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WJH covers topics concerning arrhythmia, heart failure, vascular disease, stroke, hypertension, prevention and epidemiology, dyslipidemia and metabolic disorders, cardiac imaging, pediatrics, nursing, and health promotion. Priority publication will be given to articles concerning diagnosis and treatment of hepatology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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Xiu-Xia Song, Vice Director
World Journal of Hepatology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: editorialoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
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TOPIC HIGHLIGHT

2016 Liver Transplantation: Global view

Management issues in post living donor liver transplant biliary strictures

Manav Wadhawan, Ajay Kumar

Manav Wadhawan, Ajay Kumar, Fortis Escorts Liver and Digestive Diseases Institute, Okhla, New Delhi 110025, India

Author contributions: Wadhawan M designed research, collected data, performed ERCP's, analyzed data, and wrote paper; Kumar A designed research, performed ERCP's and wrote paper.

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Correspondence to: Ajay Kumar, MD, DM, MAMS, FRCP (Glasgow), Chief and Executive Director, Fortis Escorts Liver and Digestive Diseases Institute, Okhla, New Delhi 110025, India. ajaykge@hotmail.com

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Abstract

Biliary complications are common after living donor liver transplant (LDLT) although with advancements in surgical understanding and techniques, the incidence is decreasing. Biliary strictures are more common than leaks. Endoscopic retrograde cholangiopancreatography (ERCP) is the first line modality of treatment of post LDLT biliary strictures with a technical success rate of 75%-80%. Most of ERCP failures are successfully treated by percutaneous transhepatic biliary drainage (PTBD) and rendezvous technique. A minority of patients may require surgical correction. ERCP for these strictures is technically more challenging than routine as well post deceased donor strictures. Biliary strictures may increase the morbidity of a liver transplant recipient, but the mortality is similar to those with or without strictures. Post transplant strictures are short segment and soft, requiring only a few session of ERCP before complete dilatation. Long-term outcome of patients with biliary stricture is similar to those without stricture. With the introduction of new generation cholangioscopes, ERCP success rate may increase, obviating the need for PTBD and surgery in these patients.

Key words: Living donor liver transplant; Biliary complications; Biliary strictures; Endoscopic retrograde cholangiopancreatography; Percutaneous transhepatic biliary drainage

Core tip: Biliary complications are the Achilles heel of liver transplantation and are more common in live related liver transplant than cadaver liver transplant. Endoscopic retrograde cholangiopancreatography along with percutaneous transhepatic biliary drainage is successful in managing more than 90% of biliary complications after liver transplant. Although strictures increase morbidity after liver transplant, the mortality rates are not influenced by biliary strictures. This review provides diagnostic approach and management algoritham of these biliary structures in the setting of right lobe liver transplant.

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INTRODUCTION

Liver transplantation has become a well-established treatment for end stage liver disease[1]. Living donor liver transplant (LDLT) is still the predominant form for transplantation in eastern part of the world including India. Biliary leaks and strictures are still recognized as the most common complications after LDLT. Biliary complications after orthotopic liver transplantation (OLT) are evaluated and treated by endoscopy; only a few require percutaneous interventions. Surgical intervention is necessary only in treatment failures as a backup option[2]. Non-anastomotic stricture (NAS) are uncommon and difficult to treat with endoscopic retrograde cholangiopancreatography (ERCP)/percutaneous transhepatic biliary drainage (PTBD). NAS's often require re-transplant as the only effective treatment option. This review will focus on the diagnosis and management of anastomotic biliary strictures (ABS) after LDLT.

MAGNITUDE OF THE PROBLEM AND CONTRIBUTING FACTORS

Incidence of biliary complications after liver transplantation has been variably reported between 5%-40% (Table 1). The incidence is higher after LDLT compared to deceased donor liver transplant (DDLT)[3]. Over last 3 decades the reported incidence of bile leaks as well strictures is decreasing (Table 1)[4-15]. This can be ascribed to better understanding of the technical causes leading to biliary complications.

Although the cause of biliary complications is mainly technical, various factors have been implicated in the development of these complications. Overview of the possible contributory factors and role of each has been listed in Table 2[12,14-22].

In LDLT the anastomosis is made between right anterior and posterior ducts of the donor with the common hepatic duct of the recipient. The various types of anastomoses are shown in Figure 1. There could be one anastomosis if common trunk of right hepatic duct is available (Figure 2) or there could be two or more anastomoses (Figure 3). Usually, in case of double duct anastomosis, native right anterior and right posterior are used to anastomose to donor ducts. If the two ducts are close together, sometimes ductoplasty with single recipient duct is done (Figure 4). In rare circumstances, surgeons have used cystic duct for anastomosis to one of the ducts of the donor. In our own experience, the use of cystic duct for anastomosis leads to stricture formation in almost all cases (unpublished data). Once the stricture develops in cystic duct anastomosis, it is technically almost impossible to handle with endoscopy (Figures 5 and 6). At our center, we have abandoned using cystic duct of the recipient for ductal anastomosis.

Table 1 Evolution of post living donor liver transplant biliary complications with the changing time

Ref.	Year	Country	*n*	Follow-up (mo)	Leaks	Strictures
Sugawara *et al*[4]	2003	Japan	92	45	20.6%	9.7%
Gondolesi *et al*[5]	2004	United States	96	24.2	21.9%	22.9%
Lee *et al*[6]	2004	South Korea	31	10.5	6.5%	12.9%
Liu *et al*[7]	2004	China	41	13.3	7.3%	24.3%
Soejima *et al*[8]	2006	Japan	182	21	11.5%	25.3%
Shah *et al*[9]	2007	Canada	128	23	14.8%	17.1%
Mita *et al*[10]	2008	Japan	231			9.5%
Marubashi *et al*[11]	2009	Japan	83	32.4	1.2%	7.2%
Kim *et al*[12]	2010	South Korea	22	51.3	0%	9.1%
Wadhawan *et al*[14]	2013	India	65	28	8.8%	10.3%
Mizuno *et al*[13]	2014	Japan	108	58.4	5.6%	13.9%
Vij *et al*[15]	2015	India	127	9.32	0.7%	0%

DIAGNOSIS

Biliary complications related to anastomosis could be leaks or strictures. The diagnosis of biliary complications is made on the basis of clinical symptoms (jaundice, itching, bilious drainage, and cholangitis), deranged liver function tests (LFT), and/or radiologic imaging. Imaging plays a very important role in diagnosis as well management of biliary problems. Ultrasonography (USG), magnetic resonance cholangiopancreatography (MRCP), hepatobiliary scintigraphy (HBS) as well as computerized tomogram (CT) have an important role in diagnosis and management of biliary problems.

The timeline for biliary complications after transplant is shown in Figure 7[23].

Most bile leaks would present early after transplant (within first few weeks), almost all would manifest in 3 mo[23]. Leaks presenting early after transplants are usually diagnosed clinically by the presence of bile in the drains. Sometimes, even early bile leaks may be tricky to diagnose as many patients produce large amount of peritoneal fluid for a few days after transplant, thus diluting the bile. On the other hand, late leaks may present after drain removal with pain abdomen and fever with or without jaundice and septicemia. Role of static imaging (USG, CT, MRCP) in diagnosis of leaks is mainly to diagnose collections. However, HBS may be useful to diagnose subclinical leaks (cut surface leaks after LDLT, minor leaks from anastomotic site not apparent on drain)[24]. Minor bile leaks may have minimal derangement of LFT's, any fever with pain abdomen should raise a suspicion of bile leak. Any undiagnosed sepsis in post-operative setting, should raise the suspicion of bile leak and all efforts should be made to diagnose it.

Biliary strictures usually present later than leaks but within first year after transplant[23]. The most common

Table 2 Overview of factors contributing to biliary complications

Ref.	Year	Factor	Inference
Dalgic *et al*[16]	2005	Corner sparing sutures	Decreased incidence of complications
Castaldo *et al*[17]	2007	Continuous *vs* interrupted sutures	No difference in two techniques
Soejima *et al*[18]	2008	Hilar dissection to preserve blood supply	Decreased incidence of complications
Lin *et al*[19]	2009	Microsurgical biliary reconstruction	Decreased incidence of complications
Kim *et al*[12]	2010	Telescopic reconstruction of bile duct	Decreased incidence of complications
Chok *et al*[20]	2011	CIT and acute cellular rejection	Higher biliary complications with increased CIT Acute cellular rejection predicted biliary strictures
Horster *et al*[21]	2013	HCV infection as etiology	Higher incidence of biliary complications in patients with HCV infection and higher viral load
Wadhawan *et al*[14]	2013	Type of anastomosis	Higher incidence of biliary complications in double duct and cystic duct anastomosis
Mathur *et al*[22]	2015	Internal biliary stenting	No difference in complications with or without stenting
Vij *et al*[15]	2015	Corner sparing sutures Bile duct mucosal eversion	Decreased incidence of biliary complications

CIT: Cold ischemia time; HCV: Hepatitis C virus.

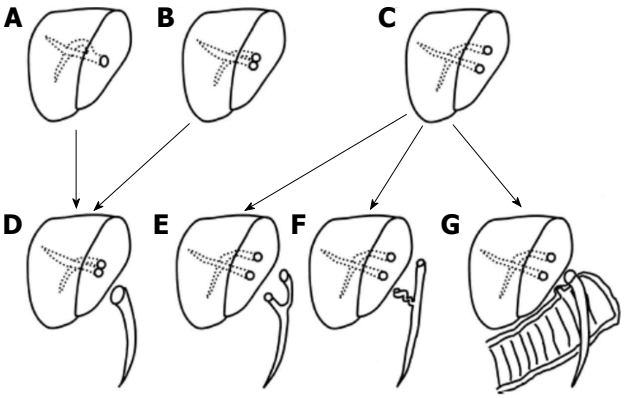

Figure 1 Types of biliary anastomoses and corresponding biliary reconstructions[54]. A: Single duct anastomosis; B: Double duct - minimum distance between two donor ducts, requires ductoplasty with recipient CBD; C: Double duct - two donor duct are far away, requires two separate duct anastomosis or a hepaticojejunostomy; D: Single duct to duct reonstruction; E: Double duct to duct reconstruction using right and left hepatic ducts; F: Double duct to duct reconstruction using cystic and CHD; G: Mixed type using duct to duct and hepaticojejunostomy. CHD: Common hepatic duct; CBD: Common bile duct.

presenting symptoms of stricture is itching with or without jaundice. LFT reveal a cholestatic pattern; bilirubin rise may be late in the course after LDLT. Less commonly, cholangitis may be the presentation of a biliary stricture. The first investigation in such cases is ultrasound of the abdomen. The presence of ductal dilatation has a high positive predictive value for the diagnosis of a stricture[25]. However, ductal dilatation is not prominent in many cases after LDLT. Absence of ductal dilatation has been previously reported to be an unreliable indicator of adequate biliary drainage[26]. It has been shown that donor bile ducts do not respond to the distal obstruction by same extent of dilatation as the non transplant liver[27]. MRCP has a sensitivity and specificity of 85%-90% in diagnosing biliary strictures after transplant[28,29]. The phenomenon of limited dilatation of donor ducts further underestimates the diagnosis of strictures on MRCP imaging. Specific criteria have been proposed for diagnosis on MRCP imaging[30]. The variables that need to be studied in MRCP include type of anastomosis, length of stricture, length of common stump proximal to the anastomosis and differential diameters of recipient and donor ducts, *etc.* These help in the diagnosis as well as planning of endoscopic treatment.

Acute cellular rejection is an important differential when we have graft dysfunction. In fact rejection has been shown to be associated with stricture[21]. In our own experience, in the presence of ductal dilatation patients should be first taken for biliary decompression and if graft dysfunction persists, they should be treated for rejection. In the absence of dilatation, a liver biopsy may help in diagnosing the predominant cause of graft dysfunction.

HBS has been used in diagnosing the biliary obstruction with variable results[31,32]. It has a high positive predictive value but low sensitivity and specificity. Hence it is not widely used in the diagnosis of strictures.

Despite the fact that diagnostic ERCP is on its way out, it still remains an important modality to diagnose and confirm suspected biliary strictures after transplant. Sometimes in doubtful cases, a direct cholangiography [ERCP, percutaneous transhepatic cholangiography (PTC)] is required for the diagnosis. Thus direct cholangiography is the gold standard not only in establishing the diagnosis but also in allowing therapeutic intervention in the same setting. ERCP being less invasive with lower complication rates, is the modality of choice and is preferred over PTC[2,14].

MANAGEMENT

Biliary strictures can be managed by either endoscopic access (ERCP) or by percutaneous access (PTBD). All over the world, ERCP is the treatment of choice for management of biliary strictures after LDLT and is preferred over PTBD. Only one trial has compared the two modalities head to head[33]. The results of this study showed similar success and complication rates for

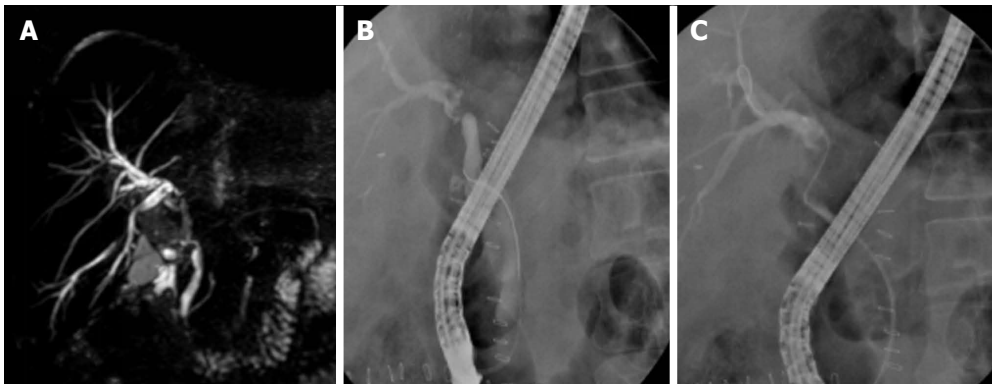

Figure 2 Anastomotic stricture - single duct anastomosis. A: Magnetic resonance cholangiopancreatography shows stricture at the anastomotic site of a single duct anastomosis; B: Endoscopic retrograde cholangiopancreatography (ERCP) in the same patient shows the stricture; C: ERCP in same patient shows guide wire negotiated across the stricture.

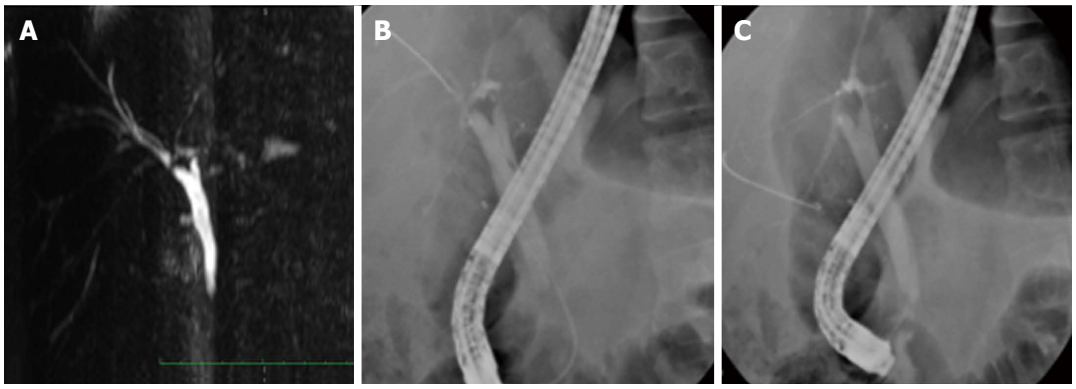

Figure 3 Anastomotic stricture - double duct anastomosis. A: Magnetic resonance cholangiopancreatography image shows stricture across both RASD as well as RPSD ductal anastomosis; B: Endoscopic retrograde cholangiopancreatography (ERCP) image shows guide wire negotiated across RPSD in this patient; C: ERCP image shows guidewire negotiated across RASD in this patient.

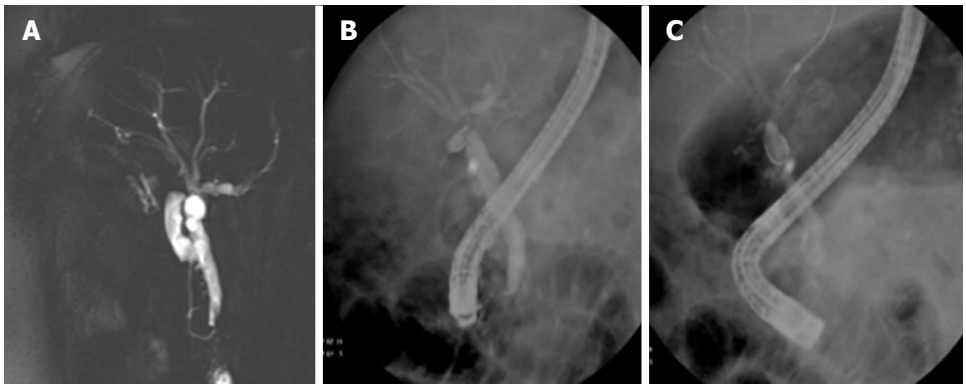

Figure 4 Anastomotic stricture - ductoplasty. A: Magnetic resonance cholangiopancreatography image of a ductoplasty of RASD and RPSD to common hepatic duct; B: Endoscopic retrograde cholangiopancreatography (ERCP) image shows stricture at ductoplasty site; C: ERCP image shows guide wire across one ductal system.

both approach. However, the number of interventions required was higher in the percutaneous arm. Despite sparse comparative data, ERCP is the preferred approach with PTBD being reserved for rescue in cases of failed ERCP/stenting. PTBD is considered more invasive, with a higher incidence of complications like hemorrhage, bile leak from entry site and need to keep an external stent that is liable to be displaced inadvertently.

Definitions of stricture and endoscopic outcomes

Classical definition of anastomotic biliary stricture on cholangiography is a dominant narrowing at the anastomotic site without effective drainage of the contrast material[34]. However, the diagnosis of stricture is nowadays made on MR cholangiography rather than direct cholangiography. The parameters to be studied on MRCP examination include the presence and location of

Table 3 Definitions

Term	Definition
Anastomotic biliary stricture	ERCP/PTC - Dominant narrowing at the anastomotic site without effective drainage of the contrast material MRCP - More than 50% reduction in anastomotic diameter compared to intrahepatic duct
Successful initial endoscopic outcome	Stricture negotiated with stent with continuous improvement in liver functions
Successful long-term endoscopic outcome	Persistent patency of the anastomotic site on cholangiography after stent removal (anastomotic site > 80% of intrahepatic ductal diameter)
Initial endoscopic treatment failure	Inability to negotiate the stricture on ERCP
Endoscopic treatment failure	Persistence of the stricture after 12 mo of therapy
Persistent ABS	Visible stricture on cholangiography after stent removal, measuring less than 80% of the diameter of the intrahepatic duct or hindering effective drainage of contrast medium
Recurrence of stricture	Biochemical derangement with ERCP documented recurrence of stricture after initial success

ERCP: Endoscopic retrograde cholangiopancreatography; PTC: Percutaneous transhepatic cholangiography; ABS: Anastomotic biliary strictures; MRCP: Magnetic resonance cholangiopancreatography.

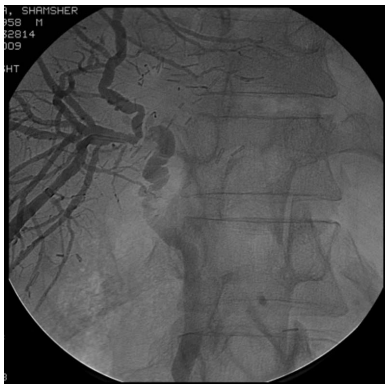

Figure 5 Anastomotic stricture - cystic duct anastomosis (endoscopic retrograde cholangiopancreatography failed, patient underwent percutaneous transhepatic biliary drainage).

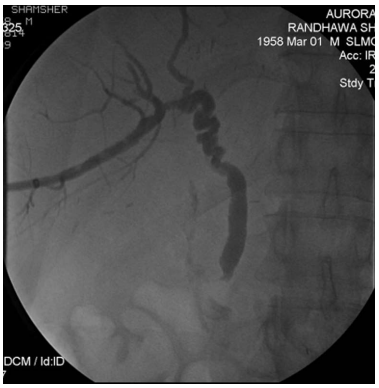

Figure 6 Cystic duct anastomosis after dilatation. This patient developed stricture again and underwent a hepaticojejunostomy.

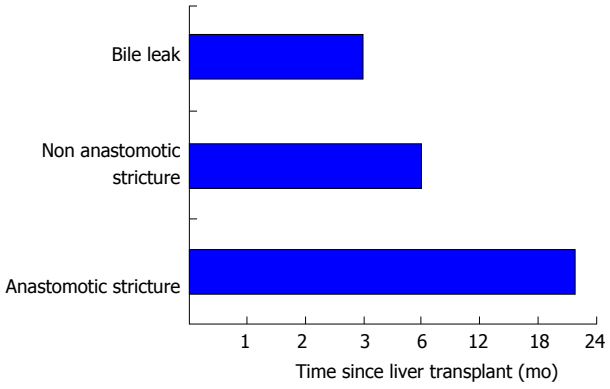

Figure 7 Timeline of biliary complications after transplant.

any strictures, upstream duct dilatation, the diameter of the ducts proximal to the anastomotic site (donor duct), distal to the anastomotic site (recipient duct) (Table 3).

ABS is diagnosed when the diameter of the anastomosis is less than 50% of the proximal (donor) bile duct[35]. If ABS is diagnosed, the length and diameter of the stricture is recorded. Also to be noted is the size discrepancy as well as angulation between donor and recipient ducts[34]. The percent stenosis is calculated as the difference between the donor duct diameter and the stricture diameter, divided by the donor duct diameter. In case of multiple duct anastomoses, details of each anastomosis have to be recorded as it has implications on number of stents to be placed. Also in case of a single duct anastomosis, the possibility of stricture extending intrahepatic is to be considered (this can convert a single duct anastomosis similar to double duct thus mandating more than one stent). Although more than 50% change in diameter of anastomosis to intrahepatic (donor duct) is taken as suggestive of stricture, this has not been validated in any of the trials. There are no studies comparing the relative diameters in asymptomatic individuals compared to those with biochemical derangements.

Successful initial endoscopic outcome is defined as the continuous improvement in LFT. Successful long-term endoscopic outcome refers to persistent patency of the anastomotic site on cholangiography after stent removal. The biliary anastomosis is considered patent on cholangiography when the narrowest diameter at the anastomosis is greater than 80% of the upstream intrahepatic (donor) duct diameter, and spontaneous emptying of contrast medium is seen on fluoroscopy[2]. Initial endoscopic treatment failure is defined as inability to negotiate the stricture on ERCP[14]. Endoscopic treatment failure is defined as persistence of the stricture after 12 mo of therapy. A persistent ABS is defined as a visible stricture on cholangiography after stent

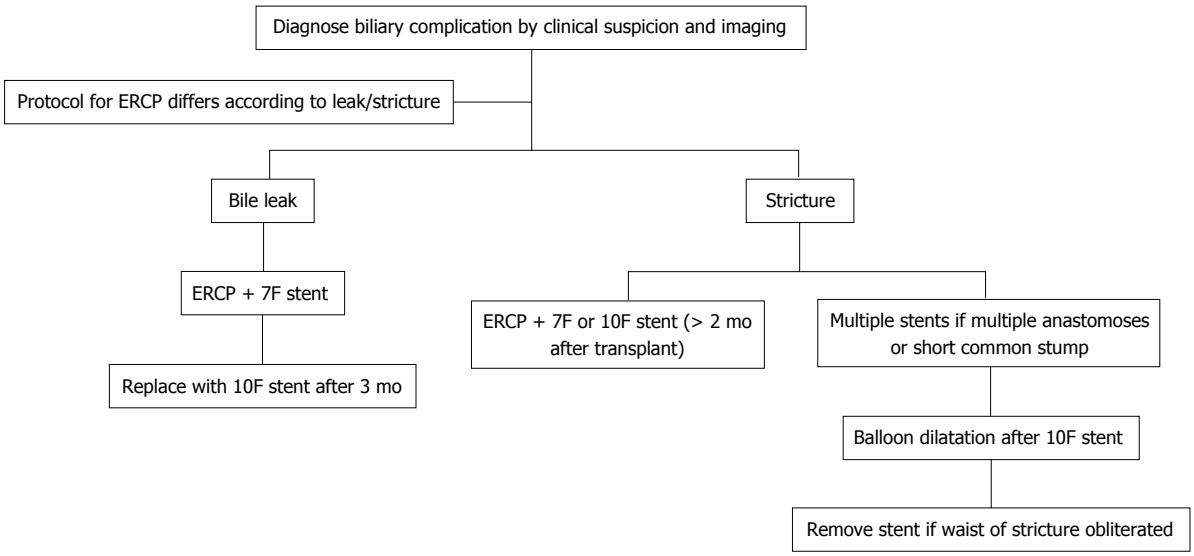

Figure 8 Protocol for endoscopic intervention (please see text also). ERCP: Endoscopic retrograde cholangiopancreatography.

removal, measuring less than 80% of the diameter of the intrahepatic duct or hindering effective drainage of contrast medium. Recurrence of stricture is defined as biochemical derangement with ERCP documented recurrence of stricture after initial success.

Timing of intervention

There is no data available on the timing of intervention after development of biliary stricture. There is a fear of disrupting the anastomosis if ERCP is done early (first few weeks). However, there is no published data to substantiate that fear. In our own center, we tend to delay ERCP for at least 3 wk. Also we feel that early biliary leaks may merit surgery than ERCP.

The success rate also depends on the time gap between the transplant to the presentation of biliary stricture. We have found that strictures that present early have a higher rate of successful outcome after ERCP. This is probably explained by the fact that early strictures are often soft involving short segment and hence are easily negotiable on ERCP. Those presenting late (generally in such cases there is a lag period between onset of symptoms and time of presentation) often have very tight strictures, making the negotiation of stricture difficult. The rate of salvage PTBD as well requirement of surgical intervention is higher in these cases.

Protocol of endoscopic intervention

The intervention protocols vary between institutions. Most centers would only stent the stricture after initial sphincterotomy at the first ERCP. Balloon dilatation is usually done in subsequent ERCP's. But there are institutions where balloon dilatation is carried out at the first ERCP itself[36]. Usually after the initial ERCP, stents are replaced every 3 mo with larger stents. Stents placed for longer time are more likely to get blocked predisposing to cholangitis[37-39]. Use of multiple stents has shown better long-term success than single stents[40,41].

There are 4 published trials comparing stenting alone *vs* stenting and balloon dilatation. Three of these are in post DDLT biliary strictures and only one was in LDLT patients[42]. This trial showed better long-term outcomes with a combination of both strategies compared to either alone.

The protocol we follow at our center is described as follows (Figure 8)[14]. The initial stenting is done with 7F/10F plastic stent depending on the timing of presentation after transplant. We use 7F stents initially for those with biliary leak in addition to stricture, and in those presenting very early after transplant (within 2 mo). Patients presenting after 2 mo of transplant usually undergo either a 10F stent (single duct anastomosis) or two 7F stents (double duct anastomosis). We always place stents across all anastomoses even if only one has a stricture as we feel stenting only one duct may block the other biliary system leading to cholangitis. We use the same strategy of stenting both anterior and posterior duct in a single duct anastomosis if the common duct of donor is small. We do not use balloon dilatation in first ERCP for the fear of anastomotic disruption. The stents are usually exchanged after 3 mo. We perform balloon dilatation with 6 mm or 10 mm biliary dilatation balloon (depending on the size of intrahepatic ducts) during second ERCP. The stents are removed if the waist of stricture is completely obliterated. Each patient requires about 2-3 stent exchanges over 6-12 mo. This is quite less than what is seen in other cases of benign biliary strictures (Iatrogenic post cholecystectomy and strictures associated with chronic pancreatitis). This could be due to the fact that these patients are on immunosuppression and thus do not have significant fibrosis.

The success rate of ERCP in post LDLT biliary strictures has been reported between 60%-75%[9,14,41-45].

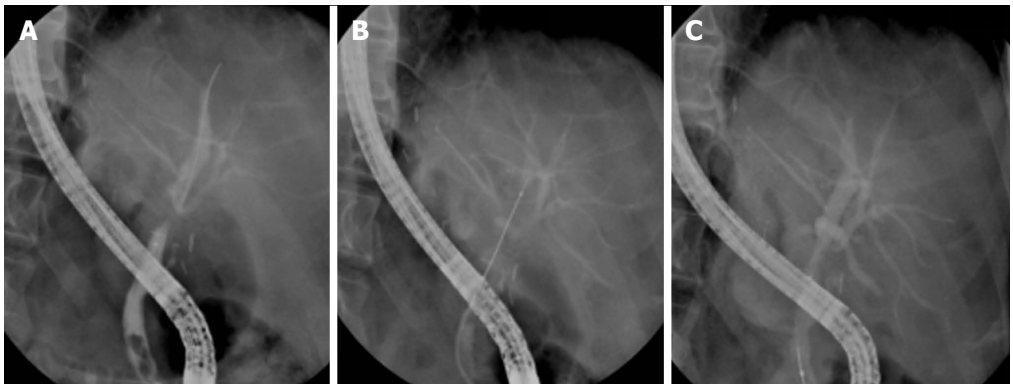

Figure 9 Balloon dilatation of biliary stricture. A: Endoscopic retrograde cholangiopancreatography (ERCP) images show stricture at the anastomotic site; B: ERCP image showing balloon dilatation of the stricture; C: Successful obliteration of the waist of stricture after balloon dilatation.

This is lower than reported success rate for post DDLT strictures (80%-90%)[3]. The reasons for lower success in LDLT strictures are multiple and will be discussed in detail in technical challenges section.

There are reports of use of covered self-expandable metal stents (SEMS) in treatment of biliary leaks and strictures after transplant. However, most of the data is in post DDLT strictures[46,47]. The smaller size of donor liver ducts as well as very short common duct stump and discrepancy between recipient and donor duct size make it unsuitable for use in LDLT strictures. Moreover, using a fully covered stent in LDLT strictures will compromise the patency of the contralateral duct. We do not use SEMS in post LDLT strictures.

Technical challenges

The ERCP procedure is much more challenging in post LDLT compared to DDLT recipient. The anastomosis is much higher and peripheral making the access difficult[44]. There is also a size discrepancy between donor and recipient ducts adding to the difficulty. The role of ischemia element at the anastomotic site often leads to the stricture extending intrahepatic, hence often converting single duct anastomosis akin to double duct anastomosis (separation of anterior and posterior segments of the donor liver)[48]. The hypertrophy of the partial liver in LDLT often creates a sharp angulation between donor and recipient ducts. This angulation when complicated by a stricture often leads to a very difficult situation less amenable to successful endoscopic treatment[49]. Kyoto group has described a similar anomaly as crane neck deformity, in which the biliary anastomosis is located at a point that is far below the highest portion of the recipient duct[43]. This is particularly difficult to negotiate with ERCP, but salvage PTBD is often successful in such cases.

A peculiar problem arises when strictures are associated with leaks also. In this scenario, the guide wire repeatedly slips preferentially into the leak area without negotiating the stricture (path of least resistance). In such cases also, ERCP is often unsuccessful and PTBD is required.

Newer techniques like cholangioscopy (spyglass) have been described in LDLT for traversing difficult strictures[50,51]. However in our limited experience of three cases, we did not find it of any additional benefit. We found that limited visibility and steering ability of the currently available devices is the major problem hindering the usefulness. With the improving technology and introduction of better cholangioscopes, this may help in negotiating difficult strictures.

Another novel technique using magnets to traverse difficult biliary strictures after LDLT has been described[52,53]. This was initially described in LDLT from Korea by Jang *et al*[52]. Subsequently, a through the scope magnet has been used by Turkey group with very good results. A similar technique with use of EUS-ERCP interface has also been successfully used to repair biliary anastomosis after LDLT[54]. Ersoz *et al*[55] described a novel technique using standard balloon to negotiate S shaped difficult strictures. With further refinement of these technique, it may help prevent surgery in difficult to negotiate biliary strictures after LDLT.

Long-term outcomes

Long-term data after removal of stents is sparse. The only published paper which discusses long-term outcome, reported a stent free status in 42.5% of patients at a median follow-up of 33 mo[38]. In our own experience, 90% of the patients after balloon dilatation are free of stents at a median of 1 year after initial ERCP (Figure 9). The recurrence rate after stent removal is around 20% at a median follow-up of 30 mo after last balloon dilatation (unpublished data). Most patients who have recurrence of stricture after balloon dilatation are successfully treated by repeat ERCP only[33].

Failure of endoscopic treatment

The failure rate of endoscopic management in LDLT strictures varies from 25%-40% in various studies. The reasons for higher failure rate compared to DDLT have been discussed in technical considerations section. Patients who fail ERCP are usually successfully managed by PTBD. We at our center always do a rendezvous ERC procedure after a successful PTBD (Figure 10)[14]. Stenting *via* PTC route has been described but is not

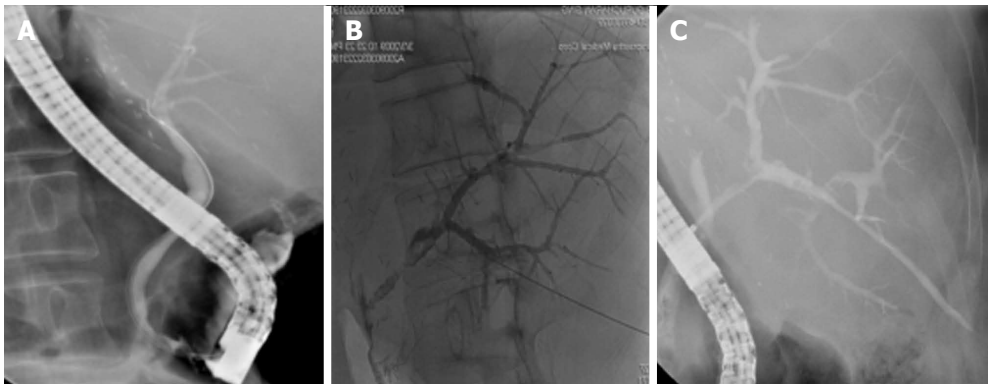

Figure 10 Rendezvous procedure. A: Endoscopic retrograde cholangiopancreatography opacified only RASD; B: RPSD accessed *via* percutaneous transhepatic biliary drainage; C: Rendezvous procedure being performed.

widely practiced as it entails dilatation of liver tract of PTBD[33]. In our series the technical success rate of ERCP was 75%, majority of the failures were managed by PTBD (combined success rate of 91%). However, in small number of patients, where both ERCP and PTBD fail (about 9%), surgical intervention in the form of hepaticojejunostomy is required[14].

Complications

Complication rates after ERCP have been variably reported between 10%-24%[14,32,39-44]. Due to altered duodenal anatomy (upper abdominal surgery), the approach to the papilla becomes difficult as after any other upper abdominal surgery. The incidence of complications including pancreatitis rates are similar as in non-transplant ERCP's. We have seen proximal migration of plastic stents in significant number of patients. Removal of these becomes quite difficult. To avoid that, we have started using single pigtail stents.

Biliary complications and graft survival

Most of the biliary strictures are now managed successfully with non-surgical approach (ERCP or PTBD). The success rate of these interventions is very high with minimum morbidity and almost no mortality. At least two trials have analyzed the effect of biliary complications on graft survival in LDLT[14,37]. Both concluded that there is no effect of biliary complications on patient or graft survival. However, both these trials had analyzed strictures in relation to mortality. We believe that if the data on bile leaks is analyzed separately, the results may be different as bile leaks predispose to sepsis and graft dysfunction early after liver transplant.

Future directions

Biliary strictures are the commonest complication of liver transplant both OLT and LDLT. Despite that there is no consensus on numerous management issues in it. We need more evidence to show what is the best protocol, *i.e.*, only balloon or balloon dilatation plus stent, how many stents, for how long. Natural history of treated biliary strictures needs to be further studied. Newer devices to facilitate difficult stricture cannulation during endoscopy need to be developed. Digital spyglass may be one such modality. Any bad/favorable prognostic signs for endoscopic treatment need to be defined. Above all more effort is required to refine the surgical techniques to avoid these strictures.

CONCLUSION

Biliary complications are common after LDLT, strictures seen more commonly than leaks. With refining surgical skills and better understanding of factors predisposing to biliary strictures, the incidence of biliary complications is decreasing. ERCP is the first line modality of treatment of post LDLT biliary strictures with a technical success rate of 75%-80%. Most of ERCP failures are successfully handled by PTBD. A minority of patients may require surgical correction. ERCP for post LDLT strictures is technically more challenging than routine ERCP's as well post DDLT strictures ERCP's. With the introduction of new generation cholangioscopes, ERCP success rate may increase, obviating the need for PTBD and surgery in the management.

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REVIEW

Hepatocellular carcinoma: Review of disease and tumor biomarkers

Jin Un Kim, Mohamed I F Shariff, Mary M E Crossey, Maria Gomez-Romero, Elaine Holmes, I Jane Cox, Haddy K S Fye, Ramou Njie, Simon D Taylor-Robinson

Jin Un Kim, Mohamed I F Shariff, Mary M E Crossey, Maria Gomez-Romero, Simon D Taylor-Robinson, Division of Digestive Health, Department of Surgery and Cancer, Imperial College London, London W2 1NY, United Kingdom

Elaine Holmes, Division of Computational Medicine, Department of Surgery and Cancer, Imperial College London, London W2 1NY, United Kingdom

I Jane Cox, the Foundation for Liver Research, Institute of Hepatology, London WC1E 6HX, United Kingdom

Haddy K S Fye, Ramou Njie, MRC Gambia, Fajara 273, The Gambia

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Correspondence to: Jin Un Kim, BSc, Division of Digestive Health, Department of Surgery and Cancer, Imperial College London, St Mary's Campus, South Wharf Road, London W2 1NY, United Kingdom. juk11@ic.ac.uk
Telephone: +44-207-8866454
Fax: +44-207-7249369

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Abstract

Hepatocellular carcinoma (HCC) is a common malignancy and now the second commonest global cause of cancer death. HCC tumorigenesis is relatively silent and patients experience late symptomatic presentation. As the option for curative treatments is limited to early stage cancers, diagnosis in non-symptomatic individuals is crucial. International guidelines advise regular surveillance of high-risk populations but the current tools lack sufficient sensitivity for early stage tumors on the background of a cirrhotic nodular liver. A number of novel biomarkers have now been suggested in the literature, which may reinforce the current surveillance methods. In addition, recent metabonomic and proteomic discoveries have established specific metabolite expressions in HCC, according to Warburg's phenomenon of altered energy metabolism. With clinical validation, a simple and non-invasive test from the serum or urine may be performed to diagnose HCC, particularly benefiting low resource regions where the burden of HCC is highest.

Key words: Hepatocellular carcinoma; Biomarker; Metabonomics; Warburg hypothesis; Serum; Plasma; Urine

Core tip: Many independent authors have utilized

quantitative techniques, such as ^{1}H nuclear magnetic resonance and mass spectrometry to discover novel biomarkers to aid early diagnosis, following the removal of alpha fetoprotein from international surveillance guidelines. However, relatively little effort has been directed to translate these findings to the clinical setting. hepatocellular carcinoma (HCC) is a global issue and the vast majority of the burden is placed upon resource-limited regions, where presentations are late and management techniques for advanced tumors are unavailable. Early identification through a simple serum or urinary investigation, therefore, may be a pivotal step in addressing the global burden of HCC.

Kim JU, Shariff MIF, Crossey MME, Gomez-Romero M, Holmes E, Cox IJ, Fye HKS, Njie R, Taylor-Robinson SD. Hepatocellular carcinoma: Review of disease and tumor biomarkers. *World J Hepatol* 2016; 8(10): 471-484 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i10/471.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i10.471

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth commonest malignancy and arises most frequently in patients with cirrhosis[1]. The global distribution of HCC is disproportionate, being most common in areas where chronic hepatitis B virus (HBV) infection is highly prevalent (Figure 1). However, HCC is an increasing problem in the western world, due to migration from HBV-endemic regions, hepatitis C virus (HCV) infection, alcoholic cirrhosis and non-alcoholic steatohepatitis, related to the obesity epidemics[2,3] (Figure 2).

Curative treatments, such as hepatic resection and orthotopic liver transplant, offer good prognosis, but are limited to early HCC[4]. In developing countries, medical advice is often sought late, resulting in delayed, end-stage presentation. More than two-thirds of HCC patients in the developed world are diagnosed at advanced stages[5]. The high global incidence and late presentation of HCC make it the second global cause of cancer-related mortality with 1.6 million global deaths, annually[6]. The key and as yet, unmet need is to identify small tumors, amenable to curable treatments, in an otherwise nodular cirrhotic liver parenchyma.

Improved surveillance of populations at-risk by adding a sensitive biomarker investigation to complement current imaging studies has the potential to detect tumors at an early stage, when curative interventions can be implemented. Furthermore, designing a simple and accessible investigative test for a set of HCC biomarkers may not only improve diagnosis and management of liver cancer, but pioneer proteomic or metabonomic diagnosis for other diseases in developing countries, where technical and human resources are limited.

PATHOGENIC MECHANISMS WITH METABOLIC IMPLICATIONS

Altered tumor metabolism

There is increasing evidence that altered metabolism in tumor cells is both a cause and effect of carcinogenesis. Tumor cells require increased amounts of energy and substrates for *de novo* synthesis of nucleotides, lipids, and proteins for rapid proliferation. Otto Warburg, in the 1920s, pioneered the theory of altered tumor metabolism. Recent evidence both supports and disputes his original conclusions.

"Warburg effect" and glycolysis

In 1924, Warburg, through placing a section of rat carcinoma in nitrogen-saturated Ringer's solution (to simulate anaerobic conditions), observed that the tumor could be transplanted to a live donor if sugar was included in the Ringer's solution, but not if the solution was left plain[7]. Following this work, Warburg discovered that even in the presence of oxygen, cancer cells preferentially metabolize glucose by glycolysis as oppose to oxidative phosphorylation, a vastly more inefficient route for energy production. He hypothesized that the increase in glycolysis under normal oxygen conditions arose from a deficiency in the mitochondrial oxidative phosphorylation[8] (Figure 3). He thus established that tumor cells take up glucose at high rates to fuel heightened glycolysis. Indeed, it is upon this basis that tumors can be identified with glucose-labeled positron emission tomography[9]. Glycolysis generates adenosine triphosphate (ATP) with lower efficiency, but at a faster rate than oxidative phosphorylation, which may be of benefit for rapidly dividing cells. The role of mitochondria in tumor cells is contentious. Primary defects in oxidative phosphorylation (which occurs within the mitochondrial membrane) have been invoked to explain the Warburg phenomenon because tumor mitochondria are often small, lack cristae and are deficient in the β-F1 subunit of the ATPase[10,11]. However, many groups have demonstrated that tumor cell mitochondria are actually functional and even Warburg admitted that despite their high glycolysis rate, oxygen consumption by cancer cells is not diminished[12]. Furthermore, HCC is a highly vascular tumor that, certainly in the early stages, is likely to be adequately supplied with oxygenated blood. Importantly, glycolysis also provides intermediates for the pentose phosphate pathway and subsequent biosynthesis of nucleic acids. Which of these functions heightened glycolysis serves is, as yet, unresolved.

There is now some consensus that the major role of heightened glycolysis in tumor cells is to provide substrates to the pentose phosphate pathway for nucleotide synthesis, rather than energy provision in the form of ATP[12,13]. In essence, the tumor is maximizing production of cellular constituents for proliferation at the expense of energy production.

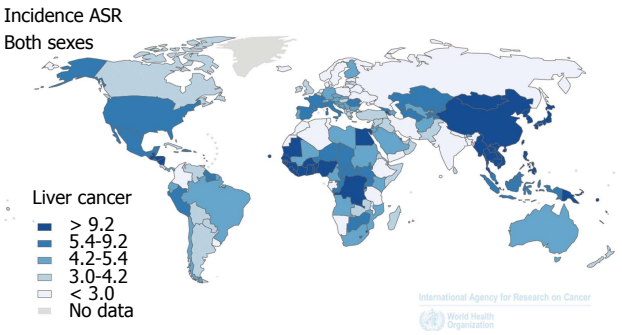

Figure 1 Global incidence of hepatocellular carcinoma. Sourced from GLOBOCAN 2012.

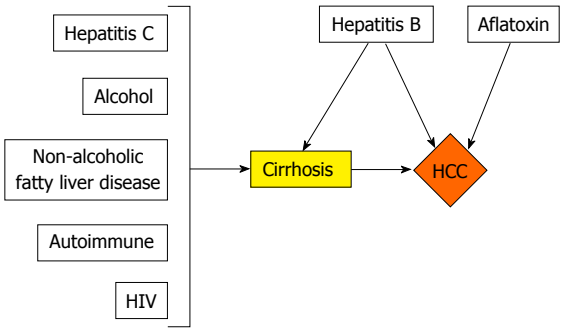

Figure 2 Independent risk factors of cirrhosis and hepatocellular carcinoma. HIV: Human immunodeficiency virus; HCC: Hepatocellular carcinoma.

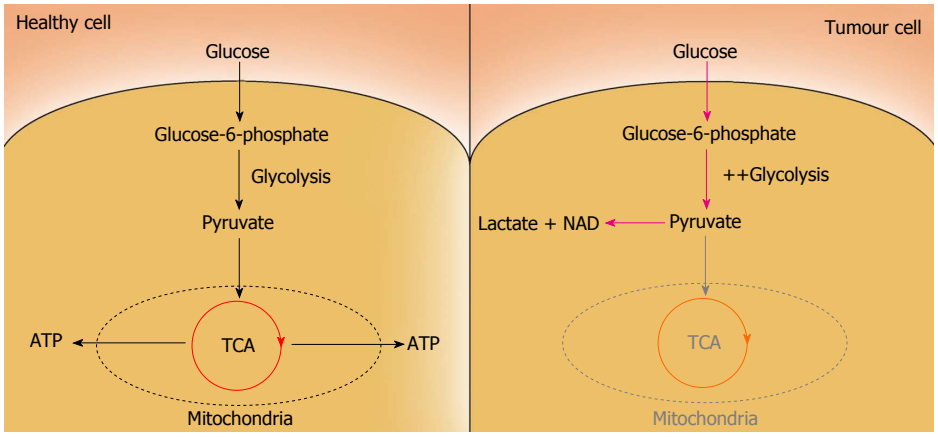

Figure 3 Warburg theory of heightened glycolysis in tumor cells. TCA: Tri-carboxylic acid cycle; ATP: Adenosine triphosphate; NAD: Nicotinamide adenine dinucleotide.

MOLECULAR EFFECTORS AND TUMOUR METABOLISM

Several oncogenes and tumor suppressor genes have been implicated in altered tumor metabolism. Sequential mutations are common in HCC and two effectors in particular, hypoxia inducible factor 1 (HIF 1) and p53, may be responsible for some of the metabolic changes arising in HCC.

HIF

HIF 1 is a heterodimeric protein complex transcription factor that is activated by hypoxic, inflammatory, metabolic and oxidative stress[10,12,14]. The HIF 1 heterodimeric complex (HIF 1α + HIF 1β) is stabilized at low oxygen levels, but degraded by the proteasome in normoxic conditions. The HIF 1 heterodimer stimulates glycolysis by increasing the expression of pro-glycolytic uptake enzymes and transport molecules, such as glucose transporter 1 (GLUT 1) and hexokinase[12]. HIF 1β deficient hepatoma cells grown as solid tumors in mice were found to have reduced rates of growth and glycolytic intermediates compared to wild type hepatoma cells[15]. It would therefore appear that HIF 1 may play a central role in the Warburg model. However, HIF 1 is only stable in hypoxic conditions and Warburg's model describes heightened glycolysis in normoxic conditions. Only a minority of cancers display aberrant HIF 1 function in normoxia, such as renal cell carcinoma[16]. The role of HIF 1 in HCC is still under investigation but a number of recent studies, mostly in animal models, have observed high HIF 1 activity and its downstream counterparts, such as GLUT1, in hepatoma cells[17-19]. Recent studies have also identified association between HIF 1 and the prognosis of HCC, where HIF 1α levels have been found to be significantly raised in HCC, compared to benign liver disease[19]. Furthermore, it appears that HIF 1 inhibition may be a potential target of therapeutic benefit in HCC by down-regulating its role in tumorigenesis. There have been several proposals to incorporate HIF 1 inhibition as adjunct to the current treatment pathways, but further investigations are required before its clinical application[20].

p53

Tumor suppressor genes, such as *p53*, have also been implicated in alterations in metabolism. Inactivation of *p53* can cause the Warburg phenomenon. *p53* positively regulates the expression of the protein synthesis of cytochrome C oxidase 2, which is required for the assembly of the oxidative phosphorylation enzyme, cytochrome C oxidase[21] and also negatively regulates phosphoglycerate mutase, a key glycolytic enzyme[22]. In addition, *p53* transcriptionally activates TP53-induced

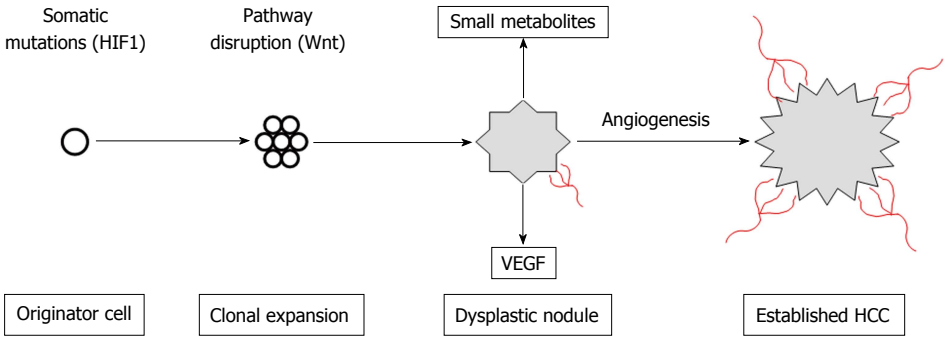

Figure 4 "Angiogenic switch" in hepatocellular carcinoma. VEGF: Vascular endothelial growth factor; HCC: Hepatocellular carcinoma; HIF 1: Hypoxia inducible factor 1.

glycolysis and apoptosis regulator an inhibitor of phosphofructokinase activity which in turn lowers the level of fructose 1,6-biphosphate which acts as an allosteric activator of glycolytic enzymes[23].

These examples illustrate the evidence that genetic alteration through tumor-driven mutation can affect metabolism. It is likely that many genes and proteins are involved in altered tumor metabolism, with a few taking a lead role.

METABOLITE EFFECTS ON CARCINOGENESIS

Metabolites can affect carcinogenesis and may not be mere by-products of cellular reactions. Lactate, thought to be a "waste" product of glycolysis, may be such a signal. Lactate may stimulate HIF 1 independently of hypoxia[24] and may condition the tumor environment and suppress anticancer immune effectors[10,25,26]. HIF 1 can also be stimulated by the buildup of tricarboxylic acid (TCA) cycle intermediates, fumarate and succinate. This is evidenced through tumorogenic germline mutations of TCA cycle enzymes fumarate hydratase and succinate dehydrogenase, resulting in an accumulation of fumarate and succinate which competitively inhibit the α-ketoglutarate-dependent HIF 1α prolyl hydroxylase, the enzyme that targets HIF 1 for destruction[27]. Through high-throughput liquid-and-gas-chromatography-based mass spectrometry of urine and plasma from patients with prostate carcinoma, Sreekumar *et al*[28] identified sarcosine, a metabolite derivative of glycine, as a marker of the cancer. Furthermore, exogenous addition of sarcosine to tumor cells, or knockdown of sarcosine degrading enzymes, caused a shift of benign prostatic cells into a malignant phenotype.

OTHER PATHOGENIC MECHANISMS

Genetic profiling studies of HCC tissue have shown several genes to be disrupted through somatic mutations, chromosomal disruption and epigenetic aberration through methylation abnormalities including *p53*, *Rb1*, *β-catenin, CMYC* and *survivin*. The Wnt-β catenin pathway is the most commonly disrupted pathway, usually as a result of mutations in *CTNNB1*, *AXIN1* genes, *CDH1* epigenetic silencing and changes in expression of Wnt receptors from the Frizzle family[29]. Activation of the pathway induces translocation of β-catenin into the nucleus where it regulates specific oncogenes such as *CMYC* and *CCND1*. An initial somatic mutation in an oncogene or tumor suppressor gene is likely to generate a clonal expansion of cells which then have the potential, through further "proliferation advantageous" mutations and chromosomal disruptions, to develop into pre-neoplastic lesions. These lesions, often < 1 cm, have been identified in patients with cirrhosis and have been sub-classified into low or high grade dysplastic nodules[30]. The former carry a low risk and the latter a very high risk, of malignant transformation.

"Angiogenic switch"

Dysplastic nodules are often hypoechoic on ultrasound imaging and derive their blood supply from the portal vein. These nodules may, less frequently, appear as either hyperechoic or isoechoic. Established HCC displays typical arterial phase uptake on contrast imaging. At a critical point, an "angiogenic switch" is activated which stimulates arterial neo-vascularization of the nodule and development of an established HCC (Figure 4). Japanese groups have identified this as a critical moment before which total cure with resection is likely and after which prognosis deteriorates rapidly[31]. Certain factors may contribute to "neo-angiogenesis" of HCCs. Vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) have been implicated as angiogenesis modulators. HCC cell lines may produce VEGF by themselves and increased concentration of VEGF in the serum of patients with HCC has been correlated with outcome after surgical resection[32,33]. HIF 1, a factor commonly expressed in HCC and heavily influential upon cellular metabolism, has been shown to induce expression of VEGF. A number of oncogenes have also been implicated in angiogenesis such as *ras* and *myc*[34].

It has been shown that chemotherapeutics active against HCC such as the multikinase inhibitor, sorafenib, exert their effects through inhibition of pro-angiogenic factors such as VEGF and PDGF, establishing neo-

Table 1 Comparison of nuclear magnetic resonance and mass spectrometry

Variable	NMR	MS
Sensitivity	Lower than MS (nanomolar)	Higher than NMR (picomolar)
Sample degradation	No	Yes
Reproducibility	High	Moderate
Metabolite identification	Well categorized	Labor intensive

NMR: Nuclear magnetic resonance; MS: Mass spectrometry.

angiogenesis as a major therapeutic target in HCC[30]. With the onset of neo-angiogenesis, there is likely to be a rapid change in the metabolism of tumor cells and also the surrounding stroma[35]. The importance of the interaction between tumor and stromal cells is becoming increasingly recognized. Vizan *et al*[36], studied the metabolic adaption of endothelial cells, to stimulation by VEGF and fibroblast growth factor. Glycogen synthesis, the pentose cycle and glycolytic pathways were shown to be essential for endothelial cell proliferation and inhibition of these pathways decreased endothelial cell viability and migration[36]. The interaction of cellular metabolism and neo-angiogenesis is therefore crucial to tumor development.

CURRENT SURVEILLANCE AND DIAGNOSIS

HCC is likely to originate from hepatic stem cells[37], with internal and external stimuli, such as viral DNA integration, inflammation and fibrosis, likely inducing alterations in tumor originator cells leading to apoptosis, cell proliferation, dysplasia and eventually, neoplasia[34]. The global alteration of metabolites that arise during, or as a consequence of tumorigenesis, then, may measure both the presence and the severity of disease.

Unfortunately, HCC surveillance lacks reliable biomarkers. Serum alpha fetoprotein (AFP) historically has been the most used biomarker. However, not all HCCs secrete AFP. Furthermore, it may be elevated in chronic liver disease in the absence of HCC[38], and its use is no longer recommended by international authorities. Ultrasonography (US) at 6 monthly intervals is the currently recommended screening and surveillance modality for patients with established liver cirrhosis[39]. Diagnosis is based on the fact that HCCs are highly arterialized, in contrast to the remainder of the liver. The most recent American Association for the Study of Liver Disease guidelines require the presence of features typical of HCC (arterial hypervascularity and venous phase washout) in just one imaging modality for lesions > 1 cm[39]. Previous guidelines suggested that diagnosis was made by the confirmation of two contrast-enhanced imaging modalities (contrast-enhanced ultrasound, computed tomography or magnetic resonance imaging) with characteristic features or one imaging modality suggestive of HCC with an AFP level of > 400 ng/mL[40].

Diagnostic imaging techniques for HCC require a combination of equipment availability, infrastructural support and technicians to perform and interpret the results, which unsurprisingly, are limited in the majority of developing regions with high HCC burden. Alternative solutions to HCC diagnosis, therefore, are urgently required, as AFP measurement lacks sensitivity and specificity. An acceptable alternative requires the diagnostics to be quick, inexpensive, accessible and adequately sensitive and specific to the disease. Blood and urine tests are extremely simple methods of investigation, which are widely utilized in developing regions. For example, designing a urine dipstick test that can quantify and score the severity of HCC from a set of candidate biomarkers may significantly reduce cancer-related morbidity and mortality, and revolutionize the surveillance process in developing regions.

METABOLIC PROFILING TO FIND BIOMARKERS

Metabolic profiling is a general term encompassing "metabonomics", which is the study of global metabolic responses to physiological, drug and disease stimuli[41] and "metabolomics", which aims to characterize and quantify all the small molecules in biofluid samples[42]. The most commonly used methods of metabolite characterization are proton nuclear magnetic resonance (^{1}H NMR) spectroscopy and mass spectrometry (MS). These techniques are complimentary and each has advantages and disadvantages (Table 1). Sensitivity of MS is high, with some forms of gas chromatography (GC)-MS reaching femtomolar levels, but samples are degraded during the run and metabolite identification can be challenging[43,44]. Nuclear magnetic resonance spectroscopy displays lower sensitivity (nano to millimolar), but samples remain intact and NMR spectral profiles have been extensively categorized making metabolite identification more straightforward[37-39].

PROTON NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

Nuclear magnetic resonance is based on the behavior of nuclei subjected to a magnetic field. Hydrogen is the most abundant element in living organisms and using high power magnetic fields of *in vitro* samples, high-resolution metabolic NMR spectra can be obtained with clearly defined metabolite peaks of small mobile molecules (< 2 kDa). Comprehensive metabolic profiles have been generated from biofluids, including urine[45,46], serum[47-50], bile[51] and intact tissue[52].

MASS SPECTROMETRY

Mass spectrometry has been utilized for metabolic profiling since the 1970s[53]. Metabolites, or their constituent fragments, are detected and distinguished

by their molecular weight and ionic charge. Owing to their complex nature, biological fluids require separation prior to mass spectrometric analysis to achieve detection of as many metabolites as possible. The most common separation methods are GC or liquid chromatography (LC). Gas chromatography requires extensive sample pre-treatment and derivatization steps. In contrast, LC requires minimal sample preparation and is immediately amenable to biofluid analysis. Ultra performance LC utilizes separation columns with much smaller particle size packing material (1.4-1.7 μm) than traditional columns, permitting the injection of liquids at pressures exceeding 10000 psi, thus allowing for improved metabolite resolution. Once ionized, the particles are detected usually by a time-of-flight analyzer, which allows the detection of analytes over the range of *m/z* 50-1000 Da.

CLINICAL APPLICATION OF BIOMARKERS

The development and progression of HCC underscores complex molecular and metabolic interactions, involving several stages of disease over a prolonged period of time. A single reliable biomarker to assess both presence and severity of disease, such as it was for AFP, is likely to be unfeasible in this setting. Therefore, a panel that reliably assesses HCC tumorigenesis from a selection of candidate biomarkers may be better suited to tackle the situation. The candidate biomarkers must show adequate sensitivity and specificity by validation-based experiments, and demonstrate diagnostic synergism when individual biomarker results are combined. Such a new biomarker panel must then be assessed in comparison studies for the current diagnostic methods, such as US and biopsy, for different disease states of HCC, and its utility in surveillance protocols must then be considered, particularly in the developing world context. Biomarkers are also heterogeneous in their quantification and analysis, as different equipment and techniques are utilized. This practical issue must be addressed with thorough cost-benefit analyses that compare biomarker analysis to the local investigative methods.

SERUM MARKERS OF HCC

Serum AFP

Serum AFP is the most widely used marker of HCC. It is a fetal glycoprotein, which is synthesized *in utero* by the embryonic liver, cells of the vitelline sac and the fetal intestinal tract. Serum AFP is usually undetectable in healthy adults[54]. The production of AFP by HCC cells has been seen as confirmation that the tumor arises from hepatic stem cells as a form of maturation arrest, akin to an embryonic state[55]. Not all HCCs secrete AFP and its diagnostic accuracy is variable. A meta-analysis of AFP for HCC surveillance found that it displayed a sensitivity of 39% to 65% and a specificity of 76% to 94% for tumor diagnosis[56]. The cut-off level of AFP was important in determining the diagnostic power. A cut-off of 20 ng/mL resulted in a sensitivity of 64% and specificity of 91%[57], while a cut-off of 400 ng/mL resulted in a sensitivity of 17% and specificity of 99%[58]. Values of over 400 ng/mL are generally considered diagnostic of HCC, although only about 20% of patients with HCC display values this high. Furthermore, patients with chronic viral hepatitis may display a raised AFP during viral flares without the presence of HCC. In a study of 290 Chinese patients with chronic HBV, 44 were found to have elevated serum AFP levels (> 20 ng/mL) and only six (13%) had HCC. The remaining 38 had elevated serum AFP, either due to viral flares or due to unknown causes[59]. Trevisani *et al*[58] also observed that an AFP elevation in non-infected patients could be more indicative of HCC when compared to infected patients.

Lens culinaris agglutinin-reactive AFP

Lens culinaris agglutinin-reactive AFP (AFP-L3) is a glycoform variant of AFP and is expressed as a percentage of the total AFP level. It can be detected in the serum of approximately one third of patients with small HCCs (< 3 cm) where cut-off levels of 10% to 15% are used. At higher cut-off levels of > 15%, AFP-L3 displays a sensitivity of 75% to 96.9% and specificity of 90% to 92%[60,61]. The usefulness of this marker is limited as studies have only been conducted in East Asian populations in whom AFP levels are already raised.

Des gamma carboxyprothrombin

Des gamma carboxyprothrombin (DCP) is an abnormal prothrombin protein and is also known as prothrombin induced by vitamin K absence Ⅱ. It is produced as a result of an acquired defect in the post-translational carboxylation of the prothrombin precursor in malignant cells, the gene responsible being gamma-carboxylase[62]. In several large studies, serum DCP was found to display poor diagnostic sensitivity (48% to 62%), but good specificity (81% to 98%) for HCC[62,63]. A study comparing the performance characteristics of AFP, DCP and lens culinaris agglutinin-reactive AFP in the diagnosis of HCC observed that DCP was significantly better than the other markers in differentiating HCC from cirrhosis, with a sensitivity of 86% and a specificity of 93%[64]. There are conflicting reports, however, with a study by Nakamura *et al*[65] reporting that the efficacy of DCP was lower than that of AFP in the diagnosis of small tumors, although higher than AFP for large tumors.

Alpha-l-fucosidase

Alpha-l-fucosidase (AFU) is a glycosidase found in cellular lysozomes and increased activity is found in the serum of patients with HCC. Studies of its diagnostic accuracy have displayed high sensitivity (82%) and specificity (70.7%-85.4%)[66-68]. A comparative study of AFP and AFU in an Egyptian cohort found AFU to have a higher sensitivity (81.8% *vs* 68.2%) but lower specificity (55% *vs* 75%) with a combined AFP + AFU sensitivity of 88.6%[69]. Unfortunately, AFU has been

Table 2 Diagnostic performance of serum markers of hepatocellular carcinoma

Serum marker	Sensitivity	Specificity
AFP	39%-65%	79%-94%
AFP-L3	75%-97%	90%-92%
DCP	48%-62%	81%-98%
AFU	82%	71%-85%
AFP-L3 + DCP	85%	98%

AFP: Alpha fetoprotein; AFP-L3: Lens culinaris agglutinin-reactive AFP; DCP: Des-gamma-carboxy prothrombin; AFU: Alpha-l-fucosidase.

found to be elevated in other tumors and is therefore not specific to HCC. The diagnostic performance of these serum markers is outlined in Table 2.

Glypican-3

Glypican-3 (GPC3) is a heparin sulfate proteoglycan and has been shown to be capable of promoting the proliferation of tumor cells by modulating Wnt pathways and affecting cellular adhesion. As a tumor marker, GPC3 expression has been shown to be elevated in HCC tissue and in serum of 40% to 53% of patients with HCC[69].

Vascular endothelial growth factor

VEGF is a homodimeric cytokine associated with tumor neovascularization. HCC is often diagnosed by imaging evidence of a highly vascularized mass in the liver, and HCC patients have been shown to have increased expressions of VEGF compared to those with normal liver tissues[70]. Furthermore, two previous studies have shown mortality in HCC increases with over-expression of VEGF[71,72].

Interleukin-8

Interleukin-8 (IL-8) is a multifunctional CXC chemokine, which may exert numerous effects on tumor proliferation, angiogenesis and migration. High serum IL-8 has been indicated in HCC patients compared to healthy controls, and its levels correlate to tumor size, absence of tumor capsule, presence of venous invasion, advanced pathological tumor-node-metastasis staging, and poorer disease-free survival[73,74].

Transforming growth factor-beta 1

Transforming growth factor-beta 1 (TGF-β1) is a negative autocrine growth factor that regulates cell proliferation and differentiation. Comparison studies against AFP (200 ng/mL) have shown TGF-β1 to have higher sensitivity at 68% (800 pg/mL cut-off), and a specificity of 95%[75]. Raised TGF-β1 also detected 23% of HCC patients with normal serum AFP[76].

Tumor-specific growth factor

Tumor-specific growth factor (TSGF) is released by malignant tumors, and has been shown to correlate with tumor growth and surrounding vascularization. Therefore, it is reasonable to suggest that TSGF could be a potential biomarker that may be used for HCC grading in populations around the world. TSGF has been approved for use by the Chinese government following study results that showed a sensitivity of 82% in HCC diagnosis at the cut-off of 62 U/mL[77].

Squamous cell carcinoma antigen

Squamous cell carcinoma antigen is part of a family of serine protease inhibitors, or serpins, and has been utilized to diagnose a variety of squamous cell carcinomas[78]. It has also been found to have a diagnostic role in HCC, where the sensitivity and specificity were 77.6% and 84%, respectively[79].

Heat shock proteins

Another potential biomarker for HCC are heat shock proteins (HSP), which are cellular molecules that are expressed under non-specific stress stimuli, including carcinogenesis[80]. In particular, HSP70 has been identified as a potentially sensitive marker to differentiate early HCC from precancerous lesions[81].

Serum metabolites

Metabolic profiling using proteomic techniques mentioned above, such as *in vitro* proton ^{1}H NMR spectroscopy[49,82-85] and MS[85-99] have been incorporated to identify a specific metabolic pattern that may be utilized for identifying HCC. Lysophosphatidylcholines (LPC) have been reported in several studies to be significantly decreased in HCC sera compared to healthy controls[88,89,91-93,96-98]. LPCs have been described in endothelial cell migration[100], which may contribute to the hypervascularized state in HCC. Two LPCs in particular, LPC 16:0 and LPC 18:0, were significantly altered in HCC compared to cirrhotic patients[91-93,97]. Morita *et al*[101] confirmed the overexpression of LPC acyltransferase 1 (LPCAT1) which converts LPC C16:0 to phosphatidylcholine 18:1. The up-regulation of LPCAT1 could be the reason for the reduction in LPC C16:0. A careful interpretation is required, as expression of LPC species has been found to be significantly different between hepatic compensation and decompensation. Free fatty acid (FFA) species have been markedly different in HCC groups compared with control groups, but study results have been conflicting, perhaps due to patient heterogeneity regarding age, gender, ethnicity, diets and existing comorbidities[91,93-95,97,98,102]. The European Prospective Investigation into Cancer and Nutrition study additionally described an extensive interaction between HCC and modifiable lifestyle factors in a large European cohort[85], and FFA levels have been linked to the severity of liver disease and disease etiology[103]. FFA species that have been identified include FFA C16:0, C18:0, C20:4 and C24:1.

Metabolites of energy production were broadly altered in HCC, particularly concerning products of beta-oxidation and other alternative metabolic pathways[49,82-84]. This may point to Warburg's phenomenon in HCC tumo-

rigenesis, where a shift of oxidative glucose metabolism to anaerobic glycolysis takes place to contribute a higher rate of energy production in tumor cells[8]. The increase in very low density lipoprotein, as seen in Gao *et al*[49] study, may explain the global lipid mobilization for the lipolytic pathway. Studies have also identified a rise in ketone bodies, such as acetone and beta-hydroxybutyrate, which are formed as by-products of beta-oxidation[84]. Furthermore, components of the normal TCA cycle such as 2-oxoglutarate, succinate and glycerol also were significantly altered in HCC groups against controls[49,102,103]. The elevation of 2-oxoglutarate may be a consequence from a decreased mitochondrial respiration. Overall, the observed effect of reduced TCA, increased beta-oxidation and increased ketone bodies suggest a heightened alternative metabolic response in tumorigenesis.

Elevated levels of serum bile acids, such as glyco-chenodeoxycholic acid, glycocholic acid, deoxycholic acid and cholic acid, have long been recognized in many hepatobiliary diseases[104]. A study by Chen *et al*[105] identified cirrhotic patients have significantly higher levels of bile acids than those without. Interestingly, levels are significantly different even when comparing compensated against decompensated cirrhosis. It is no surprise that HCC metabonomic studies have identified elevated bile acids in HCC patients when compared to the healthy population[91-94,96-98,102]. Bile acids may have a role in tumorigenesis, as reports have described their involvement in glucidic metabolism and acting as signaling molecules[106,107]. However, the studies have not controlled for possible confounding factors such as the compensation/decompensation profile, or the prandial state of patients, where certain bile acids are elevated after food intake[108], and therefore, bile acids would not be suitable HCC biomarkers until specific studies are performed to address this issue.

URINARY MARKERS OF HCC

For a urinary biomarker to be widely applicable three central attributes are necessary. First, the biomarker, if produced pre-renally, needs to be small enough and of the correct ionic charge to be filtered by the renal glomerulus and not re-absorbed by the tubules. Therefore, it has to be roughly less than 20 kDa in atomic weight. Second, the marker should be specific to the cancer in question and not secondary to the effects of cancer on general physiology. Finally, the marker should be secreted in adequate amounts for accurate, repeatable detection in early disease. Large, complex proteins are unlikely to enter the urinary stream, so are not candidates for urinary biomarkers.

Nucleosides

Studies in the 1970s observed elevated levels of the methylated purines 7-methylguanine, 1-methylguanine, *N*-dimethylguanine, 1-methylhypoxanthine and adenine in the urine of patients with HCC. In 1976, it was found that urine levels of cyclic guanosine 3':5' monophosphate (cGMP) were elevated in rats with transplanted liver and renal tumors[109]. In 1982, Dusheiko *et al*[110], found parallels in human studies, observing elevated urinary cGMP levels in patients with HCC. In the same study, cGMP was also elevated in the urine of patients with liver disease and other non-HCC tumors, reducing the specificity of the marker considerably.

In 1986, Tamura *et al*[111] observed that urinary levels of pseudouridine, a C-glycoside isomer of the nucleoside uridine, to be elevated in patients with HCC. When combined with serum AFP, sensitivity for HCC detection was 83%. Disappointingly, this marker was also non-specific and found to be similarly elevated in patients with other malignancies such as non-Hodgkin's lymphoma. In a Taiwanese patient study, it was observed that the urinary nucleosides adenosine, cytidine and inosine were elevated in patients with HCC[112]. When combined with serum AFP, sensitivity for tumor diagnosis was 80%. The study was flawed in that controls consisted of healthy patients with no liver disease and ideally the finding should have been confirmed in comparison to a group of patients with cirrhosis.

TGFα and β

TGFα and β have both been detected in the urine of patients with HCC. The first report was from 1990, observing elevated TGFα levels in urine[113]. In 1991, a TGF-related protein was found in HCC patient urine and this was confirmed as TGFβ1 in 1997 by the same group[114,115]. In these studies, TGFβ1 correlated with prognosis and survival. A functional link was attractive as TGFs are known to stimulate non-transformed cells reversibly to grow as colonies *in vitro*.

Neopterin

In 1998, a study performed in Japan found neopterin, a protein now known to be released from macrophages following inflammatory stimulation, to be elevated in the urine of patients with advanced HCC[116,117]. Similar to other potential markers, neopterin has since been shown to be elevated in a number of malignancies and pro-inflammatory conditions such as human immuno-deficiency virus related disease, reducing its validity as a specific marker for HCC[118].

Polyamines

The polyamines, organic compounds containing two or more amine groups, include putrescine, spermine, and spermidine. Their exact cellular role is unclear but they are required for cellular proliferation. Putrescine acts on *S*-adenosylmethionine (SAMe), a methylating molecule, to produce spermine which in turn acts on further SAMe molecules to produce spermidine[119]. Antoniello *et al*[120] reported increased urinary levels of free and acetylated polyamines using HCC patients compared to healthy controls and patients with cirrhosis, although the sensitivity of these markers was found not to be high enough for early tumor detection.

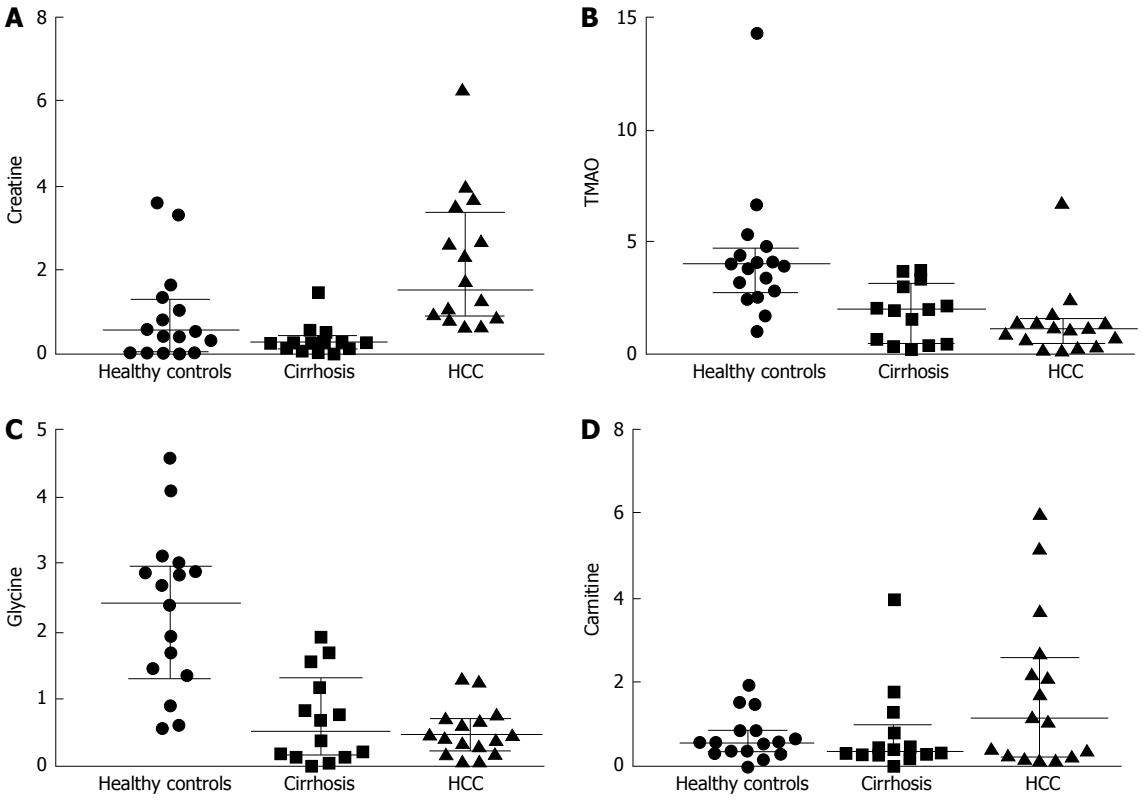

Figure 5 Univariate analysis of discriminatory urinary variables from an Egyptian cohort, comparing values from healthy controls, cirrhosis group and hepatocellular carcinoma group. Discriminatory variable A: Creatine; B: Trimethylamine N-oxide (TMAO); C: Glycine; D: Carnitine. Adapted from Shariff *et al*[125]. HCC: Hepatocellular carcinoma.

Urinary trypsin inhibitor

Urinary trypsin inhibitor (UTI) is a 25 kDa protein thought to be produced by hepatocytes. In 2004, an enzyme-linked immunosorbent assay-based study observed that urinary UTI was elevated in patients with HCC, albeit not significantly when compared to patients with cirrhosis[121]. Follow-up studies have found correlations with severity of liver disease and patient prognosis in general, but not specifically with HCC[122].

Soluble urinary metabolites

Recently, Chen *et al*[102] analyzed the serum and urine from 82 patients with HCC and compared these profiles to patients with benign liver tumors and healthy volunteers. Forty three serum and 31 urine metabolites were differentially present in samples of patients with HCC. These included bile acids, free fatty acids, inosine and histidine.

Wu *et al*[103] reported a urinary GC-MS study of 20 HCC patients which identified a marker panel of 18 metabolites discriminating HCC and healthy Chinese controls. This panel included octanedioic acid, glycine and hypoxanthine. In the same year, Chen *et al*[123] utilized mass spectroscopy techniques with hydrophilic interaction chromatography and reverse phase liquid chromatography in a comparison of 21 urine samples of patients with HCC to 24 healthy volunteer samples. In this set, hypoxanthine, creatinine, betaine, carnitine, acetylcarnitine, leucylproline and phenylacetylglutamine were altered between groups.

The most recent studies of urinary HCC metabolites to date have been performed within the African populations in Nigeria, Egypt and Gambia[124-126]. These studies compared the profiles of HCC with cohorts with cirrhosis without HCC, and healthy control, allowing further differentiation and insight into the metabolic difference in HCC tumorigenesis (Figure 5). Urinary creatinine was lowered in all three African cohorts. Urinary creatinine excretion is has been associated with muscle mass[127], and the results seen in the studies may reflect cancer cachexia rather than a specific marker for HCC.

Urinary carnitine levels were also elevated in HCC compared to cirrhosis in all three African groups. Carnitine is a hydrophilic compound, mainly absorbed from the diet and in part synthesized by the body. It is an essential compound for mitochondrial transport of long-chain fatty acids from the cytosol for beta-oxidation. Well-functioning kidneys efficiently reabsorb carnitine, a high urinary level inferring excess carnitine ingestion, biosynthesis or poor reabsorption. Increased urinary acylcarnitines have previously been reported in specific FFA oxidation disturbances and after intense exercise[128]. In the context of HCC, Shariff *et al*[125] hypothesized its elevation may be explained by increased metabolic activity and high cell-turnover, causing carnitine overproduction to fuel beta-oxidation and rapid energy production[127].

Urinary creatine levels were significantly elevated in

the Egyptian cohort with HCC, but were non-significantly elevated in the Nigerian compared to the respective cirrhosis groups[124,125]. Creatine is a nitrogenous organic acid, synthesized mainly in the liver by its constituent parts arginine, glycine and methionine. It has a direct function in cellular energy transport, interacting directly with ATP to produce phosphocreatine and adenosine diphosphate. It is likely that the heightened cell turnover increases cellular energy transport demand, and subsequently raises creatine levels.

Dimethylglycine (DMG), choline, and trimethylamine-N-oxide (TMAO) are metabolites involved in choline intermediary metabolism. Urinary DMG and choline were elevated but a lower concentration of TMAO was noted in the Gambian population. Overexpression of choline has been well established in a series of different tumors. TMAO is typically formed by bacterial degradation of choline, it is likely that this alteration reflects dysregulation of intestinal microbiota, as suggested by Ladep *et al*[126]. The metabolic alterations that have been observed may be explained by the Warburg phenomenon and its preferential glucose metabolism *via* anaerobic glycolysis.

Urinary glycine levels were reduced in the Egyptian population, but have been unreported in the other studies[125]. Glycine's normal cell function involves the methylation of DNA. Its reduction in HCC may be explained by the widely noted phenomenon of hypomethylation within the tumorigenic process. In addition, the Nigerian and Egyptian studies have seen an increase in creatine, as mentioned above. Glycine is a molecular constituent of creatine, which is upregulated in the high cell turnover environment of HCC, which may also explain the decline in glycine observed from the Egyptian study[124,125].

CONCLUSION

This review provides an overview of HCC pathogenesis and from it, a large selection of potential biomarkers that correlate to the complex molecular and metabolic interaction in its tumorigenesis. HCC is a significant global health issue, which primarily affects countries where there is an infrastructural limitation on community-based surveillance for early disease, and therapeutic options in later stages of tumor presentation. Various diagnostic techniques that have been successfully utilized in developed countries, such as US surveillance, cannot be introduced in resource-limited regions where their application is fundamentally unsuitable. In the current absence of a simple and effective diagnostic investigation in those regions, we highlight the need for research progression in designing clinical diagnostic techniques that may be cheaply and effectively administered. In particular, we emphasize the potential of metabolomics identification of candidate metabolites through the development of a simple urine dipstick, which may be easily performed even in the lowest-income settings.

In considering biomarker application, there must be a careful and a realistic consideration as to the heterogeneous metabolic profiles of varying ethnic groups. It is unlikely that a single panel of metabolites that have adequate sensitivity and specificity in the developed population would be appropriate for the developing world population. Previous research has shown that there are clear racial differences in the diagnostic value of AFP, where a minority of Asian, Eurapoid, and Hispanic patients with HCV-related HCC had a normal AFP (18%), close to half the African American patients had a normal AFP level (43%), and furthermore, there was an observed difference between underlying etiology of liver disease, where HCV-related HCC had a stronger association with raised AFP, compared to HBV-related HCC[129]. The clear etiological, dietary, genetic and environmental factors that differ between populations suggest the need for specific metabolomic studies, or at least validation studies, in the very regions of the world where better diagnostics or screening tools are required.

To address the pressing issue of identifying novel biomarkers that are sensitive, practically applied, and ethnically specific, the most recent African urinary studies may present the most relevant biomarkers, which can be translated to a simple urine dipstick test[124-126]. The significant metabolites include urinary creatine, carnitine and creatinine, among others. Again, these metabolites reflect the molecular changes that happen as part of Warburg's hypothesis of altered energy metabolism. The close fit of the results to the hypothesis should encourage researchers to study the molecular pathway closer in relation to HCC.

In conclusion, success in the field of proteomics and metabonomics will ultimately depend on its clinical application, and this requires a greater emphasis on validation-based experiments of early HCC identification.

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REVIEW

Host nucleotide polymorphism in hepatitis B virus-associated hepatocellular carcinoma

Shilu Mathew, Hany Abdel-Hafiz, Abbas Raza, Kaneez Fatima, Ishtiaq Qadri

Shilu Mathew, Center of Excellence in Genomic Medicine Research, King Abdul Aziz University, Jeddah 21589, Saudi Arabia

Hany Abdel-Hafiz, University of Colorado Denver AMC, Aurora, CO 80045, United States

Abbas Raza, Department of Immunobiology, University of Vermont, Burlington, VT 05405, United States

Kaneez Fatima, IQ-Institute of Infection and Immunity, Lahore 54000, Pakistan

Ishtiaq Qadri, King Fahd Medical Research Center, King Abdul Aziz University, Jeddah 21589, Saudi Arabia

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Correspondence to: Ishtiaq Qadri, PhD, Professor, King Fahd Medical Research Center, King Abdul Aziz University, PO Box 80216 Jeddah 21589,
Saudi Arabia. ishtiaq80262@yahoo.com
Telephone: +966-12-6400000
Fax: +966-12-6952067

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Abstract

Hepatocellular carcinoma (HCC) is etiologically linked with hepatitis B virus (HBV) and is the leading cause of death amongst 80% of HBV patients. Among HBV affected patients, genetic factors are also involved in modifying the risk factors of HCC. However, the genetic factors that regulate progression to HCC still remain to be determined. In this review, we discuss several single nucleotide polymorphisms (SNPs) which were reportedly associated with increased or reduced risk of HCC occurrence in patients with chronic HBV infection such as cyclooxygenase (COX)-2 expression specifically at COX-2 -1195G/A in Chinese, Turkish and Egyptian populations, tumor necrosis factor α and the three most commonly studied SNPs: PAT-/+, Lys939Gln (A33512C, rs2228001) and Ala499Val (C21151T, rs2228000). In genome-wide association studies, strong associations have also been found at loci 1p36.22, 11q22.3, 6p21 (rs1419881, rs3997872, rs7453920 and rs7768538), 8p12 (rs2275959 and rs37821974) and 22q11.21. The genes implicated in these studies include *HLA-DQB2*, *HLA-DQA1*, *TCF19*, *HLA-C*, *UBE2L3*, *LTL*, *FDX1*, *MICA*, *UBE4B* and *PG*. The SNPs found to be associated with the above-mentioned genes still require validation in association studies in order to be considered good prognostic candidates for HCC. Screening of these polymorphisms is very beneficial in clinical experiments to stratify the higher or lower risk for HCC and may help in designing effective and efficient HCC surveillance programs for chronic HBV-infected patients if further genetic vulnerabilities are detected.

Key words: Hepatitis B virus; Hepatocellular carcinoma; Subtypes; Genetic polymorphism; Liver cirrhosis

Core tip: In this review, we discuss various common associations between hepatitis B virus (HBV) and host polymorphisms. These single nucleotide polymorphisms which have been found to be associated with various genes still require validation in association studies in order to be considered good prognostic candidates for hepatocellular carcinoma (HCC). Screening of these polymorphisms is very beneficial in clinical experiments to stratify the higher or lower risk for HCC and may help in designing effective and efficient HCC surveillance programs for chronic HBV-infected patients if further genetic vulnerabilities are detected.

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HEPATITIS B VIRUS

Hepatitis B virus (HBV) infection is the third most common cause of cancer-related deaths in relation to hepatocellular carcinoma (HCC) with a high incidence in Asian countries. HCC is responsible for approximately 660000 deaths worldwide each year and 85%-90% of these deaths are due to primary liver cancers[1]. It is recognized that these cancers are mainly due to HBV infection with 60% of HCC cases seropositive for this virus[2]. Many risk factors including viral factors (*e.g.*, genomic mutations, genotypes, HBV-DNA levels), host factors and unhealthy lifestyles all contribute to the development of liver diseases[3].

Both epigenetic and genetic factors play a role in the malignant transformation of liver cells[4]. Multiple cellular signaling genes are enhanced by the incorporation of HBV into the host's genome which promotes transactivation of HBx protein[5]. This process activates/inactivates suppressor genes (*e.g.*, *p53*), oncogenic genes (*e.g.*, *c-fos* and *c-myc*), induces loss of heterozygosity and activates transcriptional factors [*e.g.*, nuclear factor kappa-B (NF-κB) and AP-1][6].

However, underlying disease and the duration of severity vary significantly between each phase. Moreover, clinical progression varies between patients. Liver injuries in patients with HBV infection are thought to be the outcome of the host's immune responses against HBV. For example, cytotoxic T lymphocyte-mediated, an HLA-class Ⅰ antigen-restricted, response to the HBV antigen expressed on hepatocytes results in necrosis and apoptosis[7].

Several genome wide association studies have identified candidate single nucleotide polymorphisms (SNPs) by comparing the SNPs present in HCC patients and those present in asymptomatic HBV carriers[8]. Therefore, to specifically evaluate genetic factors, it is vital that the controls and patients are well matched regarding these factors to identify the correct SNP. The results of many studies suggest that several SNPs are associated with HBV clearance and persistent infection. Functional analyses are necessary to confirm these results[6,7]. In this review, we discuss several SNPs which are reportedly associated with increased or reduced risk of HCC occurrence in patients with chronic HBV infection[9].

INFLAMMATORY GENETIC POLYMORPHISM

It has been reported previously that SNPs can affect disease progression after HBV infection. Cytokines, such as tumor necrosis factor-α (TNFα) and interleukin (IL)-10, have a significant role in regulating viral infection. Genetic variation of these cytokines is linked with the outcome of HBV infection[10-16].

Several studies have shown that genetic polymorphisms in multiple genes such as *TP53*[17], *IL-6*[18], and DNA repair genes[19], are associated with the development of chronic HBC infection, progression of the infection and increased risk of HCC. These may serve as biomarkers in identifying HCC risk[20]. However, these studies were predominantly performed in HBV-positive populations or populations with a high infection rate.

Genetic variation in tumor suppressor genes or oncogenes is capable of altering gene function and, consequently, may contribute to the development of cancer. Significant research has been conducted to investigate the association between polymorphisms in tumor suppressor genes and oncogenes and the risk of HCC; however, the results are controversial.

ASSOCIATIONS BETWEEN HBV AND THE HOST POLYMORPHISM

Cyclooxygenase-2

Cyclooxygenase-2 (COX-2) is involved in many cellular functions, including inflammation, inhibition of apoptosis, carcinogenesis, angiogenesis, invasion and metastasis[21,22]. COX-2 is overexpressed in many cancers including HCC, indicating that there is an association between COX-2 expression and the development of cancer[23,24]. Selective COX-2 inhibitors have been shown to suppress the growth of HCC cells *in vitro* and *in vivo*[25]. A polymorphism in the promoter region of the *COX-2* gene could functionally upregulate the transcriptional activity of COX-2, indicating a possible mechanism by which COX-2 may contribute to genetic susceptibility to HCC[21]. Several studies have reported that COX-2 point mutations including -1195G/A, -765G/C and +8473T/C were correlated with liver diseases and

HBV-related HCC[26]. COX-2-765G/C is related to the risk of skin, esophageal, colorectal, breast and gastric cancers[27-29]. With regard to HCC, contradictory and inconclusive results were found. Some studies have reported a correlation between COX-2-765G/C and HBV-related HCC risk[30-32], but other studies reported that no such correlation exists[26,33,34]. It has been reported that these inconsistent results were possibly due to limited sample sizes and ethnic variation in those studies. COX-2 + 8473T/C is associated with oral and breast cancers[35,36], but is not associated with HCC[37]. A recent meta-analysis by Chen *et al*[26] on Chinese, Turkish and Egyptian populations, concluded that COX-2-1195G/A may be associated with HCC risk, but not COX-2-765G/C or COX-2 + 847T/C.

IL-1alpha and 1beta

IL-1α is a potent pro-inflammatory cytokine and has many different biological functions, including cell survival, proliferation, and anti-apoptosis[38,39]. IL-1β is also reported to inhibit interferon-induced antiviral activity[40] and is assumed to be closely associated with the pathogenesis of chronic hepatitis C. Several polymorphisms of the *IL-1* gene that are thought to affect IL-1β production have been reported[41]. -31T SNPs of IL-1β have been shown to enhance IL-1β transcriptional activity[42] and several studies reported that -511C/-31T is a risk factor for the development of cancer and liver diseases[43-45]. Wang *et al*[41] showed that IL-1β-31 polymorphism was associated with HCC, after controlling for other confounding clinical parameters.

E-cadherin (CDH1)

E-cadherin is a transmembrane protein that mediates cell-cell adhesion and is expressed in most normal epithelial cells. Downregulation of E-cadherin may lead to a loss of E-cadherin-mediated adhesion, resulting in increased susceptibility to tumor development and is associated with poor prognosis in various carcinomas including HCC[45-52]. In addition, HBV and HCV reduce E-cadherin expression and promote tumor recurrence in HCC patients. One of the mechanisms that have been proposed for reduced E-cadherin expression is SNPs in the promoter region of the *CDH1* gene. CDH1-160 C/A and -347G/GA polymorphisms result in the downregulation of E-cadherin protein and is associated with cancer susceptibility[53]. Several studies demonstrated that CDH1-347 SNPs are significantly associated with HCC risk[52,54-57]. However, the correlation between CDH1-160 SNPs showed conflicting results. Some studies[58,59] have shown that CDH1-160 SNP carriers have an increased risk of prostate and bladder cancer, while others showed that it was not associated with the development of prostate, HCC, colorectal or gastric cancer[60].

Peroxisome proliferator-activated receptor gamma

Peroxisome proliferator-activated receptor gamma (PPARγ) is a hormone receptor, present in adipose tissue and plays a critical role in the regulation of fatty acid storage and glucose metabolism[61]. PPARγ has been shown to be associated with type 2 diabetes mellitus (T2DM)[62]. PPARγ contains two isoforms, PPARγ1 and PPARγ2 and several variants in the *PPARγ* gene have been identified[63]. The A allele of PPARγ2 is associated with a significant decrease in the development of T2DM[64]. The relationship between PPAR and HCC is not clear. Although experimental studies have shown that PPAR may have a role in HCC[65,66], the implications of these findings are unclear. Koytak *et al*[66] investigated the effect of the PPARα L162V polymorphism on clinical outcome in a patient with HCC caused by hepatitis viruses. They concluded that there was a relationship between the PPARα L162V polymorphism and HBV-induced HCC and was associated with advanced HCC. This polymorphism was shown to enhance PPARα transcriptional activity and is associated with lipid abnormalities and an increased body mass index[67-70].

TNFα-inducible protein 3

TNFα-inducible protein 3 (TNFαIP3), a cytoplasmic zinc finger protein with ubiquitin-modifying activity, has been shown to inhibit NF-κB activity and TNF-mediated apoptosis[71-74]. TNFαIP3 polymorphisms have been linked to inflammatory, autoimmune and malignant diseases. A recent study reported that there was no association between TNFαIP3 rs2230926 polymorphism and susceptibility to chronic HBV infection or the progression of HBV-related diseases[75].

Cytotoxic T lymphocyte-associated factor 4

Cytotoxic T lymphocyte-associated factor 4 (CTLA-4) is a protein receptor expressed in T cells and it functions as a negative regulator of the immune system. Several *CTLA-4* gene polymorphisms have been identified including -318C>T, A49G and CT60[76]. CTLA-4 polymorphisms are associated with several autoimmune diseases, including thyroid and liver diseases[77,78]. It has been shown that SNPs in CTLA-4 may be associated with HBV progression and viral persistence[79]. CTLA-4 SNPs can be used as a marker for predicting treatment outcome in chronic HCV-infected patients[80-82].

TNFα

TNFα is a multifunctional cytokine that regulates the inflammatory reaction and has an important role in the development and progression of a number of diseases, including liver disease[83,84]. It has been suggested that genetic polymorphisms of TNFα may contribute to the pathogenesis of liver diseases, infectious diseases and inflammatory disorders[43,85]. For example, TNFα SNPs affect TNFα production leading to a greater risk of HCC. The polymorphism at site -1031T/C, -863C/A, -857C/T, -376, -308G/A and -238G/A of the TNFα promoter is associated with the outcome of HBV infection and disease progression[86-89].

IL-10

IL-10 is an important anti-inflammatory cytokine produced in macrophages. Three SNPs in the *IL-10* gene promoter, at -1082, -819 and -592, are associated with IL-10 production and secretion by peripheral blood monocytes. It has been shown that IL-10-592 A/C polymorphism was associated with susceptibility to HBV infection[90].

Glutathione S-transferases

The glutathione S-transferases (GSTs) enzymes play an important role in maintaining the cellular defense mechanism against the effects of reactive oxygen species and various exogenous toxins, and have been shown to be overexpressed in several cancers[91,92]. Deletion polymorphism of *GST* genes results in diminished enzyme activity leading to the insufficient defense of cells from metabolites and free radicals, elevated concentration of endogenous mutagens and a high risk of various tumors, including HCC[93-96]. GSTs polymorphisms have been shown to be associated with colorectal cancer, lung cancer, squamous cell carcinoma of the head and neck, HBV-related HCC, and various urogenital and gastrointestinal disorders[97-99]. For example, meta-analyses have shown that GSTM1, GSTP1 and GSTT1 are associated with an increased risk of HCC[100,101].

Epidermal growth factor

Epidermal growth factor (EGF) and its respective receptor (EGFR) signaling are important regulators of proliferation and the pathogenesis of many human carcinomas[102,103]. Upon ligand binding, the two EGFR domains undergo trans-autophosphorylation at specific tyrosine residues[104]. These phosphotyrosines are recognized by Src homology 2 domain containing proteins[105] and activate a diverse signaling network that includes the RAS/extracellular signal-regulated kinase pathway[106], the phosphatidylinositol 3-kinase pathway[107] and the Janus kinase/Signal transducer and activator of transcription pathway[108].

Activation of EGF has also been shown to be required for hepatocyte growth during liver regeneration[109]. In addition, many viruses such as Epstein Barr virus and HBV can tweak EGF receptor expression in their favor[110-112]. The role of EGF polymorphism has been explored in numerous meta-analyses[113-116] and was shown to be highly associated with susceptibility to HCC[117]. Prominent among these is the EGF + 61A > G transversion (rs4444903) which was shown to regulate expression of the *EGF* gene[118,119]. This SNP is found in the 5′ untranslated regions of the *EGF* gene and was shown in cell lines to enhance the stability of EGF mRNA[119]. The G/G allele is associated with higher serum levels of EGF compared with the A/A allele[119,120]. Numerous follow-up studies have validated the positive association between this G/G and G/A genotype with HCC in diverse genetic populations[117,121-123] and thus can be considered a good prognostic marker for the genetically susceptible population.

Murine double minute 2

Murine double minute 2 (MDM2) is a ubiquitin ligase that controls the turnover rate of an important tumor suppressor, p53, which is deleted or mutated in 50% of all human tumors[124]. P53 is also referred to as the guardian of the genome because it can activate DNA repair pathways[125], arrest cell cycle at the G1/S regulation checkpoint[126] or initiate apoptosis if the damage cannot be repaired[127]. All these important networks converge in the active form of p53, which is kept in check by MDM2. The addition of ubiquitin subunits to critical lysine residues transfers the active p53 to 26S proteasome for degradation along with MDM2[128,129]. In addition, the binding of MDM2 can block p53-mediated transactivation functions[130]. The activity of MDM2 protein is equally important in regulating this DNA repair-cell cycle-apoptosis nexus and variation in the expression levels of this protein was shown to have serious consequences in cells or organisms[131]. Bond *et al*[132] showed that the SNP 309T > G (rs 2279744) located in the promoter region of MDM2 can enhance the transcriptional levels of this protein and subsequent perturbation of p53 functions in the cell. This T > G mutation is thought to generate a binding site on the MDM2 promoter for Sp1 transcription factor[133] and thus enhances the levels of MDM2 protein in the cell.

The positive association between this SNP 309T > G (rs 2279744) in the *MDM2* gene and HCC was shown by numerous ethnic-based studies[134-136] and meta-analyses[137,138]. This epidemiological finding together with functional assays of MDM2 levels point to the relevance of MDM2 SNP 309T > G polymorphism as an important player in susceptibility to HCC development.

T cell immunoglobulin mucin-3

T cell immunoglobulin mucin-3 (TIM3) negatively regulates the autoimmune and allergic responses and has been linked to T cell dysfunction associated with HBV-related HCC[139]. The 280 aa mature TIM3 is selectively expressed on $CD4^+$ Th1 and $CD8^+$ Tc1 cells, but not on $CD4^+$ Th2 cells[140]. It interacts with its ligand galectin-9 and drives death Th1 T cells[141,142]. Blocking TIM3-mediated signaling restores dysfunctional CD4 and $CD8^+$ T cell-specific adaptive immune responses[143]. TIM3 is upregulated on CD4 and $CD8^+$ T cells in chronic HBV infected individuals[144].

Numerous potential SNPs (-1541C/T, -1516G/T, -882C/T, -574G/T and +4259T/G) in TIM3 have been tested for their association with chronic HBV and HCC[145]. TIM3-1516 G/T (rs10053538) polymorphism has been shown to predispose individuals to cirrhosis and/or HCC[146,147]. One study reported that TIM3 SNPs do not have a functional effect[148], whereas others have reported a significant effect of these TIM3 polymorphic variants[149]. Further studies are needed to determine the functional relevance of this polymorphism.

Xeroderma pigmentosum complementation group C

Xeroderma pigmentosum complementation group C (XPC) protein along with seven other core members (ERCC1, XPA, XPB, XPC, XPD, XPE, XPF and XPG) constitutes the nucleotide excision repair pathway (NER). This pathway is required for the repair of DNA damage including pyrimidine dimers, photo products, chemical adducts and cross-links[150,151]. XPC requires an association with HR23B in order to recognize damaged DNA[152]. The protein HR23B is a human homolog of *Saccharomyces cerevisiae* RAD23 and binding of XPC-HR23B to a DNA lesion unwinds the helix[153]. The XPA protein can then bind and the whole repair machinery of the NER can be recruited onto the damaged base.

Many studies have investigated the association between XPC sequence variants and cancer risk[154-158]. The three most commonly studied SNPs in the literature are: PAT-/+[159], Lys939Gln (A33512C, rs2228001)[155] and Ala499Val (C21151T, rs2228000)[160]. The poly (AT) insertion/deletion polymorphism (PAT) is located on intron 9 and has been shown to be linked to head and neck cancer risk[161] and to lung cancer[162], but no studies have found an association with HCC risk. The XPC codon Lys939Gln alleles, on the other hand, significantly increased HCC risk[163,164]. The Ala499Val variant homozygous genotype is a risk factor for bladder cancer[158], but has not been studied for HCC.

IL-16

IL-16 is a pro-inflammatory cytokine and was initially called lymphocyte chemoattractant factor[165]. It can activate a diverse set of immune cells such as $CD4^+$ T cells, monocytes, macrophages, eosinophils and dendritic cells[166-169]. In addition to inducing activation and chemotaxis of immune cells, IL-16 can upregulate the IL-2 receptor[170] and HLA-DR4 expression[171]. Upon CD4 receptor binding, IL-16 signaling increases intracellular calcium and inositol triphosphate, and translocation of protein kinase C from the cytosol to the plasma membrane[172,173]. Moreover, IL-16 can stimulate the production of further pro-inflammatory mediators including LI-1β, IL-6, IL-15 and TNFα, *e.g.*, by monocytes[174] thereby initiating and/or sustaining the inflammatory response.

Genetic polymorphisms in IL-16 have recently been reported and shown to affect susceptibility to a range of cancers including colorectal, gastric and prostate cancer and nasopharyngeal carcinoma[175-178]. Data regarding HCC and IL-16 polymorphisms are scarce in the literature and only two studies were found to have assessed three SNPs (rs11556218T > G, rs4778889T > C, and rs4072111C > T)[179]. In the study by Li *et al*[180], no association with HCC was found for all three SNPs (rs11556218T/G $P = 0.511$, rs4072111C/T $P = 0.308$ and rs4778889T/C $P = 0.070$). The other study by Thomas *et al*[178] did not include HCC patients. However, this study did include chronic hepatitis B patients who showed a positive association between rs11556218T > G, a negative association between rs4778889T > C and a positive association between rs4072111C > T polymorphisms and patient susceptibility to chronic hepatitis B infection[179].

Genome-wide association studies

Numerous genome-wide association studies (GWAS) have been carried out with chronic HBV and HCC patients to identify novel susceptible loci contributing to disease[6,181-186]. Of these, strong associations were found at 1p36.22, 11q22.3, 6p21 (rs1419881, rs3997872, rs7453920 and rs7768538), 8p12 (rs2275959 and rs37821974) and 22q11.21. The genes implicated in these studies include HLA-DQB2, HLA-DQA1, transcription factor 19 (TCF19), HLA-C, ubiquitin-conjugating enzyme E2 (UBE2L3), LTL, ferredoxin 1 (FDX1), MICA, UBE4B and PG.

HLA-DQ is an MHC class Ⅱ cell surface receptor found on antigen presenting cells, whereas HLA-C is an MHC class Ⅰ receptor expressed by all cells. TCF19, as the name suggests, is an important transcription factor during cell cycle G1/S transition[187]. UBE2L3 is a typical E2 ligase that accepts ubiquitin from the E1 complex and transfers it to targeted proteins[188]. Leukocyte telomere length (LTL) has been associated with the risk of developing many malignancies[189] and LTL-related SNPs are potential targets for such GWAS studies. FDX1 is a gene that codes for a small iron-sulfur protein that transfers electrons from NADPH through ferredoxin reductase to mitochondrial cytochrome P450[190]. In addition, it is involved in steroid, vitamin D, and bile acid metabolism[191].

These SNPs found to be associated with the above-mentioned genes still require validation in association studies in order to be considered good prognostic candidates for HCC.

Tumor growth factor beta

Tumor growth factor beta (TGFβ) is a tumor suppressor gene located on chromosome 19q13.1-13.39. The protein TGFβ is involved in pleiotropic biological processes such as cell growth[192], differentiation[193], extracellular matrix synthesis[194], hematopoiesis[195], angiogenesis[196], and cellular apoptosis[197]. TGFβ1 is one of TGFβ isoforms and is upregulated in HCC tissues correlating with the carcinogenesis and prognosis of HCC[198,199]. TGFβ1 also suppresses HBV replication by reducing hepatocyte nuclear factor-4-alpha[200]. Thus, the relevance of this cytokine and its single nucleotide polymorphism in HBV-associated HCC is of paramount importance.

Seven TGFβ1 polymorphisms have been described in the literature, of which three lie in the upstream region of the gene at positions -988C > A, -800G > A, and -509C > T, one insertion in a nontranslated region at position +72C, two in exon 1 (Leu10Pro and Arg25Pro); and 1 in exon 5 (Thr263Ile)[201]. Numerous studies have investigated the association between these

Table 1 List of polymorphic genes and their contribution to hepatocellular carcinoma

Polymorphism	Genotype	Significance	Ref.
COX-2	-1195G > A	$P < 0.00$[26]	He *et al*[31]
	-765G > C	$P < 0.05$[31] and 0.41[26]	Chen *et al*[26]
	+8473T > C	$P = 0.83$[26]	
IL-1α, β	511C > T	$P = 0.02$[41]	Wang *et al*[41]
	-31C > T	$P = 0.02$[41]	
CDH1	-347G > A	$P = 0.171$[209] and < 0.05[60]	Li *et al*[209], Chien *et al*[60]
PPARγ	L162V	$P = 0.071$[66]	Koytak *et al*[66]
TNFAIP3	F127C	$P = 0.15$[75]	Zhang *et al*[75]
TNFα	-1031T/C	$P = 0.85$[86]	Wei *et al*[86]
	-863C/A	$P = 0.006$[86]	
	-857C/T	$P = 0.09$[86]	
	-308G/A	$P = 0.046$[86]	
	-238G/A	$P = 0.003$[86]	
GST	GSTM1 + GSTT1	$P = 0.001$[210]	Liu *et al*[210]
EGF	+61A > G	$P < 0.001$[117]	Jiang *et al*[117]
MDM2	309G > T	$P = 0.001$[133]	Ezzikouri *et al*[133]
TIM3	-1516G > T	$P = 0.001$[146]	Li *et al*[146]
XPC	K939Q	$P = 0.001$[163]	Long *et al*[163]
1p36.22, 11q22.3, 6p21, 8p12 22q11.21	Include genes *HLA-DQB2*, *HLA-DQA1*, *TCF19*, *HLA-C*, *UBE2L3*, *LTL*, *FDX1*, *MICA*, *UBE4B* and *PG*	$P = 1.7 \times 10^{-18}$	Al-Qahtani *et al*[181]
		$P = 4.3 \times 10^{-8}$	
		$P = 0.0266$	
		$P = 0.0067$	
		$P = 1.71 \times 10^{-12}$	
TGFβ1	-509C > T	$P = 0.01$[206] and 0.318[207]	Qi *et al*[206]
	R25P	$P = 0.472$[207]	Hosseini Razavi *et al*[207]
	L10P	$P < 0.02$[208]	Kim *et al*[208]

COX-2: Cyclooxygenase-2; IL-1α, β: Interleukin-1α, β; CDH1: Cadherin 1; PPARγ: Peroxisome proliferator-activated receptor γ; TNFAIP3: Tumor necrosis factor alpha-induced protein 3; TNFα: Tumor necrosis factor α; GST: Glutathione S transferase; EGF: Epidermal growth factor; MDM2: Mouse double minute 2 homolog; TIM3: T-cell immunoglobulin 3; XPC: Xeroderma pigmentosum; TGFβ1: Transforming growth factor beta 1.

SNPs and HCC[202-205]. There are contrasting reports with some studies reporting a positive association between -509C > T (rs1800469) and HCC risk[206], whereas another study reported a weak or no association[204]. In addition, the Arg25Pro change at +915G/C (rs1800471) was not correlated with HCC risk[207]. The mutation in codon 10 (Leu > Pro) was very strongly correlated with HCC according to one study[208]. There is still limited information regarding other polymorphisms of TGFβ1 and further studies are required to draw firm conclusions on their association with HCC. Table 1 lists the polymorphic genes and their contribution to HCC.

DISCUSSION

In this article, we discuss the association between the HBV genotype and its mutations in the development of liver cancer and the possibility that individuals with inherited genetic mutations have a hereditary predisposition for HBV-related HCC. Such individuals can inherit a germ-line mutation in one allele of the gene; somatic mutation of the second allele facilitates tumor progression. Although the inherited germ-line mutation may not be adequate to affect tumor development, it is likely that HBV proteins also induce many alterations in the genome. Analysis of the whole transcriptome in these individuals with genetic predisposition would be a useful indicator. It is now well understood that host genetic differences significantly influence susceptibility and resistance to HBV infection and the development of liver cancer, thus it is important to identify these genotype-phenotype associations for better treatment of the disease (Figure 1). Genome-wide sequencing studies have identified numerous germline mutations associated with liver cancer predisposition and large numbers of somatic alterations. It is difficult to assess the difference between background and HBV-related mutations as HBV infection plays an important role in the development of host genetic mutations, due to impairment in the DNA repair process. To elucidate the role of HBV-related genetic variations, researchers have used traditional biological methods to identify genetic mutations. More recently, advanced techniques such as next generation sequencing technology have been used to identify key mutations involved in the development of HCC. Important HCC-associated mutations have been found in key regulatory genes including COX-2, IL-1α and 1β, E-cadherin (CDH1), PPARγ, TNFαIP3, CTLA-4, TNFα, IL-10, GSTM1/GSTT1 Deletion Oxidative stress, EGF, MDM2, TIM3), XPC, IL-16, TGFβ, 1p36.22, 11q22.3, 6p21, 8p12 and 22q11.21 candidate SNPs in GWAS. The association between each locus and the outcome of liver disease is discussed in detail in this article.

Based on these findings, we predict that advanced sequence analysis of host genome will provide us with a better understanding of the viral and host genetic factors involved in the development of HCC. Further studies are needed to evaluate and understand the role

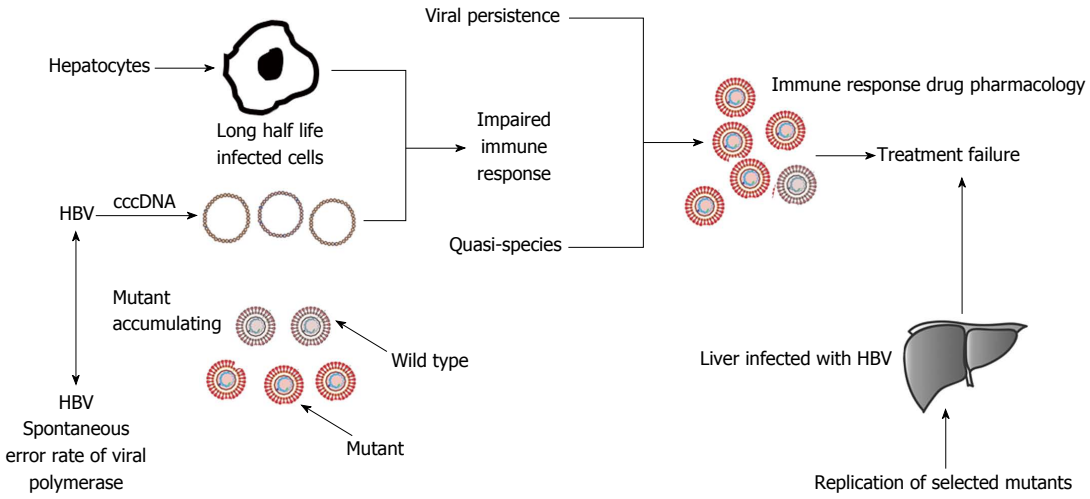

Figure 1 Mechanisms of selection and emergence of hepatitis B virus drug-resistant mutants. HBV: Hepatitis B virus; cccDNA: Covalently closed circular DNA.

of host-HBV interactions in HBV-related HCC to generate effective diagnostic and therapeutic treatments.

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ORIGINAL ARTICLE

Basic Study

Metabolomics studies identify novel diagnostic and prognostic indicators in patients with alcoholic hepatitis

Mona Ascha, Zeneng Wang, Mustafa S Ascha, Raed Dweik, Nizar N Zein, David Grove, J Mark Brown, Stephanie Marshall, Rocio Lopez, Ibrahim A Hanouneh

Mona Ascha, Mustafa S Ascha, Nizar N Zein, Department of Gastroenterology and Hepatology, Cleveland Clinic, Cleveland, OH 44195, United States

Zeneng Wang, J Mark Brown, Stephanie Marshall, Department of Cellular and Molecular Medicine, Cleveland Clinic, Cleveland, OH 44195, United States

Raed Dweik, David Grove, Department of Pulmonary, Allergy, and Critical Care Medicine/Respiratory Institute, Cleveland Clinic, Cleveland, OH 44195, United States

Rocio Lopez, Department of Quantitative Health Science, Cleveland Clinic, Cleveland, OH 44195, United States

Ibrahim A Hanouneh, Minnesota Gastroenterology, Minneapolis, Minnesota, PA 55414, United States

Author contributions: Ascha M, Ascha MS and Hanouneh IA performed the writing and critical revision of the manuscript; Wang Z, Dweik R, Grove D, Brown JM and Marshall S performed the majority of data collection; Zein NN and Hanouneh IA conceived and implemented the design of the project; Lopez R performed the statistical analysis.

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Correspondence to: Ibrahim A Hanouneh, MD, Minnesota Gastroenterology, P.O. Box 14909, Minneapolis, Minnesota, PA 55414, United States. ibrahim.hanouneh@mngastro.com
Telephone: +1-612-8711145
Fax: +1-612-8705491

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Abstract

AIM: To identify plasma analytes using metabolomics that correlate with the diagnosis and severity of liver disease in patients with alcoholic hepatitis (AH).

METHODS: We prospectively recruited patients with cirrhosis from AH (n = 23) and those with cirrhosis with acute decompensation (AD) from etiologies other than alcohol (n = 25). We used mass spectrometry to identify 29 metabolic compounds in plasma samples from fasted subjects. A receiver operating characteristics analysis was performed to assess the utility of biomarkers in distinguishing acute AH from alcoholic cirrhosis. Logistic regression analysis was performed to build a predictive model for AH based on clinical characteristics. A survival analysis was used to construct Kaplan Meier curves

evaluating transplant-free survival.

RESULTS: A comparison of model for end-stage liver disease (MELD)-adjusted metabolomics levels between cirrhosis patients who had AD or AH showed that patients with AH had significantly higher levels of betaine, and lower creatinine, phenylalanine, homocitrulline, citrulline, tyrosine, octenoyl-carnitine, and symmetric dimethylarginine. When considering combined levels, betaine and citrulline were highly accurate predictors for differentiation between AH and AD (area under receiver operating characteristics curve = 0.84). The plasma levels of carnitine [0.54 (0.18, 0.91); P = 0.005], homocitrulline [0.66 (0.34, 0.99); P < 0.001] and pentanoyl-carnitine [0.53 (0.16, 0.90); P = 0.007] correlated with MELD scores in patients diagnosed with AH. Increased levels of many biomarkers (carnitine P = 0.005, butyrobetaine P = 0.32, homocitrulline P = 0.002, leucine P = 0.027, valine P = 0.024, phenylalanine P = 0.037, tyrosine P = 0.012, acetyl-carnitine P = 0.006, propionyl-carnitine P = 0.03, butyryl-carnitine P = 0.03, trimethyl-lisine P = 0.034, pentanoyl-carnitine P = 0.03, hexanoyl-carnitine P = 0.026) were associated with increased mortality in patients with AH.

CONCLUSION: Metabolomics plasma analyte levels might be used to diagnose of AH or help predict patient prognoses.

Key words: Metabolomics; Biomarkers; Liver disease; Model for end-stage liver disease; Cirrhosis; Alcoholic hepatitis; Liver biopsy

Core tip: The model for end-stage liver disease score, which is commonly used to predict outcomes in patients who have liver disease, is far from perfect. We report results from a study that uses metabolomics biomarkers as a means for assessing diagnosis and prognosis in patients who have liver disease. Plasma analytes from fasted subjects have provided information regarding 3 and 6 mo transplant free survival. This study is one of the first to employ the novel metabolomics approach as it relates to patient outcomes. These results can pave the way for future research that can enhance the way we assess patients with liver disease.

Ascha M, Wang Z, Ascha MS, Dweik R, Zein NN, Grove D, Brown JM, Marshall S, Lopez R, Hanouneh IA. Metabolomics studies identify novel diagnostic and prognostic indicators in patients with alcoholic hepatitis. *World J Hepatol* 2016; 8(10): 499-508 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i10/499.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i10.499

INTRODUCTION

Generally, clinical assessment is sufficient to generate a diagnosis of alcoholic hepatitis (AH). However, sole dependence on clinical signs and symptoms is not specific, and further confirmation is usually needed. Thus, the gold standard for the diagnosis of AH is liver biopsy. Liver biopsy is considered an expensive and invasive procedure, and 1%-5% of patients require post-procedural hospitalization[1]. In addition, sampling error and inter-observer variability contribute to the limitations of liver biopsy as a procedure[1]. Therefore, it behooves practitioners to utilize alternative non-invasive tools to diagnose AH. Hanouneh *et al*[1] have shown promise in the possibility of analyzing volatile compounds in breath samples as a useful diagnostic test in patients with AH. Consequently, a rapid, non-invasive, accurate, and precise test would greatly benefit AH diagnosis.

Furthermore, prognosis of AH is determined by several scoring systems, including the model for end-stage liver disease (MELD), which is primarily based on serum lab values and is one of the chief parameters in evaluation of long-term outcome and qualification for liver transplant. While the MELD score can detect short-term survival in patients with AH with good accuracy, its prediction of long-term survival is still debated[2]. Palaniyappan *et al*[2] evaluated several scoring systems and their ability to predict long-term outcome of AH and concluded that all scoring systems were uniformly poor in predicting long term survival beyond six months. In addition, the cut-off value for the MELD score in detecting severe AH has not been agreed upon, with various studies employing different values[2]. Therefore, MELD score may not accurately reflect the risk of death in some groups of patients with liver disease such as AH awaiting liver transplantation.

Metabolomics was originally defined as the detailed qualitative and quantitative analysis of the metabolites present in complex biological samples[3]. Metabolites are both the intermediate and end result of all the biological processes taking place in a cell, tissue, or organism, thereby serving as the most proximal reporters of the body's response to a disease process or drug therapy[4]. By identifying and quantifying metabolites, one can gather a picture of the genetic variations and environmental influences (such as diet, lifestyle, drug use, and toxicological exposure) in a biological specimen. In more recent years physicians have been exploring the potential of metabolite profiling in providing diagnostic and prognostic information for many diseases, such as AH. For example, Rachakonda *et al*[5] demonstrated *via* metabolomics profiling that specific biomarkers could be used to determine disease prognosis in patients with severe AH. Thus, the potential of utilizing biomarkers in diagnosis of liver disease, assessing liver disease severity, and determining long-term survival in patients with AH is worth investigating; further exploration is warranted as there is limited information on this subject. Herein, we used a targeted metabolomics approach to identify plasma analytes that may provide improved diagnostic and prognostic value in patients with alcoholic hepatitis and end-stage liver disease.

MATERIALS AND METHODS

Patients

We recruited patients with liver cirrhosis awaiting liver transplantation from a single tertiary care center. The study population was divided between those with AH with cirrhosis (n = 25) and those with acute decompensated (AD) cirrhosis from etiologies other than alcohol (n = 23). The diagnosis of AH with cirrhosis was based on clinical and laboratory features: A patient with a history of heavy alcohol use, exclusion of other causes of liver disease, elevated aspartate aminotransferase that remained under < 300 IU/mL, a ratio of aspartate aminotransferase (AST) level to alanine aminotransferase (ALT) level that is > 2, total serum bilirubin level of > 5 mg/dL, an elevated international normalized ratio, and neutrophilia. Significant alcohol intake was defined as a consumption of > 2 drinks daily or > 6 drinks daily on weekends for the past 5 years. We used the definition of the American Association for the Study of Liver Disease guidelines of what constitutes a standard drink: 12 g of alcohol with range 9.3-13.2 g.

The diagnosis of liver cirrhosis was based on the histologic features of cirrhosis on liver biopsy and/or a composite of clinical signs and findings of cirrhosis provided by laboratory tests, endoscopy, and radiologic imaging. AH was defined by the acute development of one major complication of liver disease including acute kidney injury, ascites, encephalopathy, or gastrointestinal hemorrhage secondary to gastrointestinal varices or portal hypertensive gastropathy and enteropathy. Hepatic encephalopathy was assessed by a single individual using Conn score and asterixis grade. Acute kidney injury was defined as an abrupt (arbitrarily set at 48 h) reduction in kidney function manifested by an absolute increase in serum creatinine of 0.3 mg/dL or more, equivalent to a percentage increase in serum creatinine of 50% or more (1.5-fold from baseline)[6].

Among patients with acute decompensated liver cirrhosis, only those who remained abstinent from alcohol use for at least 6 mo before admission were included, whereas all patients with AH were (by definition) actively abusing alcohol before admission. The data was collected at the time of diagnosis and admission with alcoholic hepatitis - subjects were not drinking alcohol following admission. We also excluded all individuals with ongoing tobacco use. Patients with liver cancer or other malignancies were excluded, as were those with prior history of transplantation.

Data collection

Mass spectrometry identified and measured 29 metabolomics compounds related to amino acid and intermediary metabolism in plasma samples from fasted subjects. Samples and associated clinical data were collected from fasting subjects undergoing community health screens. All subjects gave written informed consent and the Institutional Review Board of the Cleveland Clinic approved all study protocols.

Quantification of plasma analytes by liquid chromatography/mass spectrometry/mass spectrometry

Stable isotope dilution liquid chromatography/mass spectrometry (MS)/MS was used to quantify plasma analytes. Four volumes of methanol containing isotope-labeled internal standards was added to one volume of plasma for protein precipitation. After centrifugation, supernatant was analyzed by injection onto a silica column that was interfaced with an atmospheric pressure ionization 4000 Q-TRAP mass spectrometer (AB SCIEX, Framingham, MA)[7]. A discontinuous gradient was generated to resolve analytes by mixing 0.1% propanoic acid in water with 0.1% acetic acid in methanol[7]. Analytes and the isotope-labeled internal standards were monitored in positive multiple reaction monitoring MS mode using characteristic precursor-product ion transitions (Table 1). Parameters for ion monitoring were optimized for each analyte. Various concentrations of analytes were spiked into a control plasma sample to prepare calibration curves for quantification of analytes.

Statistical analysis

Data are presented as mean ± SD, median (25^{th}, 75^{th} percentiles) or n (%). Univariable analysis was performed to compare clinical characteristics and biomarker levels between the two groups. Analysis of variance or the non-parametric Kruskal-Wallis test were used to assess differences in continuous variables and Pearson's χ^2 tests or Fisher's exact tests were used for categorical factors. Analysis of covariance was used to assess differences in biomarker levels while adjusting for MELD; the logarithm of each compound was modeled as the outcome variable with group and MELD as the independent variables. Receiver operating characteristics (ROC) analysis was performed to assess the utility of biomarkers in distinguishing acute alcoholic hepatitis from alcoholic cirrhosis; the area under the ROC curves [area under receiver operating characteristics curve (AUC)] and corresponding 95%CI are presented.

We used various statistical analyses to compare clinical characteristics and plasma levels of compounds among groups and to test the correlation between levels of compounds and severity of liver disease. Correlations between 0.0-0.3 are considered low, between 0.3-0.5 are considered moderate, and between 0.5-0.7 are considered high, and between 0.7-1.0 are considered very high. Spearman's correlation coefficients were also used to assess correlations between biomarkers and severity of liver disease for each group separately. Finally, logistic regression analysis was performed to build a predictive model for AH.

Lastly, a survival analysis was done to evaluate transplant-free survival. Kaplan-Meier product-limit estimates were used to assess transplant-free survival. Follow-up time was defined as time from sample collection to death and subjects were censored at time of

Table 1 Characteristic precursor-product transitions

	Name	Precursor	Product
Analytes	Trimethylamine N-oxide	76	58
	Choline	104	60
	Betaine	118	59
	Valine	118	72
	Leucine	86	43
	Isoleucine	86	56
	Ornithine	133	70
	Crotonobetaine	144	59
	Butyrobetaine	146	60
	Lysine	147	84
	Methyl-lysine	161	84
	Carnitine	162	60
	Phenylalanine	166	120
	Arginine	175	70
	Citrulline	176	70
	Tyrosine	182	136
	Methyl-arginine	189	70
	Symmetric dimethyl-arginine	203	70
	Asymmetric dimethylarginine	203	70
	Acetyl-carnitine	204	85
	Propionyl-carnitine	218	85
	Butyryl-carnitine	232	85
	Pentanoyl-carntine	246	85
	Hexanoyl-carnitine	260	85
	Octenoyl-carnitine	286	85
Internal standard	Trimethylamine N-oxide-d_9	85	66
	Choline-trimethyl-d_9	113	69
	Betaine-trimethyl-d_9	127	68
	Valine-$^{13}C_5$, $^{15}N_1$	124	77
	Leucine-$^{13}C_6$, $^{15}N_1$	139	92
	Ornithine 3, 3, 4, 4, 5, 5-d_6	139	76
	Crotonobetaine-trimethyl-d_9	153	68
	Butyrobetaine-trimethyl-d_9	155	69
	Lysine-u-$^{13}C_6$, $^{15}N_2$	155	90
	Phenylalanine-$^{13}C_6$	172	126
	Citrulline 2, 3, 4, 5-d_4	180	74
	Arginine-$^{13}C_6$	181	74
	Tyrosine-u-$^{13}C_9$, $^{15}N_1$	192	145
	Asymmetric dimethylarginine 2, 3, 3, 4, 4, 5, 5-d_7	210	77
	Acetyl-carnitine-d_3	207	85
	Propionyl-carnitine-d_3	221	85
	butyryll-carnitine-d_3	235	85
	Pentanoyl-carnitine-d_9	246	85
	Hexanoyl-carnitine-d_3	263	85

orthotopic liver transplantation (OLT), if applicable, or last follow-up visit. Cox regression was used to assess associations between biomarker levels and transplant-free survival. In addition, inverse probability of censoring weighting estimation of cumulative/dynamic time-dependent ROC curve was used to assess the role of novel biomarkers in prediction of 3 and 6-mo LT-free survival[8,9]. Each marker was compared to the MELD score and markers with AUC of at least 0.70 were further assessed to see if any of these improved prediction of survival in combination with MELD. A $P < 0.05$ was considered statistically significant. A 95%CI encompassing 0.5 was considered to indicate no significant predictive value. SAS (version 9.2, the SAS Institute, Cary, NC) and R (version 3.0.3, the R Foundation for Statistical Computing) were used to perform all analyses. The statistical methods of this study were reviewed by Rocio Lopez from the Cleveland Clinic Foundation.

RESULTS

Baseline characteristics

Table 2 presents a summary of patient demographic and clinical characteristics. A total of 45 subjects were included in the analysis. The average age was 53 ± 10 years, 54% were male, and 75% were Caucasian. The mean MELD score was 18.0 ± 9.3. MELD score was comparable between subjects with AH and those with AD.

Metabolomics biomarkers of alcoholic hepatitis

Table 3 presents a summary of MELD-adjusted biomarker levels in the two study groups. Betaine, creatinine, homocitrulline and citrulline, tyrosine, phenylalanine, octenoyl carnitine, and symmetric dimethylarginine (SDMA) were significantly higher in patients with AH compared to those with AD.

Table 4 presents AUC data using ROC analysis, where values greater than 0.7 are strongly predictive for differentiation between AH and AD. Citrulline, betaine, and tyrosine were all notable for their values in differentiating AH from AD. Using a combination of citrulline and betaine provided the greatest AUC, at 0.835 with a 95%CI between 0.747 and 0.978. Other significant biomarkers include homocitrulline, SDMA, octenoyl-carnitine, creatinine, and phenylalanine. The remaining biomarkers were insignificant.

Table 5 presents the correlations between biomarkers and liver disease severity for alcoholic hepatitis. There was moderate to strong correlation between several biomarkers and both MELD and Maddrey's scores. Correlations between 0.0-0.3 are considered trivial/low, 0.3-0.5 are considered moderate, 0.5-0.7 are considered high and 0.7-1.0 are considered very high/strong.

The objective of this study was to detect patterns in biomarkers or hypothesis generation. In addition, adjustments for multiple comparisons are typically somewhat conservative and it would be possible to miss many potential associations that should be further explored. Holm-Bonferroni adjustment is quite conservative when the number of tests is large or the tests are not independent[10]. Despite this, we performed the Holm-Bonferroni adjustment to provide a more complete set of data (Table 6). In this case only citrulline, phenylalanine, and homocitrulline remain significantly different between the groups.

Metabolomics biomarkers of severity of liver disease

Patients were followed over 12.5 (P25, P75: 4.3, 14.1) mo during which three subjects received a liver transplant and a total of 24% of subjects expired. As seen in Figure 1, tyrosine was strongly associated with transplant-free survival outcome in patients with liver cirrhosis [AUC for 3-mo OLT-free survival AUC = 0.91 (0.74-1.0)]. Combined MELD scores and tyrosine levels provided the best accuracy for 3-mo transplant-

Table 2 Patient characteristics

Factor	Cirrhosis with acute decompensation from etiologies other than alcohol (n = 23)		Alcoholic hepatitis (n = 25)		P-value
	n	Summary	n	Summary	
Age (yr)	14	53.8 ± 9.8	20	51.5 ± 10.4	0.51[1]
Male	21	13 (61.9)	23	10 (43.5)	0.22[3]
Caucasian	10	10 (100.0)	19	16 (84.2)	0.53[4]
AST	23	40.0 (33.0, 75.0)	25	138.0 (88.0, 161.0)	< 0.001[2,b]
ALT	23	21.0 (15.0, 30.0)	25	51.0 (42.0, 71.0)	< 0.001[2,b]
Bilirubin	23	3.8 (1.4, 6.0)	25	9.4 (6.8, 21.7)	0.005[2,b]
Albumin	23	2.8 ± 0.66	25	2.8 ± 0.68	0.86[1]
INR	23	1.5 ± 0.41	25	1.7 ± 0.59	0.16[1]
PT	23	16.4 ± 4.5	25	19.1 ± 6.1	0.095[1]
Creatinine	23	0.94 (0.74, 1.5)	25	0.72 (0.57, 1.04)	0.062[2]
MELD score	23	16.4 ± 8.8	25	20.5 ± 10.0	0.14[1]
Maddrey's score	16	22.2 (16.0, 37.0)	17	43.5 (34.0, 60.6)	0.028[2,a]
Ascites	23		25		0.14[2]
None		9 (39.1)		14 (56.0)	
Small		4 (17.4)		6 (24.0)	
Large		9 (39.1)		4 (16.0)	
Severe		1 (4.3)		1 (4.0)	
HE	23		25		0.94[2]
None		2 (8.7)		6 (24.0)	
Mild		12 (52.2)		7 (28.0)	
Severe		9 (39.1)		12 (48.0)	
Steroids	23	2 (8.7)	25	13 (52.0)	0.001[3,b]
Trental	23	3 (13.0)	25	9 (36.0)	0.067[3]
OLT	23	2 (8.7)	25	1 (4.0)	0.60[4]
Deceased	23	6 (26.1)	25	8 (32.0)	0.65[3]

P-values were calculated using the test corresponding to superscript characters: [1]ANOVA; [2]Kruskal-Wallis test; [3]Pearson's χ^2 test; [4]Fisher's exact test. [a]P < 0.05 and [b]P < 0.01. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; MELD: Model for end-stage liver disease; OLT: Orthotopic liver transplantation; PT: Prothrombin time; INR: International normalized ratio; HE: Hepatic encephalopathy.

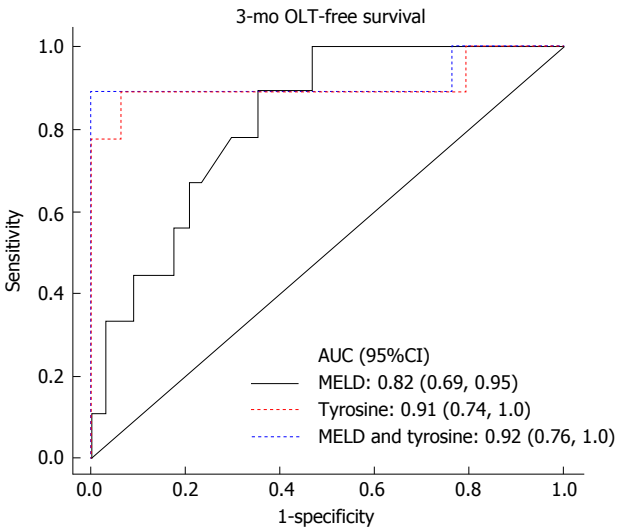

Figure 1 Tyrosine predicts 3-mo liver transplant-free survival in patients with end-stage liver disease. Results are presented as AUC (P25, P75). AUC: Area under receiver operating characteristics curve; OLT: Orthotopic liver transplantation; MELD: Model for end-stage liver disease.

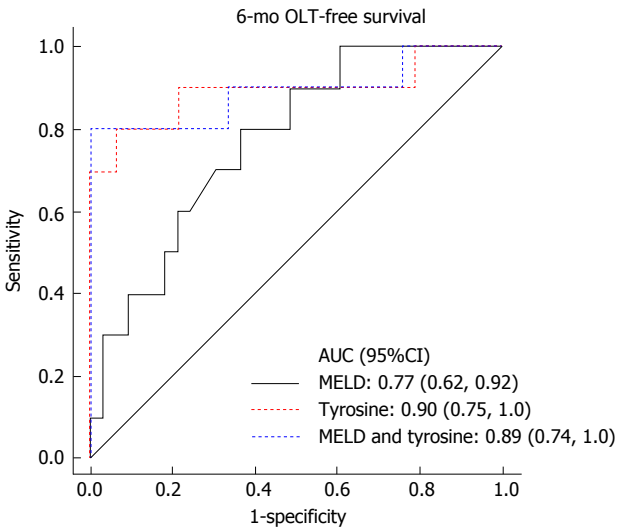

Figure 2 Tyrosine predicts 6-mo liver transplant-free survival in patients with end-stage liver disease. AUC: Area under receiver operating characteristics curve; OLT: Orthotopic liver transplantation; MELD: Model for end-stage liver disease.

free survival AUC = 0.92 (0.76-1.0). Evidently these biomarkers can be used to predict OLT-free survival with reasonable sensitivity and specificity.

Figure 2 shows the same analysis with similar results, except for 6-mo OLT-free survival. MELD provided an AUC of 0.77, tyrosine provided an AUC of 0.90, and MELD and tyrosine together provided an AUC of 0.89. Tyrosine alone as well as tyrosine in combination with MELD provided better AUC values than MELD alone, suggesting its utility in predicting OLT-free survival.

A multivariable Cox regression analysis was used to adjust for MELD, the most important predictor of mortality, and tyrosine remained significantly associated with mortality [HR = 1.02 (1.01, 1.04) for a one unit

Table 3 Model for end-stage liver disease-adjusted average biomarker levels

Biomarker (μmol/L)	Cirrhosis with acute decompensation from etiologies other than alcohol (n = 23)	Alcoholic hepatitis (n = 25)	P-value
Choline	5.8 (4.7, 7.2)	7.0 (5.7, 8.6)	0.22
TMAO	0.74 (0.34, 1.6)	0.87 (0.42, 1.8)	0.76
Carnitine	37.2 (32.1, 43.1)	32.6 (28.3, 37.5)	0.2
Betaine	83.6 (64.7, 108.2)	134.0 (104.7, 171.5)	0.012[a]
Butyrobetaine	1.6 (1.3, 1.8)	1.7 (1.4, 2.0)	0.46
Crotonobetaine	0.12 (0.10, 0.15)	0.14 (0.12, 0.18)	0.35
Creatinine	92.0 (75.0, 112.9)	59.3 (48.8, 72.1)	0.003[b]
Ornithine	71.6 (59.1, 86.9)	62.0 (51.5, 74.6)	0.29
Lysine	131.3 (112.7, 153.1)	134.8 (116.4, 156.2)	0.81
Methyl-lysine	4.0 (2.9, 5.6)	3.4 (2.5, 4.7)	0.46
Argine	61.7 (52.0, 73.2)	61.8 (52.4, 72.8)	0.99
Citrulline	40.2 (33.2, 48.6)	23.7 (19.7, 28.5)	< 0.001[b]
MMA	0.24 (0.20, 0.27)	0.21 (0.19, 0.24)	0.32
Homocitrulline	0.73 (0.55, 0.97)	0.37 (0.28, 0.48)	0.001[b]
Leucine	52.8 (44.0, 63.4)	48.8 (40.9, 58.1)	0.54
Iso-leucine	27.1 (21.7, 33.8)	28.0 (22.6, 34.6)	0.83
Valine	115.4 (100.4, 132.7)	101.8 (89.1, 116.4)	0.2
Phenylalanine	90.4 (78.0, 104.7)	60.2 (52.3, 69.3)	< 0.001[b]
Tyrosine	166.0 (126.8, 217.3)	107.4 (83.0, 139.1)	0.025[a]
Acetyl-carnitine	17.8 (14.9, 21.1)	16.9 (14.3, 20.0)	0.69
Propionyl-carnitine	1.02 (0.76, 1.4)	1.2 (0.88, 1.5)	0.5
Butyryl-carnitine	2.0 (1.6, 2.5)	2.2 (1.8, 2.7)	0.58
Trimethyl-Lysine	1.00 (0.81, 1.2)	1.1 (0.92, 1.4)	0.42
SDMA	0.81 (0.65, 1.02)	0.58 (0.47, 0.72)	0.042[a]
Dimethyl-Lysine	0.92 (0.72, 1.2)	0.74 (0.59, 0.94)	0.22
ADMA	0.90 (0.78, 1.03)	0.82 (0.72, 0.93)	0.32
Pentanoyl-carnitine	0.25 (0.20, 0.31)	0.27 (0.22, 0.34)	0.48
Hexanoyl-carnitine	0.69 (0.56, 0.85)	0.61 (0.50, 0.74)	0.38
Octenoyl-carnitine	0.05 (0.02, 0.12)	0.01 (0.00, 0.02)	0.009[b]

Values presented as mean (95%CI) and P-values obtained from analysis of covariance. The natural logarithm of each biomarker was modeled as the outcome variable with disease group and MELD as the independent variables. [a]P < 0.05, [b]P < 0.01. ADMA: Asymmetric dimethylarginine; SDMA: Symmetric dimethylarginine; MMA: Monomethylarginine; TMAO: Trimethylamine N-oxide.

increase in tyrosine; P = 0.002]. In Figures 1 and 2 it can also be seen that tyrosine performs better than MELD for prediction of 3- and 6-mo mortality, and the combination of MELD and tyrosine (in a multivariable analysis) performs more or less the same as the compound by itself.

Time-dependent ROC analysis (Table 7) shows that phenylalanine [AUC = 0.77 (0.56, 0.97)], carnitine [AUC = 0.73 (0.53, 0.93)], asymmetric dimethylarginine (ADMA) [AUC = 0.72 (0.49, 0.96)] and monomethylarginine (MMA) [AUC = 0.71 (0.47, 0.94)] all provide excellent predictive value for transplant-free survival in patients with liver cirrhosis, but there was no evidence to suggest that they were significantly better than MELD.

DISCUSSION

The purpose of this study was two-fold. First, the utility of metabolomics as an un-invasive diagnostic tool for AH was assessed. The diagnosis of AH is usually a clinical one, based on severe liver dysfunction in the context of excessive alcohol consumption, excluding other causes of acute and chronic liver disease (CLD)[1]. However, this method of diagnosis is not steadfast, as some studies that have included a liver biopsy in all patients with clinically suspected AH have shown histologic confirmation in only 70%-80% of patients[1]. Thus, liver biopsy remains the gold standard in diagnosing AH patients; however it is invasive, expensive, and burdensome for the patient. The utilization of metabolic biomarkers as an alternative, objective, un-invasive diagnostic tool is promising.

Our results demonstrated that AH patients have a specific metabolome that can be employed for diagnostic purposes. AH patients had higher levels of betaine, and lower levels of creatinine, citrulline, homocitrulline, tyrosine, phenylalanine, octenoyl-carnitine, and SDMA. Most importantly, betaine and citrulline provided excellent prediction accuracy in distinguishing AH from AD. Figure 3 shows the sensitivity and specificity of citrulline and betaine for diagnosis and acute decompensation from non-alcohol-related etiologies. Alcohol consumption in patients with alcoholic liver disease results in bacterial overgrowth and increases gut permeability and translocation of bacteria-derived lipopolysaccharides from the gut to the liver[1]. This could explain the altered levels of amino acids in these patients.

Betaine is a molecule involved in transmethylation reactions in biological systems. S-adenosylmethionine (SAM), a critical methylating agent, is crucial to maintaining the integrity of the liver. One important function of SAM is its conversion of phosphatidylethanolamine to phosphatidylcholine, the latter of which constitutes lipoproteins involved in transporting fat away from the

Table 4 Utility of biomarkers in differentiating cirrhosis with alcoholic hepatitis from cirrhosis with acute decompensation from etiologies other than alcohol: Receiver operating characteristics analysis

Biomarker	AUC (95%CI)
Citrulline and betaine	0.835 (0.747, 0.978)
Betaine and phenylalanine	0.810 (0.684, 0.937)
Citrulline and phenylalanine	0.758 (0.609, 0.907)
Citrulline	0.758 (0.610, 0.907)
Betaine	0.732 (0.588, 0.877)
Phenylalanine	0.715 (0.567, 0.863)
Crotonobetaine	0.663 (0.498, 0.827)
Tyrosine	0.650 (0.484, 0.817)
Butyrobetaine	0.649 (0.489, 0.808)
Creatinine	0.645 (0.486, 0.805)
Octenoyl-carnitine	0.631 (0.488, 0.774)
Propionyl-carnitine	0.620 (0.453, 0.787)
Homocitrulline	0.618 (0.457, 0.779)
Trimethyl-lysine	0.616 (0.447, 0.784)
Butyryl-carnitine	0.615 (0.451, 0.778)
Pentanoyl-carnitine	0.613 (0.455, 0.771)
Choline	0.597 (0.432, 0.762)
Valine	0.588 (0.424, 0.752)
Lysine	0.559 (0.393, 0.725)
Methyl-lysine	0.552 (0.383, 0.721)
Iso-leucine	0.548 (0.379, 0.716)
Acetyl-carnitine	0.543 (0.375, 0.710)
Hexanoyl-carnitine	0.529 (0.362, 0.696)
TMAO	0.521 (0.347, 0.695)
Argine	0.507 (0.339, 0.675)
ADMA	0.481 (0.314, 0.647)
Leucine	0.477 (0.309, 0.644)
Carnitine	0.470 (0.301, 0.638)
MMA	0.453 (0.286, 0.620)
SDMA	0.447 (0.277, 0.617)
Dimethyl-lysine	0.417 (0.243, 0.592)
Ornithine	0.351 (0.186, 0.517)

AUC: Area under receiver operating characteristics curve; ADMA: Asymmetric dimethylarginine; SDMA: Symmetric dimethylarginine; MMA: Monomethylarginine; TMAO: Trimethylamine N-oxide.

liver, thereby preventing hepatic fat infiltration and subsequent liver injury[11]. Betaine plays a significant role in this pathway as a methylating agent in the liver. Betaine transfers a methyl group to homocysteine *via* betaine-homocysteine methyl transferase (BHMT) in order to form methionine, which then goes on to form SAM and methylate biological molecules to protect the liver. Thus, betaine is protective against harmful fatty deposits in the liver due to alcohol abuse. While acute alcohol ingestion induces BMHT activity so that SAM levels can remain physiologically normal, chronic alcohol abuse leads to diminished SAM levels due to exhaustion of this system[11]. Consequently, this lead to increased betaine levels in the serum of AH patients, as the hepatocytes cannot compensate and regenerate SAM *via* the BHMT pathway. Furthermore, other studies have shown that dietary supplementation with betaine generated increased SAM in the liver and protected against ethanol-induced steatosis in rats[12]. However, with chronic alcohol abuse and dysfunction of the BHMT pathway, betaine cannot be metabolized.

Citrulline, in particular, is a biomarker of intestinal

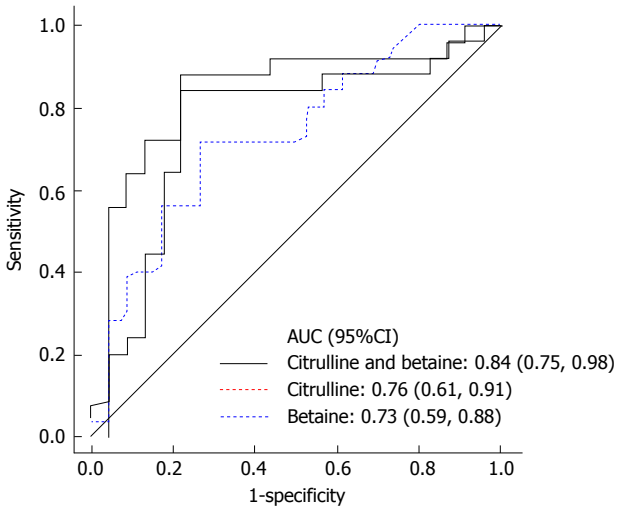

Figure 3 Citrulline and betaine serve as diagnostic biomarkers in patients with alcoholic hepatitis. AUC: Area under receiver operating characteristics curve.

functionality[13]. Consequently, changes in intestinal flora due to liver disease can lead to imbalances in citrulline. Furthermore, it has been demonstrated that portal hypertension secondary to cirrhosis stimulates nitric oxide synthase (NOS)[14]. NOS converts arginine and oxygen into citrulline and nitric oxide (NO), resulting in vasodilation and increased blood flow. Pârvu *et al*[13] found that CLD patients had an increased serum citrulline concentration, indicating increased NO production to counter the mechanisms of portal hypertension[1]. Therefore, it makes sense that acute exacerbations of liver function seen in AH patients would deplete citrulline stores in an effort to produce NO and increase blood flow to an acute on chronic liver injury.

The second goal of this study was to assess the utility of metabolomics as a marker for the prognosis of liver disease. One particularly noteworthy result is the association between tyrosine and transplant-free survival outcome in patients with liver cirrhosis. The MELD score and tyrosine level, considered together, provided the greatest sensitivity and specificity for predicting 3-mo transplant-free survival. Tyrosine and phenylalanine are aromatic amino acids whose metabolism can become impaired as a consequence of liver injury, as the enzymes that metabolize these compounds are produced by the liver. Concentrations of aromatic amino acids are increased in patients with chronic liver disease who experience an acute inflammatory event such as acute alcoholic hepatitis, gastrointestinal bleeding, infection, or encephalopathy[15]. Liver cirrhosis with a superimposed liver injury will lead to systemic inflammation resulting in elevated tyrosine levels. Systemic inflammation in the context of acute on chronic liver failure exacerbates the patient's health through the release of various pro-inflammatory cytokines[15]. Therefore, plasma tyrosine levels can be used to estimate the degree and severity of this inflammation, and provide novel information on prognosis and outcome.

Table 5 Correlations between biomarkers and model for end-stage liver disease and Maddrey's score in patients with alcoholic hepatitis

Biomarker	Alcoholic hepatitis			
	MELD		Maddrey's score	
	rho (95%CI)	*P*-value	rho (95%CI)	*P*-value
Choline	0.28 (-0.13, 0.69)	0.18	0.02 (-0.53, 0.57)	0.95
TMAO	-0.25 (-0.67, 0.17)	0.23	0.29 (-0.24, 0.82)	0.26
Carnitine	0.54 (0.18, 0.91)	0.005[b]	0.48 (-0.01, 0.96)	0.054
Betaine	0.24 (-0.18, 0.65)	0.26	-0.00 (-0.55, 0.55)	0.99
Butyrobetaine	0.30 (-0.11, 0.71)	0.15	0.29 (-0.24, 0.81)	0.26
Crotonobetaine	0.07 (-0.36, 0.50)	0.74	-0.03 (-0.58, 0.52)	0.91
Creatinine	0.44 (0.05, 0.83)	0.027[a]	0.48 (-0.00, 0.96)	0.052
Ornithine	0.28 (-0.13, 0.70)	0.17	0.26 (-0.27, 0.79)	0.32
Lysine	0.37 (-0.03, 0.77)	0.068	0.27 (-0.25, 0.80)	0.29
Methyl-lysine	0.09 (-0.34, 0.52)	0.67	-0.01 (-0.56, 0.54)	0.96
Argine	0.00 (-0.43, 0.44)	0.98	0.42 (-0.08, 0.92)	0.095
Citrulline	0.09 (-0.34, 0.52)	0.66	0.05 (-0.50, 0.60)	0.86
MMA	0.34 (-0.07, 0.74)	0.098	0.39 (-0.11, 0.90)	0.12
Homocitrulline	0.66 (0.34, 0.99)	< 0.001[b]	0.59 (0.14, 1.00)	0.014[a]
Leucine	0.03 (-0.40, 0.46)	0.89	0.50 (0.02, 0.97)	0.043
Iso-leucine	0.11 (-0.32, 0.54)	0.59	0.29 (-0.24, 0.82)	0.26
Valine	0.20 (-0.22, 0.62)	0.34	0.54 (0.08, 1.00)	0.025[a]
Phenylalanine	0.34 (-0.06, 0.75)	0.092	0.56 (0.11, 1.00)	0.018[a]
Tyrosine	0.30 (-0.11, 0.71)	0.14	0.44 (-0.05, 0.94)	0.074
Acetyl-carnitine	0.49 (0.11, 0.86)	0.014[a]	0.50 (0.03, 0.98)	0.04[a]
Propionyl-carnitine	0.40 (0.01, 0.80)	0.046[a]	0.28 (-0.25, 0.81)	0.28
Butyryl-carnitine	0.48 (0.10, 0.86)	0.016[a]	0.25 (-0.29, 0.78)	0.34
Trimethyl-lysine	0.48 (0.11, 0.86)	0.014[a]	0.38 (-0.13, 0.89)	0.14
SDMA	0.38 (-0.02, 0.78)	0.064	0.55 (0.09, 1.00)	0.023[a]
Dimethyl-lysine	0.31 (-0.10, 0.72)	0.13	0.18 (-0.37, 0.72)	0.5
ADMA	0.42 (0.03, 0.81)	0.037[a]	0.60 (0.16, 1.00)	0.011[a]
Pentanoyl-carnitine	0.53 (0.16, 0.90)	0.007[b]	0.31 (-0.22, 0.83)	0.23
Hexanoyl-carnitine	0.49 (0.12, 0.87)	0.013[a]	0.40 (-0.11, 0.90)	0.11
Octenoyl-carnitine	0.45 (0.07, 0.84)	0.024[a]	0.23 (-0.30, 0.77)	0.37

Values presented as mean (95%CI) and *P*-values obtained from analysis of covariance. The natural logarithm of each biomarker was modeled as the outcome variable with disease group and MELD as the independent variables. A superscript of a indicates [a]$P < 0.05$, [b]$P < 0.01$. rho: Spearman's correlation; MELD: Model for end-stage liver disease; ADMA: Asymmetric dimethylarginine; SDMA: Symmetric dimethylarginine; MMA: Monomethylarginine; TMAO: Trimethylamine N-oxide.

Other analytes such as phenylalanine, carnitine, ADMA, and MMA were all found to be accurate predictors of transplant-free survival in patients with liver cirrhosis. It should be noted that there was no evidence to suggest that tyrosine, phenylalanine, carnitine, ADMA, and MMA were significantly better than MELD. However, despite the lack of statistical significance, in terms of the AUC even a small jump of 0.01-0.02 is promising as it denotes clinical significance. This study was an exploratory analysis designed to assess usefulness of metabolites and given the small sample size of 45 patients, no strong conclusions can be reached. However, the promising results of this study indicate the need for future studies with larger sample sizes to evaluate and corroborate the usefulness of these analytes in predicting transplant-free survival. Future studies can also explore more complex combinations of metabolites in predicting OLT-free survival. More data can help determine if plasma analytes are superior or inferior to the MELD score, or if they should be used in combination with the MELD score as a prognostic tool.

Limitations of this study include the small number of patients in the sample size. Furthermore, no power calculations were done to determine optimum sample size. Given no power calculations and a small sample size, we are only capable of generating sufficient power for large differences and the false negative rate may be high. Further research must validate the findings from this study utilizing larger patient populations. Another limitation was the lack of control group in this study. A control group is an essential part of any experiment that seeks to find a significant difference among populations. While this project had no control group, other research has corroborated the results from this study with control groups[7]. Lastly, this study was limited in that liver biopsy was not performed in all patients to confirm the diagnosis of AH; it was only performed in a subset of patients. One final limitation of this study is the lack of biopsy confirmation of AH as a diagnosis. Since liver biopsy is considered the gold standard in diagnosing AH, it cannot be said with absolute certainty that all patients were diagnosed with AH. Further research in this area might involve standardized biopsy evaluation alongside metabolomic correlations to liver disease.

Table 6 Model for end-stage liver disease-adjusted average biomarker levels

Biomarker (μmol/L)	Alcoholic cirrhosis (n = 23)	Alcoholic hepatitis (n = 25)	Holm-bonferroni corrected P-value
Citrulline	40.2 (33.2, 48.6)	23.7 (19.7, 28.5)	0.009[b]
Phenylalanine	90.4 (78.0, 104.7)	60.2 (52.3, 69.3)	0.009[b]
Homocitrulline	0.73 (0.55, 0.97)	0.37 (0.28, 0.48)	0.029[a]
Creatinine	92.0 (75.0, 112.9)	59.3 (48.8, 72.1)	0.087
Octenoyl-carnitine	0.05 (0.02, 0.12)	0.01 (0.00, 0.02)	0.26
Betaine	83.6 (64.7, 108.2)	134.0 (104.7, 171.5)	0.35
Tyrosine	166.0 (126.8, 217.3)	107.4 (83.0, 139.1)	0.73
SDMA	0.81 (0.65, 1.02)	0.58 (0.47, 0.72)	0.99
Carnitine	37.2 (32.1, 43.1)	32.6 (28.3, 37.5)	0.99
Valine	115.4 (100.4, 132.7)	101.8 (89.1, 116.4)	0.99
Choline	5.8 (4.7, 7.2)	7.0 (5.7, 8.6)	0.99
Dimethyl-lysine	0.92 (0.72, 1.2)	0.74 (0.59, 0.94)	0.99
Ornithine	71.6 (59.1, 86.9)	62.0 (51.5, 74.6)	0.99
MMA	0.24 (0.20, 0.27)	0.21 (0.19, 0.24)	0.99
ADMA	0.90 (0.78, 1.03)	0.82 (0.72, 0.93)	0.99
Crotonobetaine	0.12 (0.10, 0.15)	0.14 (0.12, 0.18)	0.99
Hexanoyl-carnitine	0.69 (0.56, 0.85)	0.61 (0.50, 0.74)	0.99
Trimethyl-lysine	1.00 (0.81, 1.2)	1.1 (0.92, 1.4)	0.99
Butyrobetaine	1.6 (1.3, 1.8)	1.7 (1.4, 2.0)	0.99
Methyl-lysine	4.0 (2.9, 5.6)	3.4 (2.5, 4.7)	0.99
Pentanoyl-carnitine	0.25 (0.20, 0.31)	0.27 (0.22, 0.34)	0.99
Propionyl-carnitine	1.02 (0.76, 1.4)	1.2 (0.88, 1.5)	0.99
Leucine	52.8 (44.0, 63.4)	48.8 (40.9, 58.1)	0.99
Butyryl-carnitine	2.0 (1.6, 2.5)	2.2 (1.8, 2.7)	0.99
Acetyl-carnitine	17.8 (14.9, 21.1)	16.9 (14.3, 20.0)	0.99
TMAO	0.74 (0.34, 1.6)	0.87 (0.42, 1.8)	0.99
Lysine	131.3 (112.7, 153.1)	134.8 (116.4, 156.2)	0.99
Iso-leucine	27.1 (21.7, 33.8)	28.0 (22.6, 34.6)	0.99
Argine	61.7 (52.0, 73.2)	61.8 (52.4, 72.8)	0.99

Values presented as mean (95%CI) and P-values obtained from analysis of covariance. The natural logarithm of each biomarker was modeled as the outcome variable with disease group and MELD as the independent variables. A superscript of a indicates [a]P < 0.05, [b]P < 0.01. ADMA: Asymmetric dimethylarginine; SDMA: Symmetric dimethylarginine; MMA: Monomethylarginine; TMAO: Trimethylamine N-oxide.

Table 7 Utility of biomarkers in predicting transplant-free survival

Biomarker (μmol/L)	3-mo survival AUC (95%CI)	6-mo survival AUC (95%CI)
MELD	0.82 (0.69, 0.95)	0.77 (0.62, 0.92)
Tyrosine	0.91 (0.74, 1.0)	0.89 (0.74, 1.0)
Phenylalanine	0.77 (0.56, 0.97)	0.79 (0.61, 0.98)
Carnitine	0.73 (0.53, 0.93)	0.74 (0.56, 0.93)
ADMA	0.72 (0.49, 0.96)	0.70 (0.48, 0.92)
MMA	0.71 (0.47, 0.94)	0.70 (0.49, 0.91)

AUC: Area under receiver operating characteristics curve; MELD: Model for end-stage liver disease; ADMA: Asymmetric dimethylarginine; MMA: Monomethylarginine.

This is the first study that profiles plasma metabolites in patients with AH and CLD. Investigation of the human metabolome in disease states can be very useful in generating diagnostic markers and understanding the pathophysiology of those disease states. However, this study is limited in its relatively small sample size. Future larger studies are needed to confirm the diagnostic value of biomarkers in AH and CLD.

Metabolomics plasma analyte levels could help diagnose AH and determine the prognosis of patients with liver cirrhosis awaiting liver transplantation. Specifically, combined citrulline and betaine plasma levels yield a highly sensitive and specific discriminatory test of AH *vs* AD. Tyrosine, in combination with MELD score, provides even greater sensitivity and specificity for predicting 3 mo OLT-free survival than the MELD score on its own.

In conclusion, metabolomics plasma analyte levels could aid in diagnosing AH or in determining potential patient prognosis.

COMMENTS

Background

Liver biopsy remains the gold standard for the diagnosis of alcoholic hepatitis (AH). Herein, the authors use a novel metabolomics approach to identify plasma analytes that may correlate with the diagnosis of AH and the severity of liver disease in patients with AH.

Research frontiers

Metabolomics represents the analysis of metabolites present in biological samples. By identifying and quantifying metabolites, one can gather a picture of the genetic variations and environmental influences (such as diet, lifestyle, drug use, and toxicological exposure) in a biological specimen. The authors use metabolomics to assess prognostic and diagnostic factors in patients with liver disease with the hopes of developing more accurate measures of patient

outcomes.

Innovations and breakthroughs

In this study, several metabolites were found to be associated with survival in patients with liver disease.

Applications

These findings could potentially be used to develop more robust measures to provide a diagnosis and prognosis in patients with liver disease. The model for end-stage liver disease score and liver biopsy, which are currently used, are imperfect; a less invasive and more accurate measure is needed.

Peer-review

This is a very important paper and presents impact on health system. It is very well elaborated.

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