fever vaccine containing human serum[2]. In 1940's several experiments in human volunteers by Cameron (1943)[3], Mac Callam (1944)[2,3], Paul *et al*[4] (1945) confirmed the viral etiology of hepatitis. 2 distinct clinicoepidemiological forms of viral hepatitis: Serum hepatitis and infectious hepatitis was evidenced by the study of Krugman[3] in the late 1950's and 1960's at WillowBrook State Schools, NewYork[4]. But the most important exploration in the history of viral hepatitis was of Sir B. Blumberg in the year 1960's. He observed an unusual reaction between the serum of hemophiliac patient and that of Australian aborigine in immunodiffusion gel and named this unusual protein Australia Antigen (Au Ag) which was further linked to viral hepatitis[5]. In 1968 Alfred Prince also described a serum antigen (SH Ag) in the serum of post transfusion patients[6]. These Au Antigen and SH Ag were soon found to be identical[1]. In the year 1970, Dane *et al*[7] discovered 42 nm sized virus like particles while observing Au Ag immune complexes under Electron Microscope. It was obvious that Au Ag was the surface antigen, whereas the Dane particles were actual virus. Hence the Au Ag was named hepatitis B surface antigen (HBsAg). By treating these "Dane particles" with mild detergents core particles were released by Almeida *et al*[8]. Antibody present in post hepatitis serum reacted with these inner/core particles. Researchers could comprehend soon that to assess the infectivity of the disease mere presence of HBsAg is not sufficient. In 1972 hepatitis B e antigen (HBeAg) was identified by Magnius *et al*[9] which helped to differentiate between highly infectious and less infectious forms. Simultaneously hepatitis B virus (HBV) DNA was identified by Robinson *et al*[10]. In earlier days infection with HBV was detected by demonstration of antibody titer by Complement Fixation Test[2]. The first solid phase sandwich radio immunoassay named Ausria 125 was developed by Ling *et al*[11] at Abbott Laboratories (North Chicago). This highly sensitive detection method became a major discovery in the diagnosis of viral transfusion hepatitis and screening of blood donors[2]. Since then innumerable serological and molecular methods have been developed for diagnosing HBV. This article provides an overview of detection of HBV infection employing different techniques.

MATERIALS AND METHODS

Literature search

The review followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines[12]. A protocol was developed and pertinent studies were identified as per inclusion and exclusion criteria (Figure 1). A thorough search on Medline database was conducted for articles related to diagnosis of HBV infection. The search was based on the following keywords or medical subject heading terms in the database: (detection[All Fields] AND ("hepatitis B virus"[MeSH Terms] OR "hepatitis B virus"[All Fields]) AND ("infection"[MeSH Terms] OR "infection"[All Fields])) AND ("2005/04/02"[PDat] : "2015/03/30"[PDat]).

Inclusion and exclusion criteria

The inclusion criteria were (1) articles describing methods or techniques of diagnosis of HBV; (2) published in English language; and (3) published in last 10 years. Articles were excluded if (1) study not original (review or editorial or case report); (2) studies describing methods of detection of drug resistance or mutants; (3) studies describing non microbiological serum biomarkers for diagnosing hepatitis only; (4) studies describing diagnosis of patients coinfected with other viruses [hepatitis C virus (HCV), human immunodeficiency virus, *etc.*] or bacteria (*Mycobacterium tuberculosis*); and (5) full text or abstract not available in Medline.

RESULTS

Detection of HBV from samples other than serum or whole blood

HBV is most commonly detected in serum or whole blood. But we retrieved total 17 studies, which have been published in MEDLINE in last 10 years, discussing about detection of HBV from samples other than

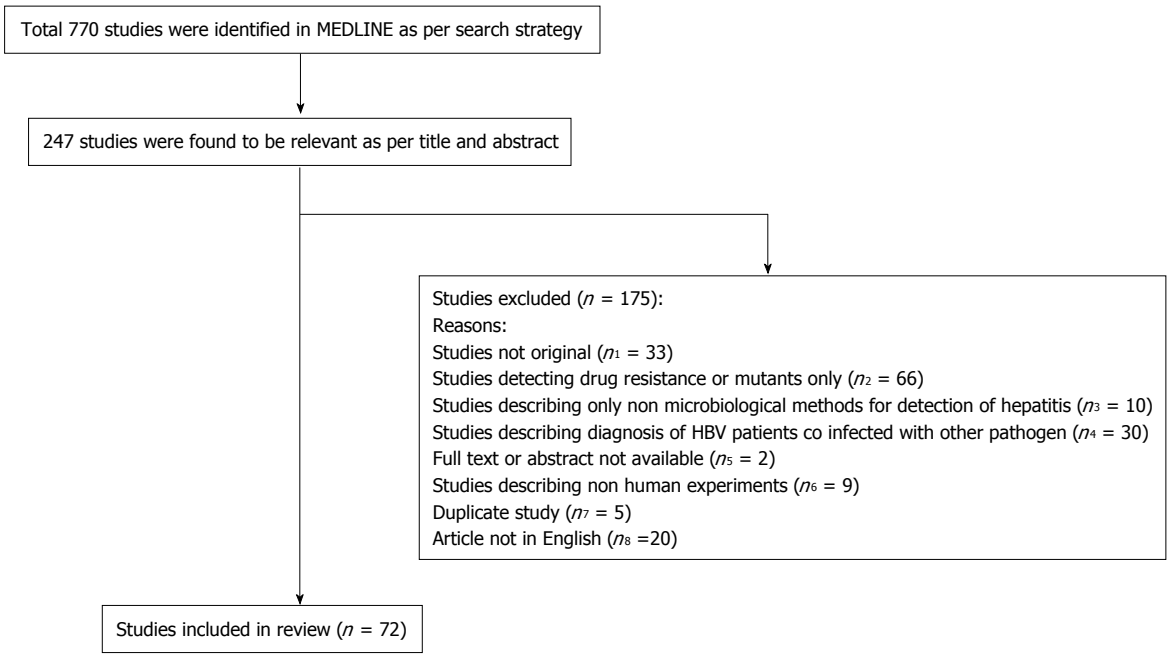

Figure 1 Flow Chart for selection of relevant articles. HBV: Hepatitis B virus.

serum or whole blood. Researchers have detected HBV from dried blood/plasma spots[13-16], hepatocytes[17-20], ovarian tissue[21], cerumen[22,23], saliva[24], parotid tissue[25], renal tissue[26], oocytes and embryos[27,28], cholangiocarcinoma tissue[29], *etc.* (Table 1).

Dried blood spots were first used in medical diagnostics by Guthrie *et al*[30] to detect Phenylketonuria. Dried blood spot (DBS) collection is much easier than taking venous blood. More over different antibodies, medications, metabolites, and nucleic acids remain stable for a longer period in these samples[15]. As researchers have validated this sample in diagnosis of HBV, it has been used much conveniently in field settings or resource poor settings. This review highlights that serological markers and nucleic acid of HBV can be detected from this sample by Point Of Care Tests (POCT), Enzyme Linked Immunosorbent Assay (ELISA) or Nucleic Acid Amplification Techniques with high sensitivity (Table 1). The combination of DBS and POCT is even more advantageous to use in resource poor settings. Sn of detection of HBsAg from saliva was 74.29% in the study of Arora *et al*[24] Presence of viral antigen in saliva makes dentistry personnel more vulnerable. Saliva can also be collected very easily without technical expertise and with the help of POCTs diagnosis can be made in resource poor settings rapidly.

In certain cases of chronic infection with low level viremia or seronegative patients, HBV DNA has been detected from hepatocytes by polymerase chain reaction (PCR) - *In situ* hybridization, while couldn't be detected from blood[18]. Other novel and highly sensitive techniques like flowcytometric quantitation, droplet digital PCR has increased the sensitivity of HBV detection from hepatocytes even more. This is especially important in diagnosing the etiology of chronic hepatitis/ hepatocellular carcinoma in seronegative or low viremic patients. Again persistent detection of covalently closed circular DNA (cccDNA) helps to predict recurrence of the disease[19]. Detection of serological markers and HBV DNA from ovarian tissue, oocytes or embryo becomes important in case of *in vitro* fertilization[21,28]. Though in one study nucleic acid couldn't be detected after culture and vitrification of oocytes or embryos from seropositive mothers during the procedure[27]. In the study of Kong *et al*[26], HBsAg and HBcAg were detected in frozen renal tissue by immunohistochemistry in 1.9% of seronegative patients with glomerulonephritis. As it is a common extrahepatic manifestation of viral hepatitis, in occult infections renal tissues can be used to detect the presence of virus.

Different methods of detection of HBV infection

The detection of HBV is very important in controlling its spread. After the discovery of Ausria 125 various serological, molecular and automated detection methods have been introduced and validated by different researchers. While searching Medline database in last 10 years total 55 studies were found describing different methods of detection.

Serological methods

Serological methods are most common, rapid and cost effective methods to detect different markers like HBsAg, anti-HBsAg, anti-HBcAg, HBeAg, anti-HBeAg, *etc*.

ELISA: ELISA is a type of solid phase immunoassay in which antigens or antibodies are covalently bound with suitable enzymes that can catalyze the conversion of a substrate into colored products. It is a validated method

Table 1 Studies describing detection of hepatitis B virus from samples other than serum or whole blood

Ref.	Year of publication	Sample used	Method used	Comments
Mendy *et al*[13]	2005	Dried blood spots along with serum	HBsAg detected by determine (TM) HBsAg	Comparison of DBS results with serum testing results: Sn 96%, Sp 100%
Chen *et al*[21]	2005	Ovarian tissue	HBsAg and HBcAg detected by immunocytochemistry and HBV DNA by PCR	Positivity rate of HBV DNA was 58.3%
van der Laan *et al*[17]	2007	Hepatocytes	Flow cytometric quantitation	A significant correlation was found between the percentage of infected hepatocytes and the intracellular expression level of HBsAg ($R = 0.841$, $P < 0.001$)
Goh *et al*[22]	2008	Cerumen and otorrhoea samples along with serum	HBsAg and HBeAg were detected by Enzyme Immunoassay and HBV DNA was detected by quantitative PCR	HBV DNA was detected in 66.7% of cerumen samples and 100% of otorrhoea samples
Chen *et al*[25]	2009	Parotid tissue	Serological markers by immunocytrochemistry and HBV DNA by PCR	Overall positivity rate was 54.5% to 58.3%
Nuriya *et al*[18]	2010	Hepatocyres	PCR - *In situ* hybridisation	All hepatocytes were infected with HBV in chronic liver disease
Villar *et al*[14]	2011	Dried blood spots	HBsAg, anti-HBc, and anti-HBs were detected by ELISA	Sn was 90.5%, 97.6%, and 78% for anti-HBc, HBsAg, anti-HBs assays, and Sp was 92.6%, 96.7%, and 97.3% for anti-HBc, HBsAg, and anti-HBs assays, respectively
Wu *et al*[29]	2012	Paraffin embedded intrahepatic and extrahepaticcholangiocarcinoma tissue	HBV DNA by nested PCR and HBV related antigens by immunohistochemistry method	HBV DNA and HBV antigens were detected significantly in cases of intrahepatic cholangiocarcinoma
Arora *et al*[24]	2012	Saliva	HBsAg was detected by ELISA	Sn 74.29% and Sp 100%
Cobo *et al*[27]	2012	Spent culture media and liquid nitrogen samples of oocytes and embryos	Reverse transcriptase PCR	Viral sequences were not detected in these samples from seropositive patients
Ye *et al*[28]	2013	Discarded test tube embryos from mothers with chronic HBV infection undergoing *in vitro* fertilization treatment	Single cell reverse transcriptase PCR	Detection rate was 13.2%
Eftekharian *et al*[23]	2013	Cerumen along with serum	HBV DNA was detected by PCR	HBV DNA was detected in 6.6% of HBsAg positive patients
Kong *et al*[26]	2013	Frozen renal tissue	HBsAg and HBcAg detected by immunohistochemistry	Found positive in 9 out of 500 patients of glomerulonephritis without serological evidence
Ross *et al*[15]	2013	Dried blood spots	HBsAG, anti HBcAg, anti HBsAg detected by Abbott Architect and HBV DNA by artus HBV LC PCR	Sensitivity was 98.6%, 97.1%, 97.5%, 93%
Alidjinou *et al*[16]	2014	Dried plasma spots	HBsAg and HBV DNA detected by ELISA and PCR	Sn and Sp 100% for serological markers and Sn 96%, Sp 100% for HBV DNA
Zhong *et al*[19]	2014	Hepatocytes	Covalently closed circular HBV DNA detected by *in situ* PCR	Helps to detect recurrence of HBV
Huang *et al*[20]	2015	Formalin fixed paraffin embedded hepatocellular carcinoma tissue	Droplet digital PCR to detect HBV copy number	Highly sensitive method

HBsAg: Hepatitis B surface antigen; DBS: Dried blood spots; Sn: Sensitivity; Sp: Specificity; HBcAg: Hepatitis B core antigen; HBV DNA: Hepatitis B virus deoxy ribonucleic acid; PCR: Polymerase chain reaction; ELISA: Enzyme linked immunosorbent assay; Anti-HBc: Antibody to HBcAg; Anti-HBs: Antibody to HBsAg.

to detect different serological markers. Various ELISA kits are commercially available. Maity *et al*[31], 2012 evaluated 3 ELISA kits (Span diagnostics Ltd., J. Mitra and Co. Pvt. Ltd., and Transasia Biomedicals Ltd.) in 300 samples. All the kits were found to be good at screening having higher specificity. Positive predictive value (PPV) and negative predictive value (NPV) were 100% when panels were tested by kits of J. Mitra and Co. Pvt Ltd. and Transasia Biomedicals Ltd, though little less in case of kit of Span Diagnostics Ltd. Though in most of the cases kits are evaluated against a pretested panel, when the results are projected to a population, PPV and NPVs depend widely on the prevalence of that infection. Different researchers have modified this method even. Yazdani *et al*[32], 2010 used novel monoclonal antibodies as capture layer and a polyclonal biotinylated antibody as detector phase to develop one new ELISA system. Sensitivity and specificity of the assay were 98.98%

and 99.6%, respectively when compared to established commercial kit. The performance of ELISA depends on concentration of coating antibody, conjugates and sera. Using different concentrations by checkerboard titration method Fatema *et al*[33], found that, optimal concentration of coating antibody to be 0.25 ng/mL and 1 in 9 diution of both conjugate and sera. Poly L lysine coated magnetic beads were used to concentrate the virus by Satoh *et al*[34]. HBsAg and Anti HBc were tested by Enzyme Immuno Assay (AxSYM, Abbott), and haemaglutination inhibition test. By HBsAg EIA they were able to detect 27 out of 40 occult HBV infection. Antigen/ antibody quality is very important for diagnostic accuracy. Recombinant HBcAg is expressed in *Escherichia coli* and *Pichia pastoris* (*P. pastoris*) by Li *et al*[35], 2007 and used in ELISA for detection of anti HBcAg. *P. pastoris* derived antigen was more specific and sensitive in detection than the other counterpart.

Chemiluminescent enzyme immunoassay and its modifications: This rapid immunoassay method uses antigen or antibodies labeled with luminescent molecules. This is more sensitive than ELISA. In comparative studies with PCR the sensitivity of chemiluminescent enzyme immunoassay (CLEIA/ CLIA) is 96%[36]. Its sensitivity is even more enhanced by different modifications by researchers. Matsubara *et al*[37], 2009 developed a highly sensitive CLEIA method for quantitative detection of HBsAg by a combination of monoclonal antibodies each specific for epitopes of HBsAg. This method was 230 fold more sensitive than existing CLIA methods. Incorporating firefly luciferase as labelling enzyme a bioluminescent enzyme immunoassay was developed by Minekawa *et al*[38]. This became 50 fold more sensitive than conventional CLIAs. Liu *et al*[39], 2013 developed an amplified luminescent proximity homogeneous assay (AlphaLISA) for HBsAg. The detection sensitivity was as low as 0.01 IU/mL, when compared with the commercial light-initiated chemiluminescence assay. The correlation coefficient of this assay was 0.921.

Automated systems: AxSYM (Abbott) is the first automated third generation immunoassay system. Abbott PRISM HBsAg assay is an *in vitro* chemiluminescent immunoassay. A new prototype assay based on magnetic micro particle was developed in this sysyem to increase its sensitivity and ability to detect mutants. Lou *et al*[40] demonstrated that it can detect more commercially available seroconversion panel members (185 of 384) than PRISM (181). Researchers have evaluated different automated CLIA systems across the world. Elecsys (Roche) and Architect (Abbott) gave comparable results for quantitation of HBsAg when assessed by Gupta *et al*[41]. Beckman Coulter's anti-HBs chemiluminescence immunoassay (Access AbHBsII) was evaluated in 1207 routine samples prescreened with AxSYM (Abbott) for detection of anti HBsAg by Motte *et al*[42]. Sn, Sp, PPV and NPV were 97.8%, 98.1%, 96%, and 99%, respectively. ADVIA centaur CP Immunoassay System is based on chemiluminescent with advanced acridinium ester technology. van Helden *et al*[43] compared its performance with AxSYM, Abbott. It's Sn and Sp was 100% and 99.5%. The automated chemiluminescent micro particle immunoassay of Abbott (Architect) detects anti-HBc. Borderline reactivity in this system was reassessed by 2 other tests: Microparticle enzyme immunoassay (MEIA, AxSYM, Abbott), and enzyme linked fluorescent assay (ELFA, VIDAS Anti-HBc Total Ⅱ, bioMérieux) by Ollier *et al*[44]. 42.99% of borderline reactive samples were found to be positive by MEIA, ELFA. So, other confirmatory tests should be done in this scenario. This commonly used Abbott's Architect system was also compared with another fully automated and closed DiaSorinLIAISON(®)XL by Krawczyk *et al*[45] and Kinn *et al*[46]. The two tests were in > 95% agreement in both the studies. In a multicentre study, automated VIDAS HBsAg Ultra [long (L) and short (S)] incubation protocol (Biomérieux) was compared to AxSYM (Abbott) by Weber *et al*[47]. Sn of the VIDAS HBsAg Ultra (L), (S) and the AxSYM HBsAg v2 were 99.07%, 97.87% and 94.14% respectively. Sp was 100% for VIDAS. The mean time of the diagnostic window was shortened with the VIDAS HBsAg Ultra (L) and (S) when compared with the AxSYM HBsAg v2 by 1.06 and 0.66 d, respectively. Sn for the VIDAS HBsAg Ultra (L), (S) and AxSYM HBsAg v2 were 99.07%, 97.87% and 94.14%. The Sp were 100% (VIDAS HBsAg Ultra L and S) and 99.6% (AxSYM HBsAg v2)[47].

Other methods: A biosensor based imaging ellipsometry was developed and validated for 169 patients by Qi *et al*[48]. They concluded that this method could detect 5 markers within 1 h with acceptable agreement when compared to ELISA. Another novel assay based on magnetic beads and time resolved fluroimmunoassay (TR FIA) was developed by Ren *et al*[49], 2014. The detection antibodies were europium labeled and capturing monoclonal antibodies were immobilized on magnetic beads. The test results had correlation with CLIA (Y = 1.182X - 0.017, R = 0.989). The same TRFIA method was also used to detect HBV Pre S_1 antigen by Hu *et al*[50] and HBsAg by Myyryläinen *et al*[51]. Burbelo *et al*[52] used Luciferase Immmunoprecipitation system to detect HBV infection. This could correctly predict the HBV status in all but 2 of 99 assays. Fletcher *et al*[53] standardised an in house neutralization test for confirmation of HBsAg. Six hundred and fifteen HBsAg samples were subjected to the test. 100% of high reactive samples and 93% of low reactive samples were neutralized by this method, whereas 100% of grey zone reactive samples were negative.

POCT: POCTs are developed to make diagnosis more rapid and accessible to patients. Njai *et al*[54] validated 3 POCTs (Determine, Vikia and Espline) for detecting HBsAg in field or laboratory setting in Gambia, Western Africa. All the 3 tests gave acceptable result when

Table 2 Studies describing different quantitative molecular methods

Ref.	Year of publication	Method of quantitation	Detection limit
Garson *et al*[58]	2005	FRET based real time PCR assay	Sn at 95% detection level was 24.2 IU/mL
Welzel *et al*[59]	2006	Novel real time PCR	Sn at 95% detection level was 56 IU/mL
Mazet-Wagner *et al*[60]	2006	Real time PCR assay to detect total HBV DNA and cccDNA from serum and peripheral blood mononuclear cells	27 IU/mL
McCormick *et al*[61]	2006	Procleix Ultrio Assay (Multiplex PCR) to detect HIV 1, HCV RNA and HBV DNA simultaneously	Sp ⩾ 99.5%, Sn is > 95% with detection limit for HBV DNA of 15 IU/mL
Cai *et al*[62]	2008	Real time fluorogenic Loop Mediated Isothermal Amplification (RtF-LAMP)	At 95% detection level 210 copies/mL
Paraskevis *et al*[63]	2010	New ultrasensitive in house real time PCR assay	Sn at 95% and 50% detection level: 22.2 IU/mL and 8.4 IU/mL
Chevaliez *et al*[64]	2010	v2.0 of the CAP/CTM assay	Highly Sn, could even detect 6.25 IU/mL HBV DNA; Sp is 99%, Intra-assay and interassay coefficients of variation ranged from 0.21% to 2.67% and from 0.65% to 2.25%, respectively
Sun *et al*[65]	2011	Duplex real-time PCR assay using two sets of primers/probes and a specific armored DNA as internal control	Detection limit 29.5 IU/mL; Sp 100%
Cha *et al*[66]	2013	ExiStation HBV diagnostic system	9.55 IU/mL
Yang *et al*[67]	2014	Colorimetric PCR with DNA zyme containing probe	Broad range of linearity and high sensitivity
Kania *et al*[68]	2014	2 in house real time PCR targeting X (qPCR1) or S (qPCR2) genes	qPCR1: 104 IU/mL; qPCR2: 91 IU/mL

FRET: Fluoresence resonant energy transfer; PCR: Polymerase chain reaction; cccDNA: Covalently closed circular DNA; Sn: Sensitivity; Sp: Specificity; HBV DNA: Hepatitis B virus deoxy ribonucleic acid; v2.0: Version 2.0; HCV: Hepatitis C virus; CAP/CTM: CobasAmpliPrep/CobasTaqMan.

compared to AxSYM HBsAg ELISA as reference test. Rapid kits (J. Mitra and Co. Pvt. Ltd., Span diagnostic Ltd., Standard Diag. Inc.) were also evaluated by Maity *et al*[31], 2012. Sn, Sp, PPV and NPV of all the kits were 100%.

Comparison of different methods

Liu *et al*[55] compared test results of 4 different types of serological tests in 116455 samples. Chemiluminescent microparticle immunoassay (CMIA), electrochemiluminescent immunoassay (ECLIA), ELISA and golden immunochromato-graphic assay (GICA) were used to test the HBsAg level. For qualitative results GICA was significantly less specific than the other 3 tests. Compared to CMIA the false negativity rate of ECLIA, ELISA and GICA were 0.2%, 1.3%, 12.3%.

Molecular methods: Molecular methods used in diagnosis can be categorized as nucleic acid hybridization, nucleic acid amplification, sequencing and enzymatic digestion of nucleic acids.

Hybridization technique: Conventional hybridization technique, though highly specific, lacks sensitivity. Yao *et al*[56] constructed a peptide nucleic acid probe which combined with target DNA sequences more efficiently than DNA probes. The detection limit was 8.6 pg/L and Sp was 94.4%.

Nucleic acid amplification technique: Amplification techniques can be: (1) target amplification: PCR, nucleic acid sequence based amplification, transcription mediated amplification, Strand Displacement amplification, *etc.*; (2) Signal amplification: Branched DNA probe (bDNA); and (3) probe amplification: Ligase chain reaction. These techniques can qualitatively or quantitatively detect minute amount of HBV DNA present in the sample. Some researchers have even combined 2 different methods to increase Sn. Combination of bDNA and HBV PCR helped in detection of HBe Ag positive chronic HBV patients by Ozdarendeli *et al*[57].

Quantitative detection is very important for monitoring of HBV infection. Molecular methods have been used for quantitation by different researchers (Table 2). In this review, of the entire in house and automated molecular techniques version 2.0 (v2.0) of the Cobas-AmpliPrep/CobasTaqMan (CAP/CTM) assay was found to be most sensitive, with a lower detection limit of only 6.25 IU/mL[63]. Commercial assays were more sensitive than in house assays.

Park *et al*[69] evaluated Magicplex™ HepaTrio Real-time Detection test, a multiplex PCR assay for the detection of hepatitis A virus, HBV and HCV. Sn and Sp was 93.8% and 98.2%. Monjezi *et al*[70] developed a Taq Man real time detection assay based on the concept of phage display mediated immune PCR for the detection of HBcAg. This method was able to detect about 10 ng of HBcAg.

A rapid real time micro scale chip based PCR system consisting of 6 individual thermal cycling modules was developed by Cho *et al*[71]. It took less than 20 min to complete 40 thermal cycles. They conducted large clinical evaluation study to detect HBV infection. The sn and sp was 94% and 93% respectively.

The persistence of HBV can be detected by demonstration of covalently cccDNA. Takkenberg *et al*[72]

Table 3 Comparison of different methods

Ref.	Year of publication	Comparison between	Remarks
Hochberger *et al*[75]	2006	Automated COBAS AmpliPrep/COBAS TaqMan system (real time PCR) and Versant HBV 3.0	Good correlation between two
Juman Awadh *et al*[76]	2008	Versant HBV 3.0 (Bayer, branched DNA mediated assay) and Biotitre B (real time PCR variant)	Both were highly specific, though reproducibility of Versant HBV 3.0 was higher
Yang *et al*[77]	2009	Real Art HBV PCR Kit (Abbott, real time PCR) and Versant bDNA 3.0	Abbott's kit was more sn, detection limit 27 IU/mL
Louisirirotchanakul *et al*[78]	2010	fully automated ElecsysHBsAg Ⅱ assay, Architect, AxSYM and Advia Centaur HBsAg assays	The later 2 tests appeared less sensitive in detecting early HBV infection
Berger *et al*[79]	2010	CAP/CTM assay v2.0 and v1.0	Comparable results for all 278 tested samples
Lunel-Fabiani *et al*[80]	2010	Access immunoassay system from Beckman coulter with Abbott AxSYM and PRISM HBsAg assays; VIDAS was used to conclude discrepant results	Sn: 100%, Sp: 99.96%
Caliendo *et al*[81]	2011	Abbott RealTime HBV IUO, the Roche CAP/CTM HBV test, the Roche CobasTaqMan HBV test with HighPure system, and the Qiagen artus HBV TM ASR	Limit of detection of artus 1.5 log(10) IU/mL, of other 3 tests 1.0 log(10) IU/mL
Ismail *et al*[82]	2011	Abbott HBV real-time PCR (Abbott PCR), artus HBV real-time PCR with QIAamp DNA blood kit purification (artus-DB), and artus HBV real-time PCR with the QIAamp DSP virus kit purification (artus-DSP)	Lower limit of detection against WHO standards were 1.43, 82 and 9 IU/mL respectively
Yeh *et al*[83]	2014	Abbott real time HBV (RealTime assay) and CAP/CTM HBV assays 2.0 (TaqMan assay)	Real time assay's Sn: 98.2%, Sp: 100%. Good level of agreement between the two

PCR: Polymerase chain reaction; Sn: Sensitivity; Sp: Specificity; WHO: World Health Organization; v2.0: Version 2.0; CAP/CTM: CobasAmpliPrep/CobasTaqMan; HBV: Hepatitis B virus; bDNA: Branched DNA; HBsAg: Hepatitis B surface antigen.

developed a sensitive, specific and reproducible Real Time PCR to detect and quantitate cccDNA in chronic HBV patients. The lower limit of detection was 15 copies/PCR. cccDNA is detected by Southern blot analysis in cell cultures by Cai *et al*[73]. Guo *et al*[74] developed magnetic capture hybridization and quantitative PCR assay to detect cccDNA with a detection limit of 90 IU/mL.

Studies have been conducted to compare different methods (Table 3). Abbott's real time PCR kit was most sensitive with lower limit of detection of only 1.48 IU/mL. In comparison most of the automated systems had good agreement.

DISCUSSION

HBV can be detected reliably from DBS (Sn > 90% in all cases). In certain cases of occult infections or seronegative patients, HBV have been detected from hepatocytes or renal tissues also. Serological and Molecular assays are predominant and reliable methods for HBV detection. CLIA is more sensitive than ELISA. Rapid tests are also dependable and useful for screening purpose, especially in resource poor settings. Quantitation is important for monitoring. Real time PCR, bDNA assays are principal methods used for this purpose. Automated systems are more sensitive when compared to in house assays. Among the molecular techniques v2.0 of the CAP/CTM assay and Abbott real time PCR were found to be most sensitive with a lower detection limit of only 6.25 IU/mL and 1.48 IU/mL respectively.

ACKNOWLEDGMENTS

We acknowledge staff of Virology Division, National Institute of Cholera and Enteric Diseases for providing support.

COMMENTS

Background

In earlier days infection with hepatitis B virus (HBV) was detected by demonstration of antibody titer by Complement Fixation Test. The first solid phase sandwich radio immunoassay named Ausria 125 was developed by Ling *et al* at Abbott Laboratories (North Chicago). This highly sensitive detection method became a major discovery in the diagnosis of viral transfusion hepatitis and screening of blood donors. Since then innumerable serological and molecular methods have been developed for diagnosing HBV.

Research frontiers

This article provides an overview of detection of HBV infection employing different techniques.

Innovations and breakthroughs

Beside serum/plasma, HBV can be detected reliably from dried blood spots (DBS) (Sn > 90% in all cases). In occult infections or seronegative patients, HBV was detected from hepatocytes or renal tissues. Serological and Molecular assays are predominant and reliable methods. Chemiluminescent immunoassay is more sensitive than enzyme Linked Immunosorbent Assay. Rapid tests are useful for screening. Real time polymerase chain reaction (PCR), branched DNA assays are principal methods for quantitation. Automated systems are more sensitive compared to in house assays. CobasAmpliprep/CobasTaqMan version 2.0 assay and Abbott real time PCR were found to be most sensitive with a lower detection limit of only 6.25 IU/mL and 1.48 IU/mL respectively. Rapid tests are also highly sensitive and specific as evaluated by different researchers.

Applications

Use of DBS and validated rapid tests can aid in initial diagnosis in resource poor settings. Quantitation is important for monitoring and prognostic evaluation and automated systems are highly sensitive and efficient for this purpose.

Peer-review

The authors have performed a good study, the manuscript is interesting.

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P- Reviewer: Changotra H **S- Editor**: Tian YL
L- Editor: A **E- Editor**: Liu SQ

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DOI: 10.4254/wjh.v7.i23.2492

World J Hepatol 2015 October 18; 7(23): 2492-2496
ISSN 1948-5182 (online)

CASE REPORT

Arterial ischemia in the deportalized liver following associating liver partition and portal vein ligation for staged hepatectomy

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Author contributions: All authors contributed to this manuscript.

Institutional review board statement: This case report has been approved by the Karolinska University Hospital's Hepatic Surgery Board.

Informed consent statement: The patient described in the case report provided verbal consent prior to submission for publication.

Conflict-of-interest statement: The authors have no conflicts of interest to declare. This work did not receive any support or funding.

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Received: June 10, 2015
Peer-review started: June 12, 2015
First decision: July 25, 2015
Revised: August 3, 2015
Accepted: August 30, 2015
Article in press: August 31, 2015
Published online: October 18, 2015

Abstract

Associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) is a novel 2-stage technique intended to induce rapid growth of the future liver remnant (FLR). Initial reports of a 12% mortality rate have sparked debate regarding the safety of the procedure. A 64 years old male was planned for a right-sided hemi-hepatectomy due to colorectal cancer liver metastases. Intra-operatively it was decided to convert to an ALPPS due to unexpectedly small segments 2-4. Post-operative serum laboratory tests indicated an acute liver failure and radiological imaging showed no sign of arterial blood flow to the right hemi-liver. A computed tomography examination on post-operative day 3 revealed that the FLR had increased from 290 to 690 mL in 3 d (138% growth). In the following days serum values gradually improved and stage 2 was carried out on post-operative day 7. The rest of the hospital stay was uneventful and the patient made a full recovery. ALPPS is a fascinating advancement in liver surgery. Despite severe post-operative complications, in properly selected cases it provides successful outcomes that other modalities of treatment cannot offer.

Key words: Associating liver partition and portal vein ligation for staged hepatectomy; Liver surgery; Acute liver failure; Future liver remnant

Core tip: Associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) is a novel

2-stage technique intended to induce rapid growth of the future liver remnant. Initial reports of a 12% mortality rate have sparked debate regarding the safety of the procedure. We here present a complication following ALPPS that to our knowledge has never been described before. Yet proper patient selection resulted in a full recovery following a potentially life threatening liver failure.

Sanjeevi S, Sparrelid E, Gilg S, Jonas E, Isaksson B. Arterial ischemia in the deportalized liver following associating liver partition and portal vein ligation for staged hepatectomy. *World J Hepatol* 2015; 7(23): 2492-2496 Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i23/2492.htm DOI: http://dx.doi.org/10.4254/wjh.v7.i23.2492

INTRODUCTION

Associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) is a novel two-stage technique intended to induce rapid growth of the future liver remnant (FLR). Classically the technique is applied in the setting of an extended right-sided hemi-hepatectomy. The stage 1 operation involves transection of the right portal vein, followed by division of the parenchyma between the portal flow-deprived segments 4-8 and the normally vascularized FLR (segments 1-3). After a short interval (in general 7 d at our institution) and having confirmed adequate growth of the FLR, stage 2 is performed with removal of the deportalized liver[1]. Viable alternatives to ALPPS include portal vein embolization, portal vein ligation and two-stage hepatectomy[2]. The decision to perform an ALPPS procedure can also be made intra-operatively when detection of additional disease makes other forms of therapy unfeasible[3]. The procedure is applicable in a variety of liver tumors, but is utilized most often for colorectal cancer liver metastasis (CRLM)[4]. The question whether ALPPS is a superior choice compared to previously established surgical procedures is still unanswered. Initial reports of a 12% mortality rate have sparked debate regarding the safety of the procedure[5]. Large multicenter retrospective studies have studied the morbidity and mortality associated with ALPPS, but a prospective randomized control trial is yet to be performed[3,4,6].

In the following case report, we present a post-operative complication following ALPPS for CRLM, to our knowledge not previously described.

CASE REPORT

A 64 years old male with no comorbidities was diagnosed with rectal cancer at a screening colonoscopy. The tumor was staged as a T3cN0M0 and was surgically resected. A scheduled follow-up at 2 mo revealed elevated carcinoembryonic antigen levels and a computed tomography (CT) thorax/abdomen showed 9 metastatic lesions in segments 5, 6, 7 and 8. The patient received 4 cycles of FOLFOX and subsequent imaging showed marked regress of the hepatic lesions (Figure 1A). It was also noted that the right hepatic artery arose directly from the superior mesenteric artery (type Ⅲ according to the Michel classification). Following 2 additional cycles of chemotherapy, the patient was planned for a right-sided hemi-hepatectomy.

Intra-operatively, pronounced chemotherapy induced liver changes were evident with signs of sinusoidal obstruction; *i.e.*, blue liver syndrome. Further exploration showed that segments 2, 3 and 4 were unusually small (post-operative calculation of the FLR (segments 1-4) 290 mL, giving a FLR/BW-ratio of 0.34% and a ratio of FLR/total estimated liver volume (sFLR) of 16.1% (Figure 1A). It was decided intra-operatively to deviate from the initial planned one-stage right-sided hemi-hepatectomy and to perform an ALPPS procedure with segments 1-4 as FLR. The right hepatic vein, a large inferior hepatic vein ("Makuuchi vein"), right hepatic artery and right bile duct were isolated and marked with rubber vessel loops (Figure 2). The right portal vein was divided and the liver was transected with a cavitron ultrasonic surgical aspiration. Intra-operative bleeding was 400 mL, no blood was administered intra-operatively and no hypotensive episodes were noticed during the operation.

On post-operative day two, serum laboratory tests showed signs of acute liver failure and hepatic ischemia [international normalised ratio (INR) of 2.1 (ref < 1.2), bilirubin 61 μmol/L (ref < 26 μmol/L), aspartaat aminotransferase (ASAT) 131 ukat/L (ref < 0.76 ukat/L), alanine aminotransferase (ALAT) 94.2 ukat/L (ref < 1.20 ukat/L) and creatinine 140 μmol/L (ref < 100 μmol/L)] (Table 1). An ultrasound of the liver showed no sign of arterial blood flow to the right hemi-liver. Following proper rehydration, a CT examination was performed on post-operative day 3, which confirmed occlusion of the right hepatic artery and showed an ischemic right hemi-liver. It was, however, noted that the FLR had increased from 290 to 690 mL in only 3 d translating into a 138% increase of the FLR volume (Figure 1B). Already at this stage the FLR/BW-ratio was 0.8% and sFLR 38.3%. Surprisingly, on the late venous phase of the CT there was contrast enhancement in the hepatic venous system of the deportalized segments, not only in the large veins close to the inferior vena cava, but also in the peripheral vessels (Figure 3A).

Despite impressive growth in terms of volume, the patient was clearly still in severe liver failure. The decision was taken to observe the patient and postpone stage 2 of the ALPPS procedure for as long as possible, closely monitoring the patient's liver function during the following days. On post-operative day 6 the serum values had gradually improved and were as follows: INR 1.7, bilirubin 52 μmol/L, ASAT 1.54 ukat/L, ALAT 11.85 ukat/L (Table 1). At this point the patient fulfilled the Balzan 50-50 criteria for post-hepatectomy liver failure[7]. However, apart from some mild confusion

Table 1 Patient's laboratory liver values during in-hospital stay

Reference intervals	INR	Bilirubin (μmol/L)	ASAT (ukat/L)	ALAT (ukat/L)	FLR (mL)	FLR/BW ratio (%)	sFLR (%)
	< 1.2	< 26	< 0.76	< 1.20			
Pre-op day (before stage 1)							
1	1.0	4	0.51	0.58	290	0.4	16.1
Post-op day (after stage 1)							
3	2.1	61	131.00	94.20	690	0.8	38.3
6	1.7	52	1.54	11.85	746	0.9	41.4
14	1.4	17	0.65	1.35	-	-	-
29	1.3	24	0.77	1.09	-	-	-

FLR: Future liver remnant; ASAT: Aspartaat aminotransferase; ALAT: Alanine aminotransferase; INR: International normalized ratio; sFLR: Standardized future liver remnant.

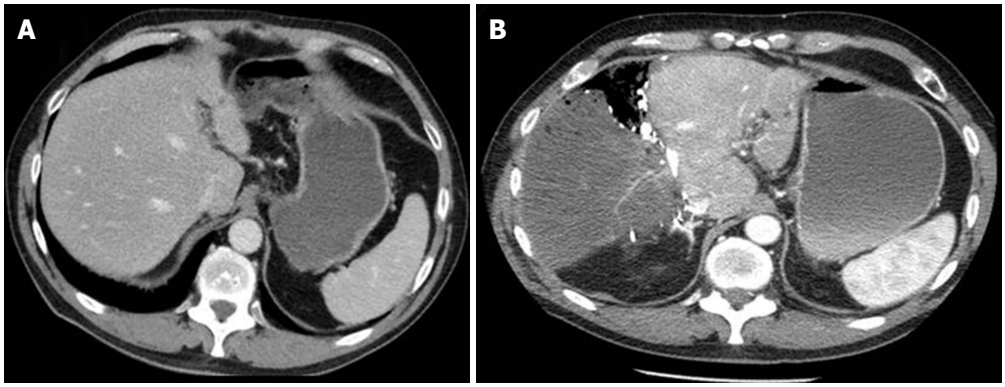

Figure 1 Pre-operative computed tomography (A) and on post-operative day 3 (B) following stage Ⅰ of the associating liver partition and portal vein ligation for staged hepatectomy procedure.

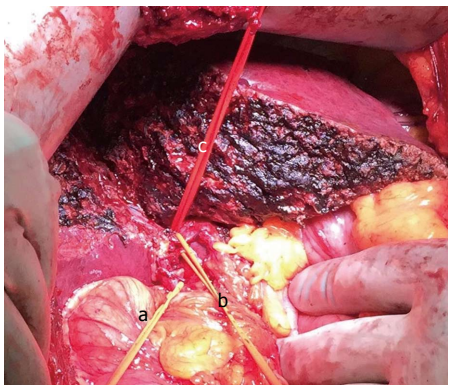

Figure 2 Vessel loops around the right hepatic artery (a), right bile duct (b) and inferior hepatic vein ("Makuuchi vein").

and fever, the patient was asymptomatic. CT images now (post-operative day 6) showed signs of complete hepatic ischemia with air in the intrahepatic bile ducts. The FLR had only hypertrophied slightly since post-operative day 3 and now measured 746 mL. Once again, on the late venous phase images there was contrast-enhancement in the hepatic venous system of the deportalized segments, both in the central and peripheral vessels (Figure 3B).

Leaving the ischemic right hemi-liver for a longer period of time was considered a clear risk and the decision to proceed to stage 2 was made. Stage 2 of the ALPPS was carried out on post-operative day 7. A dark necrotic right hemi-liver was found at laparotomy (Figure 4). The right hepatic artery, bile duct and right hepatic vein were divided by staples. The right hemi-liver was removed and the abdomen was closed. No complications were experienced post-operatively and the patient recovered well. The only complaint was recurring hiccups that that ceased spontaneously 7 d after stage 2 (the patient experienced a prolonged period of unexplained hiccups even after the colectomy of the primary tumour). At this point the serum values had further improved (INR 1.4, bilirubin 17 μmol/L, ASAT 0.65 ukat/L, ALAT 1.35 ukat/L) (Table 1). The rest of the hospital stay was uneventful and the patient was discharged on post-operative day 22.

DISCUSSION

ALPPS is a relatively new technique with the mechanisms driving growth of the FLR not fully understood. Complete ischemia of the deportalized liver following ALPPS has to our knowledge never before been described. Remarkably, despite this unforeseen complication, the patient successfully underwent even the second stage of the operation and made an uneventful recovery.

Recent reports have questioned the use of ALPPS due to a morbidity of 43%-60% and mortality of 15%-20%[8-11]. A multicenter registry study by Schadde *et al*[4] reported severe complications (Clavien-Dindo ≥ Ⅲb) in 27% of patients and a 90 d mortality of 9%.

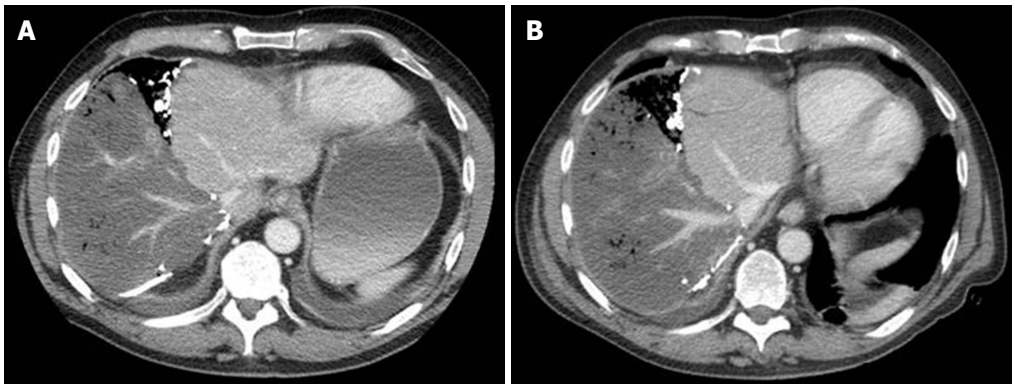

Figure 3 Post-operative late venous phase computed tomography with contrast enhancement of the hepatic venous system of the ischemic right hemi-liver on (A) day 3 and (B) day 6.

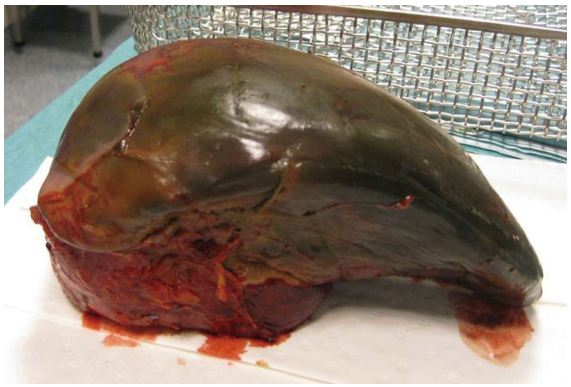

Figure 4 Ischemic right liver lobe following stage 2 of associating liver partition and portal vein ligation for staged hepatectomy.

Significantly better outcomes were noted in patients with CRLM and patients under the age of 60. Although our patient was 64 years old, he was previously healthy with no recorded comorbidities. Other independent risk factors included red blood cell transfusions and stage Ⅰ operating times longer than 300 min, two factors our patient did not fulfill. Truant *et al*[6] hypothesize that complications occurring between stage 1 and 2 of ALPPS play a significant role in morbidity and mortality. More specifically, post stage 1 biliary fistula, ascites and infected bile collections worsen overall outcome. Again, our patient experienced none of the abovementioned complications and had an uncomplicated post-operative course apart from post-hepatectomy liver failure and the described liver ischemia.

Post-operative imaging showed that the FLR of the patient increased by 157% over a period of 6 d. Of greater significance is that the major part of the growth (136%) occurred in less than 3 d after stage 1. This is an impressive increase in size, considering that metabolic support from the deportalized liver was sub-optimal. Reported increases in the FLR range from an average of 49%-80% over 7 d[3,4,12]. It should be emphasized that the increase in volume does not necessarily correlate with an increase in function of the FLR[13].

Another unique aspect of this case was the post-operative venous flow noted in the deportalized right hemi-liver (Figure 3). This phenomenon may explain the subsequent improvement of serum liver values between stage 1 and 2. Additionally, an increase in venous flow was noted between post-operative days 3 and 6 (Figure 3). The source of the venous blood is however not clearly understood, considering that blood flow in the right hepatic artery was absent on imaging. A standing theory is that the right hemi-lobe received arterial blood from bile duct associated vessels with retrograde filling of the portal veins from the hepatic sinusoids[14]. Retrograde filling of the right hepatic and inferior hepatic veins could also have been possible[15].

The present case was extensively analyzed at the institutional morbidity and mortality review board. A point of criticism was the initial decision to proceed with a right-sided hemi-hepatectomy despite the small FLR. If this was pre-operatively noticed the patient may have benefited from portal vein embolization. Despite the misjudgment, the intra-operative decision to proceed with ALPPS was considered the best option for the patient. The reasoning behind the arterial occlusion was also discussed. A technical error was considered unlikely as optimal flow was palpated in the right hepatic artery prior to closure of the abdomen. It could be hypothesized that trauma during the surgery precipitated the formation of an arterial thrombus. Occlusion of the right hepatic artery has been described following liver transplantations[16]. Damage during the Pringle maneuver and the formation of a pseudoaneurysms have been proposed as underlying mechanisms.

To conclude, ALPPS is a fascinating advancement in liver surgery, requiring further studies to better our understanding. Yet, despite post-operative complications, in properly selected cases it provides successful outcomes that other modalities of treatment cannot offer.

COMMENTS

Case characteristics

A 64 years old male that went into severe liver failure following associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) due to arterial ischemia in the deportalized liver.

Clinical diagnosis
Liver failure following stage Ⅰ of ALPPS due to arterial ischemia.

Differential diagnosis
The diagnosis of liver failure was well established by imaging and laboratory tests. The exact mechanism for the full recovery of the patient is not completely understood.

Laboratory diagnosis
An acute rise in international normalized ratio and serum bilirubin, alanine aminotransferase and aspartaat aminotransferase which gradually improved over time.

Imaging diagnosis
An ultrasound of the liver showed no sign of arterial blood flow to the right hemi-liver and a computed tomography examination confirmed occlusion of the right hepatic artery and an ischemic right hemi-liver.

Pathological diagnosis
Pathology following stage Ⅱ of ALPPS confirmed the underlying disease to be colorectal liver metastases.

Treatment
Supportive therapy.

Related reports
This is the first report to describe arterial ischemia in the deportalized liver following ALPPS.

Term explanation
ALPPS: Associated liver partition and portal vein ligation for staged hepatectomy; FLR: Future liver remnant; CRLM: Colorectal liver metastasis.

Experiences and lessons
ALPPS is a fascinating advancement in liver surgery requiring further studies to better our understanding. Despite post-operative complications, in properly selected cases it provides successful outcomes that other modalities of treatment cannot offer.

Peer-review
ALPPS is an interesting novel two stage surgical procedure for extended liver tumors with more and more applications described into literature: This article describes a case report of application of ALPPS procedure in a patient intraoperatively judged as not eligible to a right hepatectomy due to unexpected little remnant volume: The attention is focalized on a specific particular postoperative complication, never described previously into literature.

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P- Reviewer: Campos RR, Sguinzi R, Vennarecci G
S- Editor: Ji FF **L- Editor**: A **E- Editor**: Liu SQ

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