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EDITORIAL

Pathophysiological mechanisms involved in non-alcoholic steatohepatitis and novel potential therapeutic targets

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is a major health care problem and represents the hepatic expression of the metabolic syndrome. NAFLD is classified as non-alcoholic fatty liver (NAFL) or simple steatosis, and non-alcoholic steatohepatitis (NASH). NASH is characterized by the presence of steatosis and inflammation with or without fibrosis. The physiopathology of NAFL and NASH and their progression to cirrhosis involve several parallel and interrelated mechanisms, such as, insulin resistance (IR), lipotoxicity, inflammation, oxidative stress, and recently the gut-liver axis interaction has been described. Incretin-based therapies could play a role in the treatment of NAFLD. Glucagon-like peptide-1 (GLP-1) is an intestinal mucosa-derived hormone which is secreted into the bloodstream in response to nutrient ingestion; it favors glucose-stimulated insulin secretion, inhibition of postprandial glucagon secretion and delayed gastric emptying. It also promotes weight loss and is involved in lipid metabolism. Once secreted, GLP-1 is quickly degraded by dipeptidyl peptidase-4 (DPP-4). Therefore, DPP-4 inhibitors are able to extend the activity of GLP-1. Currently, GLP-1 agonists and DPP-4 inhibitors represent attractive options for the treatment of NAFLD and NASH. The modulation of lipid and glucose metabolism through nuclear receptors, such as the farsenoid X receptor, also constitutes an attractive therapeutic target. Obeticholic acid is a potent activator of the farnesoid X nuclear receptor and reduces liver fat content and fibrosis in animal models. Ursodeoxycholic acid (UDCA) is a hydrophilic bile acid with immunomodulatory, anti-inflammatory, antiapoptotic, antioxidant and anti-fibrotic properties. UDCA can improve IR and modulate lipid metabolism through its interaction with nuclear receptors such as, TGR5, farnesoid X receptor-α, or the small heterodimeric partner. Finally, pharmacologic modulation of the gut microbiota could have a role in the therapy of NAFLD and NASH. Probiotics prevent bacterial translocation and epithelial invasion, inhibit mucosal adherence by bacteria, and stimulate host immunity. In animal models, probiotics prevent obesity, decrease transaminase levels, and improve IR and liver histology in NASH.

Key words: Insulin resistance; Lipotoxicity; Non-alcoholic steatohepatitis; Physiopathology; Therapeutic targets

Core tip: Non-alcoholic fatty liver disease (NAFLD) is an important health care problem. The pathophysiology of NAFLD and non-alcoholic steatohepatitis (NASH) and their progression are multifactorial and complex processes, where multi-parallel simultaneous hits derived from the gut and adipose tissue promote a pro-inflammatory response and liver injury. All of these represent attractive therapeutic targets. Pharmacological agents such as glucagon-like peptide-1 agonists, dipeptidyl peptidase-4 inhibitors, ursodeoxycholic acid, obeticholic acid and probiotics need to be explored in clinical trials specifically for treating NAFLD and NASH.

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INTRODUCTION

Currently, non-alcoholic fatty liver disease (NAFLD) is recognized as a major health care problem, and it is the most common cause of raised transaminases in western countries, where it is considered the first cause of liver disease. Worldwide its incidence and prevalence are increasing[1-4]. By 2020, non-alcoholic steatohepatitis (NASH) could be the main cause of liver transplantation[5].

NALFD is characterized by fat accumulation, mainly as triglycerides, in the hepatocytes; it is associated with clinical factors such as obesity, dyslipidemia, and diabetes; its diagnosis requires the exclusion of other conditions that could be associated with steatosis, such as, significant alcohol consumption, viral hepatitis, use of steatogenic medications or hereditary disorders[1,6].

Histologically, NAFLD is classified as non-alcoholic fatty liver (NAFL) or simple steatosis, and NASH. NASH is characterized by the presence of steatosis accompanied by inflammatory infiltrate, ballooning of hepatocytes, and the presence of Mallory-Denk bodies; any degree of fibrosis can be present, but this is not a mandatory finding. Patients with NASH, mainly those with advanced fibrosis, are at higher risk for developing decompensated cirrhosis, hepatocellular carcinoma[7-9], or even death due to cardiovascular disease as a result of early atherosclerosis[10].

NAFLD represents the hepatic expression of the metabolic syndrome, and its physiopathology involves several mechanisms, such as, glucose intolerance, insulin resistance (IR)[11,12], enhanced lipogenesis and lipotoxicity[12,13], hepatic and systemic inflammation[14], and oxidative stress[15].

The "multi-parallel hits" hypothesis is the most accepted for understanding the pathogenesis of NAFL and NASH and their progression to cirrhosis. This hypothesis proposes that many simultaneous hits derived from the gut and adipose tissue may promote inflammation and liver injury[13].

Unhealthy lifestyles are clearly related to NAFL and NASH. Excess energy intake through a diet rich in fat and carbohydrates[14-17] leads to failure of adipocytes to adapt in terms of proliferation and differentiation[18]. In the liver, free fatty acids (FFAs) are the main source for the synthesis of triglycerides. Similarly, excess dietary fat and *de novo* lipogenesis are two main factors contributing to the production of diacylglycerol and lysophosphatidyl choline, two non-triglyceride metabolites, which are responsible for lipotoxicity[19,20]. FFAs and cholesterol can also accumulate in the mitochondria leading to inflammation and liver injury mediated by tumor necrosis factor alpha and reactive oxygen species[13,21,22].

As NAFL and NASH are frequently associated with overweight, an important objective in the treatment of NAFL and NASH is to encourage weight loss; this can be achieved through lifestyle modification including a hypocaloric diet and/or aerobic exercise. The loss of at least 5% of body weight is necessary to improve steatosis, but a loss greater than 10% may be needed to improve steatohepatitis[23].

Pharmacological agents that could be useful in NAFL and NASH include glucagon-like peptide-1 (GLP-1) agonists. GLP-1 is an intestinal mucosa-derived hormone which is secreted into the bloodstream in response to nutrient ingestion; it favors glucose-stimulated insulin secretion, inhibition of postprandial glucagon secretion and delayed gastric emptying[24]. GLP-1 agonists also promote weight loss. In one study, treatment with liraglutide 1.2 mg once daily for 12 wk improved eating behavior in obese women with polycystic ovary syndrome (PCOS) and resulted in an average weight loss of 3.8 ± 0.1 kg ($P < 0.001$)[25]. In another study, short-term combined treatment with liraglutide 1.2 mg once daily and metformin 1000 mg twice daily was associated with significant weight loss and a decrease in waist circumference in obese women with PCOS who had previously been poor responders to metformin monotherapy[26]. In a cohort of obese nondiabetic women, short-term treatment with exenatide was also associated with a modest weight loss and decreased waist circumference[27].

GLP-1 is also involved in lipid metabolism; studies in animal models and in diabetic patients have found that GLP-1 agonists suppress postprandial elevations in lipids and lipoproteins[28-30]; result in a decrease in serum triglycerides, total cholesterol, low density lipoprotein-cholesterol and serum high density lipoprotein-cholesterol levels and reduce the development of atherosclerosis[31-34]. In mice, treatment with GLP-1 agonists was related to a reduction in the hepatic content of triglycerides[35]. Besides its property of enhancing

insulin sensitivity, other possible mechanisms through which GLP-1 agonists may improve the lipid profile and metabolism are: Activation of peroxisome proliferator-activated receptor-α on the hepatic cell surface, which reduces the synthesis of apolipoprotein C, degrades fat in plasma, and removes triglycerides[36-39].

Once secreted, GLP-1 is quickly degraded by dipeptidyl peptidase-4 (DPP-4). DPP-4 inhibitors prolong the activity of incretins, GLP-1 and glucose-dependent insulinotropic polypeptide[24]. As the receptor for GLP-1 has been shown to exist in various cells, including hepatocytes[40,41], DPP-4 inhibitors may have pleiotropic effects independent of lowering plasma glucose level and stimulating insulin secretion. In a retrospective study which included diabetic patients who received treatment with DPP-4 inhibitors and with abnormal transaminase levels, Kanazawa *et al*[42] found that transaminase levels decreased after six months of treatment with DPP-4 inhibitors.

Ursodeoxycholic acid (UDCA) is not approved for treating NASH; however, it is a hydrophilic bile acid with immunomodulatory, anti-inflammatory, anti-apoptotic, antioxidant and anti-fibrotic properties. It also reduces the mitochondrial membrane permeability and the release of hydrolytic enzymes from damaged hepatocytes. In a recent study which included patients with biopsy-proven NASH, high-dose UDCA for 12 mo reduced transaminase levels, the degree of fibrosis, and improved IR independently of the change in body weight. UDCA modulated lipid and glucose metabolism through its interaction with nuclear receptors such as, TGR5, farnesoid X receptor-α, or the small heterodimeric partner[43].

Obeticholic acid is a potent activator of the farnesoid X nuclear receptor that reduces liver fat content and fibrosis in animal models of NAFLD. In a multicenter, double-blind, placebo-controlled, randomized clinical trial, treatment with obeticholic acid in adult patients with NASH for 72 wk improved the histological features of NASH, but its long-term benefits and safety require further study[44].

Recently, it was demonstrated that the gut-liver axis plays a key role in the pathogenesis of obesity, NAFL, NASH and their progression. The gut microbiota is composed of bacteria, viruses, yeasts and parasites. Gut microbes are able to interact actively with the host immune system modulating inflammation, IR, intestinal permeability, and endotoxemia[45]. Pharmacologic modulation of the gut microbiota could have a role in the therapy of NAFL and NASH. Probiotics prevent bacterial translocation and epithelial invasion, inhibit mucosal adherence by bacteria, and stimulate host immunity. In animal models, probiotics also prevent obesity, improve IR and liver histology in NASH, and decrease transaminase levels[46-50]. In patients with NAFLD the use of probiotics improved transaminases, the cytokine profile and oxidative stress[51].

In summary, NAFLD is an important health care problem. The pathophysiology of NAFL and NASH and their progression involve multifactorial and complex processes, where multi-parallel simultaneous hits derived from the gut and adipose tissue promote a pro-inflammatory response and liver injury. All of these represent attractive therapeutic targets. Pharmacological agents such as GLP-1 agonists, DPP-4 inhibitors, UDCA, obeticholic acid and probiotics need to be explored in clinical trials specifically for treating NAFL and NASH.

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REVIEW

Hemodynamic monitoring during liver transplantation: A state of the art review

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Abstract

Orthotopic liver transplantation can be marked by significant hemodynamic instability requiring the use of a variety of hemodynamic monitors to aide in intraoperative management. Invasive blood pressure monitoring is essential, but the accuracy of peripheral readings in comparison to central measurements has been questioned. When discrepancies exist, central mean arterial pressure, usually measured at the femoral artery, is considered more indicative of adequate perfusion than those measured peripherally. The traditional pulmonary artery catheter is less frequently used due to its invasive nature and known limitations in measuring preload but still plays an important role in measuring cardiac output (CO) when required and in the management of portopulmonary hypertension. Pulse wave analysis is a newer technology that uses computer algorithms to calculate CO, stroke volume variation (SVV) and pulse pressure variation (PPV). Although SVV and PPV have been found to be accurate predicators of fluid responsiveness, CO measurements are not reliable during liver transplantation. Transesophageal echocardiography is finding an increasing role in the real-time monitoring of preload status, cardiac contractility and the diagnosis of a variety of pathologies. It is limited by the expertise required, limited transgastric views during key portions of the operation, the potential for esophageal varix rupture and difficulty in obtaining quantitative measures of CO in the absence of tricuspid regurgitation.

Key words: Intraoperative monitoring; Physiologic monitoring; Liver transplantation

Core tip: Accurate hemodynamic monitoring is essential to safely navigate orthotopic liver transplantation. Although specific indications for pulmonary artery catheters exist, their use has slowly been replaced by newer technologies which offer less invasive and more accurate measurement. The latest evidence on the strengths and limitations of arterial pulse wave form analysis, intraoperative transesophageal echocardiography, peripheral *vs* central arterial blood pressure monitoring and pulmonary arterial catheters are discussed.

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INTRODUCTION

Orthotopic liver transplantation (OLT) has been performed for the past three decades with significant improvement in patient and graft survival. Despite improvements in the anesthetic and surgical techniques, it still is a challenging procedure, requiring dedicated, specifically trained providers and a collection of monitors not common to other operations.

Most classic hemodynamic monitors like radial and femoral arterial lines and a pulmonary artery catheter (PAC) are still part of the protocol at many institutions[1], but new technology has been emerging. These new devices and techniques along with evidence of the limitations of some of the classic monitors, are reshaping the way in which hemodynamics are monitored during anesthesia for liver transplantation in the 21st century.

HEMODYNAMICS DURING LIVER TRANSPLANTATION

Liver transplantation can be thought of as having 3 distinct stages: the dissection or pre-anhepatic phase, the anhepatic phase, and the neohepatic phase. Each stage has its own hemodynamic concerns.

The pre-anhepatic phase is when all the dissection occurs, and is marked by significant fluid shifts from drainage of ascites to the potential for significant blood loss in the presence of varices from portal hypertension. Additionally, manipulation of the liver and downward retraction of the inferior vena cava may intermittently obstruct venous return causing hemodynamically significant changes in preload[2].

The anhepatic stage is defined as the cessation of blood flow to the native liver until the time of reperfusion of the transplanted liver. With cross clamping of the portal vein and IVC, cardiac output (CO) may decrease by up to 50%[3]. To avoid this sudden loss of preload, volume loading should occur prior to crossclamping. An alternative is use of the "piggyback" technique by the surgeons where the inferior vena cava is only partially occluded. Other alternatives include the use of a temporary portocaval shunt or venovenous bypass. Some centers make use of one of these techniques routinely while others employ them as clinically indicated[4].

The neohepatic stage is defined as the beginning of reperfusion until the end of the case. Reperfusion is often marked by significant hemodynamic instability due to the rapid return of blood from the previously obstructed portal system and newly transplanted liver. This blood tends to be acidotic, hyperkalemic, cool, and contains a variety of inflammatory and vasodilatory mediators[3]. The result is often a transient but significant decrease in myocardial contractility, chronotropy and systemic vascular resistance[5]. Postreperfusion syndrome, defined as a decrease in mean arterial pressure by 30% for at least 1 min within 5 min of reperfusion, has been reported to occur in 12.1%-42% of patients[6,7]. After overcoming the instability of reperfusion, the remainder of the neohepatic stage tends to have relatively stable hemodynamics.

BLOOD PRESSURE

Invasive blood pressure monitoring is the standard of practice during liver transplantation. The number and location of these lines varies by center[1]. In healthy individuals, radial artery pressures have a higher systolic pressure as compared to femoral or more central pressures. This difference has been attributed to pulse amplification as a result of the impedance and harmonic resonance of the vasculature. However, the central and distal mean arterial pressures remain relatively unchanged[7,8].

Specific circumstances can create significant discrepancies between central and radial mean arterial pressures. When measuring radial arterial pressure, the presence of a proximal obstructive vascular lesions, rewarming after hypothermic cardiopulmonary bypass[9,10], and high dose vasopressor therapy in critically ill patients[7] are all known to underestimate central pressures. The cause for this discrepancy in obstructive vascular lesions is self-evident, but controversy exists regarding the etiology in cardiopulmonary bypass or vasopressor therapy. In the latter, it has been theorized vasoconstriction of the extremity conductance vessels, which are more sensitive to vasopressors than the central vasculature, significantly reduces the flow to the radial artery[10]. Regarding the use of cardiopulmonary bypass, it is theorized to cause an extreme vasodilatory state leading to proximal shunting, possibly in the splanchnic beds, in combination with distal vasoconstriction which both contribute to lower peripheral pressures[11].

Similarly, it has been suspected in OLT that radial arterial and more centrally measured pressures may not correlate well[12]. Our observations and demonstrated in unpublished data, over many years, have shown a consistent decrease in systolic, diastolic and mean pressures in radial *vs* femoral arterial pressures, most pronounced immediately after reperfusion. The theory behind this, similar to post-cardiopulmonary bypass physiology, is extreme peripheral vasodilation, seen especially during reperfusion, decreases distal pressures disproportionately to central pressure[13,14]. Studies attempting to demonstrate this effect have had conflicting results. Acosta *et al*[15] found no difference in mean arterial pressure at any point during OLT, while Arnal *et al*[16] found systolic but not mean arterial pressures differed during reperfusion which was exag-

gerated in patients receiving vasopressors. A study looking at pediatric OLT, on the other hand, did show a discrepancy is both systolic and mean arterial pressures when comparing femoral to radial pressures throughout most of the operation[17]. Interestingly, a separate study showed noninvasive blood pressure measurements of the upper extremities more closely correlated with femoral artery pressures than radial pressures 3-10 min after reperfusion. Presumably this is due to the more proximal location of the blood pressure cuff[18].

Larger trials are required to definitively determine the reliability of radial pressure monitoring during OLT, but currently the literature suggests central monitoring from femoral or brachial locations and mean arterial pressures should be followed over peripheral radial systolic pressures.

PAC

According to the Frank-Starling law, as end-diastolic volumes increase, myocardial fiber length increases which in turn increases the number of myosin-actin connections resulting in increased CO. Classically, central venous and pulmonary artery occlusion pressures (PAOPs) have been used as surrogates for volume measurements. However, numerous studies have established that these static preload measurements are poor predictors of end diastolic volume and fluid responsiveness in a wide variety of medical and surgical patients[19-26]. The compliance of the heart and vasculature, intrathoracic pressures, cardiac contractility and valvular pathologies significantly affect the pressure measured for a given preload making static pressure measurements an unreliable indicator of end diastolic volume[27,28].

Specifically during liver transplant, Costa *et al*[29] found stroke volume index did not correlate with either central venous pressure (CVP) or PAOP. Similarly, Rocca *et al*[28] looked at pre-anhepatic dissection, the anhepatic phase, and after reperfusion and found no correlation between cardiac index and CVP or PAOP during liver transplantation. A separate study by Rocca *et al*[30] again found in 244 patients undergoing liver transplantation that CVP and PAOP correlate poorly with stroke volume index.

PACs also allow for the measurement of CO *via* intermittent thermodilution. Although often used as the gold standard for CO measurements, its accuracy depends on several user-dependent techniques such as the speed, volume and temperature of the injectate as well as its timing with respect to the respiratory cycle[27]. Significant tricuspid regurgitation or intracardiac shunts also limit the accuracy of thermodilution[31,32]. Additionally, during liver transplantation, the rapid return of cooled blood during reperfusion and administration of large volumes of intravenous fluids generate thermal noise which may result in an underestimation of the true CO[33,34].

A new generation of PACs allow for continuous CO monitoring. This technology uses heat instead of cold thermodilution *via* a thermal filament connected to a specialized PAC and a distal thermistor. CO values are then continuously calculated. Several studies have shown continuous CO measurements correlate well with intermittent thermodilution in a variety of patient populations[35-37]. Although also seen to be accurate during liver transplantation[33], it has similar limitations to intermittent thermodilution with less accuracy during reperfusion and cross-clamping[34].

Mixed venous oxygen saturation (SvO_2) can be measured *via* a PAC and used as an indirect measure of CO. However, changes in SvO_2 are not very specific to CO and may be the result of changes in oxygen content, oxygen consumption or, in the case of reperfusion, return of desaturated blood[38]. Specifically, in liver transplantation SvO_2 has shown to poorly correlate with CO[39].

Perhaps the most important use of PAC during liver transplantation is in patients with portopulmonary hypertension. Patients with mean PA pressures above 50 have typically been denied liver transplantation due to an unacceptably high risk of mortality ranging from 71%-100%[40,41]. While mild (mean PA pressures 25-35 mmHg) and moderate (mean PA pressures 35-45) pulmonary hypertension are not strict contraindications for liver transplantation[42,43], these patients still do have an elevated perioperative mortality rate as high as 33%-35%[40,41]. When pulmonary artery pressures are responsive to treatment, the mortality risk significantly decreases[44-46]. Preoperative workup demonstrating increased PA pressures on echocardiography suggest portopulmonary hypertension. Additionally, hypoxemia or exaggerated respiratory alkalosis also may suggest increased PA pressures[47]. However, a PAC is the only modality that directly measures pulmonary arterial pressures. At the start of the procedure, it may unveil worse pulmonary hypertension than suspected during preoperative evaluation leading to cancellation of the procedure. Intraoperatively, the sudden volume shifts and release of vasoactive mediators seen, especially during reperfusion, may result in significant right heart strain and failure[48]. Treatment of elevated PA pressures during OLT includes the use of venovenous bypass during the anhepatic phase, phosophodiesterase-5 inhibitors, endothelin receptor antagonists, and prostacyclins[48-50].

The use of PACs does have significant diagnostic limitations however they are still one of the most accurate tools to assess CO and an essential monitor in patients with significant pulmonary hypertension.

CO WAVEFORM ANALYSIS

Beginning in 1904, Joseph Erlanger and Donald R Hooker theorized that CO could be derived from the arterial pulse pressure[51]. Logically, as more blood is ejected from the heart, there should be a greater pressure transmitted to the arterial tree. This correlation

is limited, however, due the vascular resistance which determines the runoff of blood from the conductance vessels into the arterioles. Even more complex is the nonlinear compliance of the arterial tree where a given volume of blood will decrease compliance more at higher pressures as compared to lower pressures[52]. Only recently has the technology been available to not only precisely analyze pressure waveforms but also the sufficient knowledge to create algorithms which account for the complex physiology of pulse wave morphology.

There are currently several commercially available products which calculate continuous CO from arterial waveform analysis. The PiCCO system (Pulsion Medical System, Munich, Germany) requires the placement of a thermodilution catheter into the axillary or femoral artery. A solution is then injected into any central venous catheter and CO is then determined by the arterial temperature probe. After this initial calibration, the system then continuously calculates CO based on arterial waveform analysis. Additionally, static preload indices such as global end diastolic volume and intrathoracic blood volume can be calculated. The LiDCO system (LiDCO Plus, Cambridge United Kingdom) is similar to the PiCCO system but uses lithium indicator dilution rather than thermodilution to calibrate the pulse wave form to the CO[53]. The Flowtrac/Vigileo system (Edwards Lifesciences, Irvine, CA United States) uniquely does not require intermittent CO bolus calibration. It accounts for changes in arterial compliance and resistance using a conversion factor Khi which factors in the standard deviation of the mean arterial pressure and the analysis of the skewness and kurtosis of the arterial waveform. Large vessel compliance is then estimated using patient demographics of age, gender and body surface area[54].

In patients undergoing liver transplant, PiCCO derived CO measurements were found to agree with the gold standard of PAC thermodilution CO[55]. Similarly Nissen *et al*[56] looking at dissection, anhepatic, early and late reperfusion phases during OLT also found arterial pulse wave CO measurements to correlate with thermodilution CO.

Despite a few reassuring studies, significant concerns have been raised concerning the validity of arterial waveform analysis in particular patient populations whose physiology may not be well represented by the algorithms. CO by waveform analysis was found to correlated poorly with PAC thermodilution CO in patients on a significant dose of vasopressor[57,58]. There was also poor correlation during cardiac surgery[59] and abdominal aortic aneurysm operations[60] with the uncalibrated FlowTrac system performing worse than the PiCCO system.

The hyperdynamic circulation in liver cirrhosis is characterized by low systemic vascular resistance, elevated CO and possible underlying cardiomyopathy. The transplant operation then adds large changes to preload and afterload, vasoactive mediators during reperfusion, myocardial contractility changes and the possibility of significant hemorrhage[14]. Likely as a result of this altered physiology and dynamic intraoperative changes, several studies have found poor correlation between waveform analysis CO calculations when compared to the PAC thermodilution. Krejci *et al*[59] found, in Child-Pugh class B and C cirrhotics, both FlowTrac and LiDCO systems correlated poorly with PAC thermodilution. Two separate studies found that in Child-Pugh B and C cirrhosis, the degree of FlowTrac's inaccuracy was proportional to the patient's SVR with lower resistances showing less correlation to reference thermodilution values[61,62]. Della Rocca *et al*[63] examined the effect of the high output cardiac state and found Flowtrac underestimated CO in liver transplant patients whose CO exceeded 8 L/min.

A recent software update to FlowTrac has been released (third generation) whose aim was to improve CO accuracy specifically in low systemic vascular resistance states. Some improvements have been seen in septic patients[64] and those undergoing cardiac surgery[65]. Despite these improvements, in liver transplantion the accuracy of FlowTrac CO measurements remain unreliable. Tsai *et al*[66] found a 55% discrepancy between the third generation software of FlowTrac COs as compared to PAC thermodilution. Likewise, Su *et al*[67] found a percentage error of 75% which was inversely related to the patient's systemic vascular resistance index. CO derived from waveform analysis depends on the intrarterial peripheral catheter reflecting systemic conditions. CO in cirrhosis and during liver transplantation, however, is not evenly distributed[29]. Peripheral arterial waveform analysis, therefore, cannot be recommended for liver transplant intraoperative monitoring or in Child-Pugh class B or C patients.

With the currently available technology, arterial waveform analysis cannot reliably measure CO during OLT.

STROKE VOLUME AND PULSE PRESSURE VARIATION

Although CO by waveform analysis has proven unreliable in liver transplant, the technology also allows for the continuous measurements of stroke volume variation (SVV) and pulse pressure variation (PPV).

Pulse pressure changes proportionally to left ventricular stroke volume[25]. During positive pressure ventilation, blood return to the right heart decreases. After this lower volume of blood passes through the pulmonary vasculature, left ventricular end diastolic volume decreases. The overall result is lower stroke volumes and a smaller pulse pressure after positive pressure ventilation. The magnitude of this difference is proportional to preload. Patients whose CO would be supplemented by increased intravascular volume will have a larger difference in pulse pressure during positive pressure ventilation and exhalation. A review of twenty-nine studies studying this phenomenon found SVV less than 11.6% ± 1.9% and a PPV less than 12.5% ±

1.6% predicted volume responsiveness in critically ill patients[26].

Arterial waveform analysis of the SVV has been shown to predict fluid responsiveness during general anesthesia[68] and the use of vasoconstrictors does not change the variation[69]. In liver transplantation, poor SVV was found to be a better predictor of right ventricular end diastolic volume index than CVP[70]. Kim *et al*[71] confirmed these findings and found a PPV of greater than 9% predictive of lower RVEDVI which would likely be fluid responsive. Biais *et al*[72] found, in liver transplant, a SVV of 10% discriminated fluid responders from non-responders with a 93% sensitivity and 94% specificity.

The accuracy of SVV or PPV in predicting fluid responsiveness depends on the patient meeting a specific set of criteria. Stroke volume is less predictably dependent on preload during spontaneous breathing. Breathing effort causes irregularity in both pulse rate and intrathoracic pressures[73]. Not only must the patient be mechanically ventilated with no breathing efforts, but the pressure must be adequate to decrease preload. The degree of SVV is linearly related to tidal volume with tidal volumes less than 10 mL/kg showing a lesser degree of SVV[74]. Likewise, when driving pressures (defined as the difference between the plateau and positive end expiratory pressure) are less than 20 cm H_2O, a PPV less than 13% does not rule out fluid responsiveness[75]. De Backer *et al*[76] found that PPV only reliably predicted fluid responsiveness at tidal volumes above 8 mL/kg. Finally, during cardiac arrhythmias, SVV is more dependent on the irregular diastolic time than on intravascular volume[77].

Although waveform analysis has limited utility in quantitative measurements of CO in cirrhosis and liver transplantation, it is a minimally invasive option of monitoring end diastolic volume and fluid responsiveness.

TRANSESOPHAGEAL ECHOCARDIOGRAPHY

Echocardiography has a variety of intraoperative uses. In experienced hands, it has the capability of diagnosing right ventricle (RV) or left ventricle (LV) systolic or diastolic dysfunction, volume overload, global or regional wall abnormalities, and intracardiac air or thrombosis[78-80]. In liver cirrhosis, numerous case reports have reported its usefulness in diagnosing porto-pulmonary hypertension[81], ischemic heart disease, cirrhotic cardiomyopathy[82], and intraoperative thromboembolic events[83]. More rare conditions such as pericardial tamponade[84], cardiomyopathy secondary to undiagnosed hemochromatosis[85], and Takosubo cardiomyopathy[86,87] have also been reported during OLT intraoperative transesophageal echocardiography (TEE).

One of the greatest strengths of TEE is its ability to directly visualize in real-time the preload of both the right and left sides of the heart. As previously discussed, the PAOP is a known poor measure of LV preload. The PAOP may differ dramatically despite no change in left end-diastolic volume (LVEDV) and fails to detect hypovolemia when compared to direct measurement of LVEDV by TEE[88]. The LVEDV can be directly visualized from the transgastric mid-short axis view (TG mid-SAX). Additionally, TEE has been shown to accurately determine stroke volume and left ventricular changes[88]. In a multicenter study of 244 patients undergoing OLT, stroke volume index was found to be more strongly correlated with right ventricular end diastolic volume index than either CVP or PAOP[30].

Similarly, CVP can be an unreliable indicator of stroke volume and intravascular volume[20,23,89]. During increases in intravascular volume, the RV dilates which results in no significant change in the CVP despite large increases in volumes[90]. The resulting fluid overload can result in congestion and injury to the newly transplanted liver. SVV can indicate the need for additional fluid, but depends on a regular heart rate and adequate tidal volumes without respiratory effort. These are not limitations for TEE as it directly measures end diastolic volume. Determination of preload by TEE has significant clinical consequence. Hofer *et al*[82] found intraoperative use of TEE influenced fluid therapy in 50% of OLT patients.

Hypotension in the presence of adequate preload may be a result of myocardial dysfunction. Coronary artery disease (CAD) is not uncommonly encountered in patients undergoing OLT, with one study reporting up to 32% of patients over the age of 50 having moderate to severe disease[91]. Patients with known CAD undergoing OLT have a mortality of 50%[92]. Due to this high mortality, reversible ischemia seen on stress testing is a contraindication to proceeding with OLT[93]. Intraoperative TEE allows for the real time detection of myocardial ischemia manifested by regional wall motional abnormalities. A study by Smith *et al*[94] found intraoperative TEE was more sensitive in detecting myocardial ischemia than EKG changes. A separate study showed TEE may be more sensitive than conventional monitors as 73% of patients with regional wall abnormalities had no detectable change in heart rate, blood pressure or PA pressures[95].

Pulmonary embolization can be a real and significant risk during OLT[96]. Paradoxical embolization to the systemic circulation may result in stroke and can occur as a result of a patent foramen ovale. Cirrhotic patients are at particular risk for paradoxical embolization as dilated pulmonary vasculature can allow the free passage of emboli to the systemic circulation. TEE can not only identify patients with right to left shunts but also distinguish between intracardiac and transpulmonary etiologies, thereby identifying those patients who are at higher risk for paradoxical embolism[97]. Furthermore, TEE Doppler is the most sensitive monitor for the detection of air embolism, able to detect 0.1 mL/kg of air[98]. The diagnosis of an acute pulmonary embolism, however, is more difficult with TEE, typically manifesting

Table 1 Comparative summary table of hemodynamic monitors in orthotopic liver transplantation

Monitor	Benefits and uses	Limitations
Invasive blood pressure monitoring	Beat-to-beat monitoring of blood pressure	Peripheral arteries possibly underestimate the central mean arterial pressure especially during reperfusion or use or high dose vasopressors
Pulmonary artery catheter	Accurately determines cardiac output *via* intermittent thermodilution	Invasive
	Directly measures PA pressures	Static pressure measurements are imperfect indicators of fluid status or stroke volume
Arterial pulse wave analysis - CO	Less invasive option to calculate CO	Does not reliably calculate CO in advanced cirrhosis or during OLT
Arterial pulse wave analysis - SVV	Predicts fluid responsiveness in OLT population	Requires sinus rhythm
		Requires patient does not make any spontaneous respiratory efforts
		Most accurate during tidal volumes of 8-12 mL/kg
Transesophageal echocardiography	Direct assessment of cardiac filling	Requires advanced training
	Monitors myocardial ischemia and strain	Limited views intraoperatively
	Potentially can diagnose pulmonary embolisms, shunts, effusions, and valvular pathologies	Risk of esophageal varix rupture or esophageal injury

CO: Cardiac output; SVV: Stroke volume variation; OLT: Orthotopic liver transplantation; PA: Pulmonary artery.

with signs of RV dysfunction such as RV dilation, hypokinesis and possible pulmonary hypertension[99]. However, a large burden of pulmonary embolism is required to see these effects, especially those over 30% of the pulmonary artery area, are more likely to show RV dysfunction[100]. The classic "McConnell sign" refers to hypokinesis of the RV with preservation of RV apical contractility[101]. This sign has been reported to be very specific for acute pulmonary embolism but with a sensitivity of only 19%[102,103].

Despite the many advantages of TEE, like every monitor, there are limitations and risks. The proficient use of TEE requires significant training and expertise with the American Society of Echocardiography recommending 300 transthoracic echocardiograms, 20 esophageal intubations and 50 transesophageal examinations within a 6 mo period[103]. However, the performance of "limited-scope examinations" by non-credentialed anesthesiologists is not uncommon with 88% of users lacking echocardiography certification[104].

Additionally, the presence of esophageal varices creates concern for rupture. However, while grade four esophageal varices may be a true contraindication[27], TEE has been safely performed in patients with grade Ⅰ and Ⅱ varices[105]. TEE is also limited in its ability to assess pulmonary artery pressures in the absence of tricuspid regurgitation with the far majority of centers using a PAC with or without the use of TEE[1]. As previously discussed, TEE is a very sensitive monitor for ischemia, however, the transgastric short axis view which best assesses the circumference of the left ventricle is largely unavailable during the operation due to posterior retraction of the stomach[27].

CONCLUSION

A variety of hemodynamic monitors are an essential part of the successful intraoperative management of patients undergoing OLT but each has their own indications and limitations (Table 1).

Invasive blood pressure monitors is currently the standard of care during transplantation, however, the evidence suggests that peripheral measurements are possibly not representative of central perfusion pressures especially in instances of significant vasopressor use or in patients with unequal vasodilation as is the case in significant cirrhosis or during reperfusion. When possible, more central invasive monitors at the femoral or brachial artery have a theoretical advantage of representing central perfusion pressures.

PACs continue to have controversial indications in and outside the operating room. The available evidence is clear that static cardiac pressure measurements such as CVP and PAOP are imperfect predictors of fluid responsiveness and CO. However, CVP allows for the monitoring of the backpressure of the IVC into the newly transplanted liver and may still guide the transplant anesthesiologist in fluid management or need for vasodilators to prevent injury to the liver. When using a combination of data such as the heart rate, blood pressure, CVP, urine output, a more clearly picture of the patient's hemodynamic status may emerge.

A role still exists for PACs for the accurate measurements of CO *via* intermittent or, more recently, continuous thermodilution which remains the current gold standard. In OLT, PACs also play an important role in the monitoring of patients with PA pressures in patients with pulmonary hypertension which, even in the setting of mild hypertension, carries a significant risk of morbidity and mortality.

Pulse pressure analysis is a newer monitoring technique. Although the promise of accurate and continuous CO analysis has not been delivered in the OLT patient population, continuous PPV monitoring does appear to predict fluid responsiveness and may serve as an invaluable guide.

TEE is the most direct measurement of cardiac filling that currently exists allowing for the real-time assessment of fluid status during these dynamic operations. Additionally, it also offers the unique benefit of diagnosing a variety of other intraoperative complications such as myocardial ischemia, pulmonary embolism, and pulmonary or pericardial effusions. It currently is limited by the expertise required to interpret the images, but as more anesthesiologists are trained in this technology it stands to supplant many of the indirect monitors currently in use.

The perfect hemodynamic monitor would be non-invasive, precise and accurate, and provide continuous data at all stages of transplantation. Until this device exists, adept intraoperative management requires knowledge of the applicability and known limitations of available technology. Perhaps the current best monitor is the experienced provider who can adeptly integrate the various pieces into a complete but adaptable perioperative treatment plan.

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REVIEW

Targeted proteomics for biomarker discovery and validation of hepatocellular carcinoma in hepatitis C infected patients

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Abstract

Hepatocellular carcinoma (HCC)-related mortality is high because early detection modalities are hampered by inaccuracy, expense and inherent procedural risks. Thus there is an urgent need for minimally invasive, highly specific and sensitive biomarkers that enable early disease detection when therapeutic intervention remains practical. Successful therapeutic intervention is predicated on the ability to detect the cancer early. Similar unmet medical needs abound in most fields of medicine and require novel methodological approaches. Proteomic profiling of body fluids presents a sensitive diagnostic tool for early cancer detection. Here we describe such a strategy of comparative proteomics to identify potential serum-based biomarkers to distinguish high-risk chronic hepatitis C virus infected patients from HCC patients. In order to compensate for the extraordinary dynamic range in serum proteins, enrichment methods that compress the dynamic range without surrendering proteome complexity can help minimize the problems associated with many depletion methods. The enriched serum can be resolved using 2D-difference in-gel electrophoresis and the spots showing statistically significant changes selected for identification by liquid chromatography-tandem mass spectrometry. Subsequent quantitative verification and validation of these candidate biomarkers represent an obligatory and rate-limiting process that is greatly enabled by selected reaction monitoring (SRM). SRM is a tandem mass spectrometry method suitable for identification and quantitation of target peptides within complex mixtures independent on peptide-specific antibodies. Ultimately, multiplexed SRM and dynamic multiple reaction monitoring can be utilized for the simultaneous analysis of a biomarker panel derived from support vector machine learning approaches, which allows monitoring a specific disease state such as early HCC. Overall, this approach yields high probability biomarkers for clinical validation in large patient cohorts and represents a strategy extensible to many diseases.

Key words: Hepatocellular carcinoma; Biomarkers; Early detection; Selected reaction monitoring; Targeted proteomics

Core tip: The projected rise in hepatocellular carcinoma (HCC) is largely attributed to hepatitis C virus infection with onset of HCC being a latent consequence occurring decades after the original infection. However, other environmental risk factors including alcohol, tobacco, and diet-derived insults that cause liver injury increase the incidence of HCC. The poor prognosis associated with late stage diagnosis renders successful intervention difficult. The methodology described in this review article shows the feasibility of a highly multiplexed manner using multiple reaction monitoring using internal standard peptides to more easily quantify proteins, which narrows the time between discovery and validation in the biomarker pipeline in general.

Mustafa GM, Larry D, Petersen JR, Elferink CJ. Targeted proteomics for biomarker discovery and validation of hepatocellular carcinoma in hepatitis C infected patients. *World J Hepatol* 2015; 7(10): 1312-1324 Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i10/1312.htm DOI: http://dx.doi.org/10.4254/wjh.v7.i10.1312

PROTEOMICS APPROACHES: PATH TO EARLY DIAGNOSIS

This article highlights a proteomics pipeline that is being applied to develop and validate a panel of candidate biomarkers suitable for early hepatocellular carcinoma (HCC) diagnosis. However, the strategies described are also applicable to other objectives including other diseases, drug development, and therapeutic monitoring. Given the time and money involved in bringing a drug to market, the availability of biomarkers capable of identifying potential drug failures early in development is key, but this depends increasingly on advanced proteomic technologies. The characterization of unique protein patterns associated with specific diseases as a discovery strategy to identify candidate biomarkers is one of the most promising areas of clinical proteomics. Cancer, although often classified as a genetic disease, is functionally a proteomic disease. The proteomic tissue microenvironment directly impacts the tumor-host communication system by affecting enzymatic events and the sharing of growth factors[1], so the tumor microenvironment represents a potential source for biomarkers. An example of an early disease biomarker is the prostate-specific antigen (PSA). Today, serum PSA levels are regularly used in the diagnosis of prostate cancer in men. Unfortunately, a reliance on a single protein biomarker is frequently found to be unreliable. Despite decades of effort, single biomarkers generally lack the specificity and sensitivity required for routine clinical use[2]. Due to the heterogeneity that exists from tumor to tumor, biomarker discovery is moving away from the pursuit for an idealized single cancer-specific biomarker in favor of identifying a panel of markers. Disease complexity almost dictates that accurate screening and diagnosis of HCC will require multiple biomarkers. Proteomics affords us the ability to simultaneously interrogate the entire proteome or sub-proteome in order to identify correlations between protein expression (or modifications) and disease progression. In this manner, a panel of biomarkers can be constructed that exhibit the desired sensitivity and specificity necessary for the detection and monitoring of the disease. Recent advances in proteome analysis have focused on the more accessible body fluids including plasma, serum, urine, cerebrospinal fluid, saliva, bronchoalveolar lavage fluid, synovial fluid, nipple aspirate fluid, tear fluid, and amniotic fluid[3]. These body fluids are obtained using minimally invasive procedures, are readily processed, and hence represent clinically tractable cost effective sources of biological material[4].

An effective, clinically useful biomarker should be quantifiable in a readily accessible body fluid such as serum. Since blood comes in contact with almost every tissue, it constitutes a treasure trove of potential biomarkers that provide a systemic picture of the physiological state of the entire body. Every cell in the body contributes to the blood proteome through normal metabolic processes, consequently defined changes characteristic of disease will be reflected in the blood proteome due to perfusion of the affected tissue or organ[5]. Therefore alteration of serum protein profiles can effectively reflect the pathological state of liver injury. It is also ideal to use serum because its sampling is minimally invasive and it is easily prepared with high reproducibility. The disease-related differences in the proteome profile can be attributed to altered protein levels reflecting changes in expression, or dysregulated proteolytic activity affecting protein turnover in diseased cells.

While on the surface this sounds simple to discover a biomarker in serum, the actual process is laborious and time consuming because of the inherent complexity of the human serum proteome, which is composed of thousands of individual proteins. Moreover, the prevalence of serum proteins spans a wide dynamic range-approximately 9 orders of magnitude from pg/mL to mg/mL. These properties represent substantial challenges that often prevent the development and wide-scale utilization of this treasure chest of biological information.

TARGET DISEASE BACKGROUND: HEPATITIS C AND HCC

Liver cancer accounted for 745000 deaths in 2012 alone (World Health Organization. Fact Sheet No 297: 2014). Worldwide, the prevalence of HCC is estimated to affect 180 million people and the incidence continues to rise[6], especially in the United States. It is the fifth most common cancer in men (554000 cases, 7.5% of the total) and the ninth in women (228000 cases, 3.4%)[7]. Chronic infection with hepatitis C virus (HCV)

is a major risk factor for the development of HCC with an estimated 130-150 million people having chronic HCV infection (World Health Organization. Fact Sheet No 164:2014). The prognosis for HCC is very poor with an overall 5 year survival rate below 5%, primarily because HCC frequently goes undetected prior to advanced stage disease when therapeutic options are limited. Major risk factors for HCC are infection by the HCV and HBV, alcoholic liver disease, and associated liver cirrhosis. In the developed world however, non-alcoholic fatty liver disease (NAFLD) is increasingly being recognized as a risk factor for HCC without evidence of underlying cirrhosis. Currently, HBV and HCV account for 80%-90% of all HCC worldwide[8-10]. Although HBV remains the most common HCC risk factor worldwide to date, the use of a HBV vaccine in newborns is expected to decrease the HCC incidence associated with HBV infection[8]. In contrast, despite the existence of HCV tests and moderately effective anti-viral therapies, HCV remains a major risk factor for HCC. In fact, the incidence of HCC increased from 2.7 per 100000 to 3.2 per 100000 in 5 years and an estimated 78% of this increase was attributable to latent HCV infections in the general population[11]. In the United States, the incidence of HCC is on the rise stemming from HCV exposures several decades earlier[12], and retrospective studies suggest that once cirrhosis develops, liver disease progresses to either hepatic decompensation (liver failure) or HCC occurs at a rate of 2% to 7% per year[12-16].

The absence of randomized clinical trials notwithstanding, there is compelling evidence suggesting that surgical resection, liver transplantation, or ablative therapies significantly improve survival in HCC patients[17,18]. However, few patients with advanced HCC meet the criteria for these therapeutic modalities. Hence, these clinical options are generally only available to individuals fortunate enough to have been diagnosed with early stage HCC, typically where the tumor is less than 3 cm in diameter without vascular involvement[19,20]. Since early HCC tumors are asymptomatic[17,21,22], routine surveillance of high-risk patients such as those with cirrhosis is recommended as a strategy to detect tumors at a time when therapeutic intervention still offers markedly improved survival rates[23]. Surgical resection offers a 5-year survival rate of approximately 35%, increasing to 45% for small tumors (2-5 cm), thereby highlighting the value of early detection[24]. Hence a screening modality that provides the requisite sensitivity and specificity for early HCC detection would be of significant clinical benefit[25].

Current diagnostic tools for early HCC detection are unfortunately insensitive and/or nonspecific. To date, most established serological diagnostic test for HCC is measures serum alpha-fetoprotein (AFP) levels, however the assay is insufficiently sensitive (39% to 65%) or specificity (65% to 94%) to be very reliable[26-28]. For example, HCV patients with necro-inflammation and liver fibrosis may register high serum AFP levels unrelated to HCC. AFP levels are also elevated in hyperthyroidism[29] and pancreatitis[30] limiting its efficacy as a reliable biomarker for HCC. Biopsied and histopathologically tested samples to discriminate early HCC from benign nodules can be difficult even for expert pathologists[31]. The two newer serological biomarkers, DCP and AFP-L3, fared no better than AFP as their elevation was nonspecifically common in patients without HCC and was influenced by race, gender, age, and severity of liver disease. Therefore it was concluded that screening protocols based on AFP, AFP-L3, and DCP are in rveillance[32]. More recently, screening modalities based on markers including Dickkopf-1 and Midkine designed to complement AFP, are being developed to facilitate screening and diagnosing HCC at an earlier stage[33,34]. However, rigorous validation studies are required before their clinical value is established. According to the National Cancer Institute's Early Detection Research Network guidelines for biomarker development, robust prospective, randomized, controlled, multi-center trials using a large cohort of patients with hepatitis B and hepatitis C infectious liver disease, NAFLD, and alcohol-induced liver disease are required for validation[35]. Currently, imaging with triphasic computed tomography scanning and magnetic resonance imaging with intravenous gadolinium can improve the diagnostic accuracy, but these techniques are time consuming and very expensive, and are not practical for screening the millions of people identified with known risk factors for HCC. Although ultrasound is very sensitive (in the order of 80%) it is extremely operator dependent[36-38] and is not well suited to differentiate between malignant and benign nodules in the cirrhotic liver. Consequently, development of a minimally invasive test using serum-based biomarkers with the necessary sensitivity and specificity will enhance surveillance, widespread screening, and early HCC detection among the millions who are at risk of developing liver cancer.

SAMPLE PREPARATION: FRACTIONATION AND ENRICHMENT

High abundance serum proteins comprise fewer than two dozen proteins, including albumin and the immunoglobulins, which account for approximately 99% of the total serum protein[39]. The presence of these highly abundant proteins masks the ready detection of medium and low abundance proteins that comprise the repertoire of potential biomarkers. This renders identification of the biomarkers extremely challenging. Serum contains 60% - 80 mg/mL protein, but approximately 65% of this is serum albumin, and approximately 15% are γ-globulins[40-42]. Finding a disease-related protein in such a complex mixture is like searching for a needle in a haystack. So it becomes important to compress the serum protein large dynamic range and reduce the few over-represented (*i.e.*, abundant) proteins by depleting

highly abundant proteins to allow detection of lower abundant proteins.

Developments in biomarker-based proteomics technologies are dramatically impacted by the recent realization that a high percentage of the diagnostically useful lower molecular weight serum protein entities are bound to higher molecular weight carrier proteins such as albumin[5,43-45]. In fact, these carrier proteins likely serve to amplify and protect lower molecular weight proteins from clearance by the renal system[46,47]. Conventional protocols for biomarker discovery discard the abundant high molecular weight carrier species such as albumin without realizing the valuable cargo they harbor. Albumin is a carrier/transport protein that sequesters numerous other serum components. Consequently, stripping away albumin from a serum sample risks removing potentially important species. This is like "throwing the baby out with the bath water" by failing to capture the information associated with this valuable resource. Researchers believe that the albumin-bound proteomic signature in serum can be used for early detection and staging of HCC[48]. Therefore, in choosing a method for removal of over represented proteins, the chosen strategy should protect against the nonspecific loss of unrelated proteins. A novel methodology is the aptamer-based Proteominer technology (Bio-Rad) designed to preserve the complexity of the serum proteome using a strategy that does not merely deplete carrier proteins. It constitutes a novel sample preparation protocol that narrows the dynamic range of the serum protein profile without losing the complexity of the entire proteome. This is accomplished through the use of a highly diverse hexabead-based library of combinatorial peptide ligands[49,50]. When complex biological samples such as serum are applied to the beads, the high-abundance proteins such as albumin readily saturate the finite high affinity sites on the beads. However, the retention of carrier proteins such as albumin guarantees that albumin-bound entities are retained in the enriched sample. Medium- and low abundance proteins on the other hand are concentrated by binding to the specific aptamers. As a consequence, the dynamic range of protein profile is reduced while preserving the full complexity of the protein sample. Before performing subsequent high-resolution identification strategies, the samples should be desalted (using standard cleanup kits or desalting columns) to remove electrolytes and other impurities present in the sample[50].

PROTEIN EXPRESSION PROFILING: 2D-DIFFERENCE IN-GEL ELECTROPHORESIS DISCOVERY

Expression profiling is central to proteomics and depends on methods that are able to provide accurately and reproducibly differentiate between the expression profiles in two or more biological samples. Despite recent technological advances in methods for separating and analyzing complex protein mixtures, two-dimensional gel electrophoresis (2D-PAGE) remains a widely used approach in proteomics research[51-55]. 2D-PAGE allows for the separation of thousands of proteins on the basis of both size and charge from a tissue or biological fluid. However, inter-gel variability and the extensive time and labor needed to resolve differences in protein expression have hampered the efficacy of 2D-PAGE. A major technical improvement in 2D-PAGE involves development of the multiplexed fluorescent two-dimensional-difference in-gel electrophoresis (2D-DIGE) method[56] which utilizes direct labeling of the lysine groups on proteins with cyanine (Cy) dyes before isoelectric focusing fractionation in the first dimension. 2D-DIGE is a robust technique proving fruitful in the identification of proteins exhibiting differential expression between samples. It is considered to be one of the most significant advances in quantitative proteomics technology and is crucial to the success of many proteomics initiatives[57,58]. A critical benefit of 2D-DIGE technology is the ability to label 2-3 distinct samples using specific dyes prior to isoelectric focusing of the samples on a single (non-linear) IPG strip[59]. This feature limits spot pattern variability in experimental replicates and therefore the number of gels required to establish confidence in spot pattern differences. Consequently, 2D-DIGE successfully identified statistically significant changes in the serum proteome using only 6 sets of randomly paired patient samples (*e.g.*, 6 HCV and 6 HCC) in 3-5 wk. By labeling the 6 random pairs of HCV and HCC samples with three distinct CyDyes (Cy2, Cy3 and Cy5), we generated 18 HCV and 18 HCC samples for 2D-DIGE and identified 43 significantly differentially expressed proteins. Specifically, 2D-DIGE was used to examine changes in protein abundance between HCV and HCC patient samples by performing Cy3 and Cy5 dye swap experiments under conditions involving a Cy2-labeled internal standard-comprising a pooled preparation of all the patient samples - to normalize between technical replicates[58]. Moreover the substantial sensitivity and broad dynamic range available using these dyes enhances the quantitative accuracy beyond that attainable using silver staining[60]. These advantages renders 2D-DIGE more reliable than 2D-PAGE as a qualitative and quantitative method to interrogate complex proteomes[43], and thus has found utility in proteomics studies examining several human cancers (Figure 1).

The CyDye DIGE Fluor minimal dye chemistry relies on a N-hydroxysuccinimidyl ester reactive group that readily forms an amide linkage covalent bond with the epsilon amino group of lysine in proteins. The positive charge of the CyDye substitutes the positive charge in adducted lysine residues at neutral and acidic pH, thus keeping the pI of the protein essentially unchanged. The labeling reaction is designed to be dye that the dye labels approximately 1%-2% of lysine residues. Therefore, each labeled protein typically harbors on average only one dye-labeled residue and is visualized

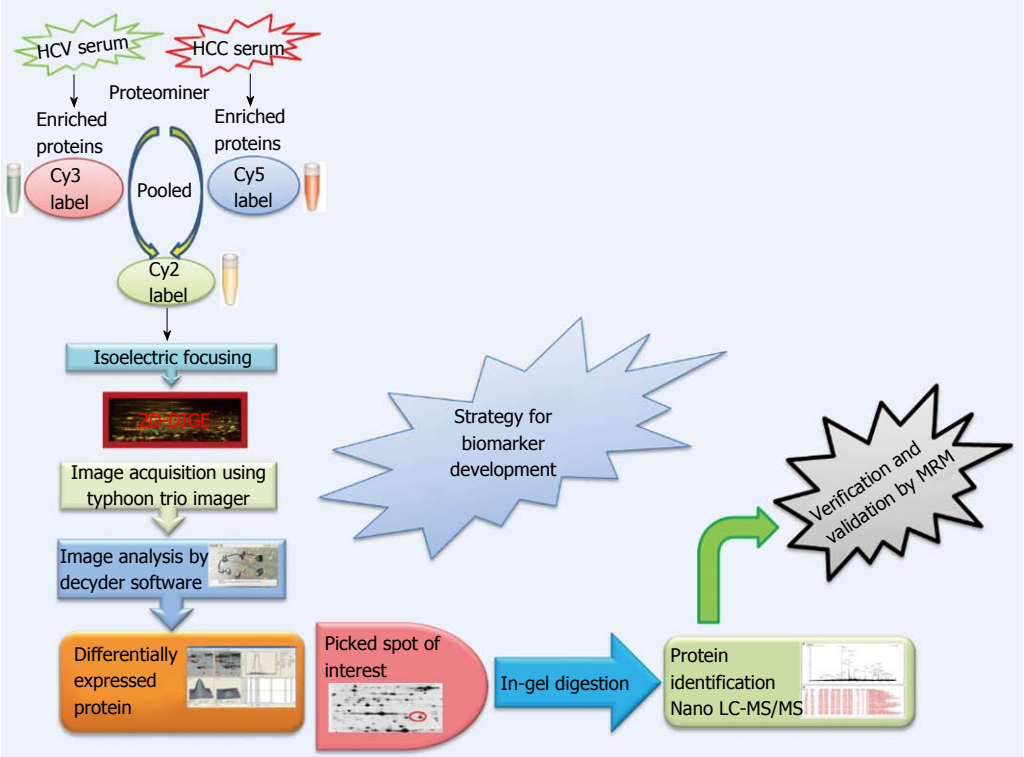

Figure 1 Proteomics strategy for biomarker discovery. HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; Cy: Cyanine; 2D-DIGE: 2D-difference in-gel electrophoresis; MS: Mass spectrometry; LC: Liquid chromatography.

as a single protein spot. The labeling reaction is rapid, taking only 30 min and is quenched by the addition of 1 μL of 10 mmol/L lysine for 10 min. It should be noted that CyDye labeling contributes approximately 500 Da to the mass of the labeled proteins; however, this modest increase in molecular weight does not corrupt the 2D gel pattern since all visualized proteins are labeled. The increased molecular weight conferred by a single CyDye labeling event per protein nor the hydrophobicity of the fluorophore substantially alter gel migration of the labeled proteins. The labeling protocol dictates that the ratio of label to protein be optimized so that less abundant proteins are tagged while keeping the highly abundant proteins in the linear dynamic range for quantitative imaging. 2D-DIGE benefits from the use of a pooled internal standard (*i.e.*, a pooled sample comprising an equal aliquot from each sample in the experiment) labeled with the Cy2 dye. The internal standard is essential for assessing biological and experimental variations and increasing the confidence of the statistical analysis. Sequential scanning of Cy2-, Cy3- and Cy5-labeled proteins (gels) is achieved by the following laser/emission filters: 488/520, 532/580 and 633/670 nm, respectively[61] using a Typhoon Trio Imager (GE Healthcare). Image analysis of the fluorescently labeled proteins requires sequential evaluation of the spot patterns using proprietary (Decyder) software for differential in-gel analysis. In order to reveal changes in protein abundance intra-gel statistical analysis, Cy5/Cy3:Cy2 normalization, and biological variation analysis-which performs inter-gel statistical analysis to provide relative abundance in various groups-are performed. Cy5 or Cy3 samples are normalized against the Cy2 dye-labeled sample (*i.e.*, Cy5:Cy2 and Cy3:Cy2). In the discovery of HCC biomarkers, log abundance ratios were compared between pre-cancerous and cancerous samples from all gels using the chosen statistical analysis (*e.g.*, *t*-test and ANOVA) using software packages such as DeCyder (GE Healthcare)[62-64]. In addition to being sensitive and quantitative, 2D-DIGE is also compatible with downstream mass spectrometry (MS) protein characterization protocols since most lysine residues in a given protein remain untagged and are accessible for tryptic digestion. Spot detection, the matching and picking of differentially expressed spots of interest among various samples, is done by identifying the spots that reproducibly show expression differences between the cancerous and pre-cancerous samples across biological replicates. Differentially expressed protein spots that satisfy the selection criteria using a statistical significance of $P < 0.05$ and a threshold of > 1.5-fold change in abundance are selected, and these protein spots are picked from preparative gels involving the 2D-PAGE fractionation of substantially greater amounts of the same protein samples for identification by MALDI-TOF and/or nano-Liquid chromatography (LC)-MS/MS. The combination of 2D-DIGE to confidently detect changes in protein abundance between two samples, with contemporary MS techniques capable of identifying proteins in complex mixtures greatly enhances the biomarker discovery pipeline.

The many advantages of this approach notwithstanding, there remain significant caveats. For example, proteins with a high percentage of lysine residues are more susceptible to multiple labeling events than proteins encoding few or no lysines. Therefore, it is conceivable that a highly abundant protein with few lysine restudies may be readily detectable by conventional 2D-PAGE but be poorly labeled by the CyDye fluorophores in 2D-DIGE and hence be underestimated. Also, while LC-MS/MS typically requires only 1-5 μg of protein, preparative 2D-gels require substantially more protein (approximately equal to 500 μg) for reliable spot detection, which may become a limiting factor in discovery proteomics. Moreover, despite recent advances in high-resolution mass spectrometers that facilitate quantitative analyzes of thousands of proteins, the technology is still not capable of comprehensively characterizing the entire proteome in complex mixtures such as serum. Thorough assessments of these complex samples require prior fractionations to reduce sample complexity using strategies including multidimensional separation (gel-based and chromatography-based technology). Some of the most common methods used for these complex mixtures are 2D-DIGE, isotope-coded affinity tags, isotope-coded protein labeling, tandem mass tags, isobaric tags for relative and absolute quantitation, stable isotope labeling, and label-free quantification. It is noteworthy that the lower abundance proteins detected by 2D-DIGE are refractory to identification by mass spectrometry due to the detection limits of currently available mass spectrometers.

Proteome analysis is often achieved by the sequential use of 2D-PAGE and MS. However, traditional 2D-PAGE techniques are hamstrung by constraints associated with detection limits of low-abundance proteins in complex samples. These limitations have been addressed by the development of sophisticated front-end separation technologies. LC in combination with tandem LC-MS/MS affords researchers the ability to directly analyze complex mixtures in much greater detail without incurring the detection issues associated with 2D-PAGE[65]. The evolution of proteomics technologies has catalyzed large-scale analyzes of differentially expressed proteins under various experimental conditions, which has greatly enriched our understanding of the global physiological processes that occur at the protein level during cellular signaling events[66]. Bottom-up or shotgun proteomics is a high-throughput strategy capable of characterizing very large numbers of proteins simultaneously. Using LC, hundreds of proteins or peptides can be efficiently separated chromatographically into much simpler protein mixtures if not individual species, prior to identification by MS. By pairing distinct prefractionation technologies with complementary MS capabilities, the researcher can customize the analytical resources to meet their specific experimental needs. For example, Orbitrap mass analyzers are frequently coupled to LC to take full advantage of the MS capabilities. Other common configurations include the quadrupole-TOF and linear ion trap quadrupole-Orbitrap to obtain mass determinations with high accuracy and resolution[67,68].

^{18}O-^{16}O LABELING: VERIFICATION

To increase the odds of success an independent, alternative strategy for biomarker development can be used. For this purpose, enriched or fractionated sera is subjected to differential $^{18}O/^{16}O$ stable isotope labeling, a quantitative MS-based proteomics technique that separates individual peptides on the basis of a 4 Da m/z change. The ratio of ^{16}O labeled (pre-cancerous) and ^{18}O labeled (cancerous) tryptic digestion products can be analyzed by nano LC-MS/MS to determine quantitative changes in peptide abundance between the samples. $^{18}O/^{16}O$ labeling can be also used in preliminary experiments of selective reaction monitoring to verify the proteins discovered by 2D-DIGE, and to identify optimal precursor and transition product ions for relative quantitation before doing more expensive absolute quantitation using AQUA peptides. Since the spots in 2D-DIGE gels have more than one protein, this approach also increases the confidence in identification of a protein with an altered expression profile.

Investigators planning to use $^{18}O/^{16}O$ labeling technique need to be aware that incorporation of ^{18}O atoms into peptides can vary when using trypsin as a catalyst[69]. This issue can be ameliorated under conditions of extensive trypsinization. Also, until recently the availability of suitable computational tools was lacking, but this deficit has been addressed with the development of computational algorithms designed to evaluate $^{18}O/^{16}O$ labeling spectra[70,71]. It is noteworthy that ^{18}O labeling is far less expensive than the stable labeling techniques, rendering it especially attractive for use in biomarker discovery where large numbers of samples are generally analyzed concurrently[71].

In general, the development and validation of biomarkers for clinical use includes four phases: discovery, quantification, verification, and clinical validation[72,73]. Discovery-phase platforms have to date generated large numbers of candidate cancer biomarkers. However, a comparable system for subsequent quantitative assessment and verification of the myriad candidates is lacking, and constitutes the rate-limiting step in the biomarker pipeline[74]. Clearly, discovery "-omics" experiments only serve to identify candidate biomarkers, which must survive rigorous verification and validation before their clinical utility becomes evident[72,75]. At present, established immunoassay platforms, in particular ELISA, are the paragon for quantitative analysis of protein analytes in sera. However, a reliance on immunological methods to verify candidate biomarkers is impractical given the time, effort and cost required to generate the necessary reagents while their value remains uncertain. These constraints restrict the development of immunoassays to the short list of already highly credentialed candidates. Instead, the large majority of new, unproven candidate biomarkers are best examined

using intermediate verification technologies with shorter assay development times, lower assay costs, suitable to multiplexing 10-100 s of candidates, small sample requirements, and a high-throughput capability for analyzing hundreds to thousands of serum or plasma samples with high precision[76,77]. The objective is to winnow down the initial list of candidate biomarkers to a more manageable number worth advancing to traditional candidate-validation.

SELECTIVE REACTION MONITORING: VALIDATION

Targeted or quantitative proteomics has emerged as a new technical approach in proteomics and is an essential step in biomarker development. Among the several types of quantification methods, selected reaction monitoring (SRM) and multiple reaction monitoring (MRM) which enable MS-based absolute quantification (also termed AQUA) are emerging as potential rivals to immunoassays[78]. Where once biomarker discovery workflows were bottlenecked at the verification step, steady improvements over the years have resolved the issues such that MS-based techniques now represent viable strategies for biomarker verification[79,80]. SRM is a powerful tandem mass spectrometry method that can be used to monitor target peptides within a complex protein mixture. The specificity surpasses that of traditional immunoassays that may not identify or distinguish between post-translationally modified peptides. The sensitivity and specificity of the SRM assay to identify and quantify a unique peptide in a complex mixture by triple quadrupole mass spectrometry, lends itself to biomarker discovery and validation. This is especially poignant where affinity-based quantitative assays for proteins are unavailable and generating one is hampered by homology between isotypes. However, even isotypic variants of proteins with high homology can be quantified using SRM[81]. Stable-isotope-dilution MRM mass spectrometry (SID-MRM-MS) is a relatively new technique that enables quantification of a protein using isotopically heavy amino acid labeled peptide internal standards that correspond to the protein of interest. By including three to five diagnostic peptides representing a particular protein, the concentration of a protein of interest can be accurately determined based on the known amount of an internal standard added to the sample. The technique is amenable to multiplexing for the simultaneous quantification of 50 or more proteins, and requires only very small samples. SID-MRM-MS is a high-throughput method and has emerged as a valuable technique for validating multiple potential biomarkers[82]. MRM-MS of peptides using stable isotope-labeled internal standards is increasingly being adopted in the development of quantitative assays for proteins in complex biological matrices. These resultant assays are precise (providing primary amino acid sequence information for the analyte), quantitative, are compatible with tandem MS/MS data, and can be developed very rapidly in comparison to immunoassays. Since hundreds of MRM assays can be incorporated into a single method, the multiplexed nature of the technique allows for parallel monitoring of many targets. This is highly attractive from both a scientific and economic perspective. Furthermore, SRM assay design can target predetermined regions within a protein sequence, which would complement methods designed to enrich targets prior to SRM analysis[83]. Even subtle changes in a protein can be readily measured using the SRM approach. Once a SRM or MRM assay is developed, its utility extends to numerous experimental situations where the target protein(s) are to be measured. This has motivated the development of public repositories containing configured MRM assays[84,85].

The availability of previous tandem mass spectrometry data provide reliable information as to which fragment ions will yield the greatest signal in an SRM assay using a triple quadrupole mass spectrometer. The concept of monitoring specific peptides from proteins of interest is well established. As the methods exhibit high specificity and sensitivity within a complex mixture, they can be performed in a fraction of the instrument time relative to discovery-based methods. In addition, the capability to multiplex measurements of numerous analytes in parallel highlights the value of this technology in hypothesis-driven proteomics[77,82,86]. Multiplexing also helps with minimizing the amount of sample needed, an important consideration when working with hard to acquire samples.

ASSAY DEVELOPMENT: BIOMARKER VERIFICATION AND VALIDATION

There are a number of criteria to consider when selecting the optimal transitions for MRM analysis[87,88]. Once the proteins of interest found in the discovery phase are selected, the following steps and considerations should be incorporated in the design of an absolute quantification assay using AQUA-peptides (Figure 2).

Peptide design

First, the peptide sequence for the synthesis of the internal standard is selected. If the absolute amount of a protein is to be quantified, theoretically, any peptide of the protein produced by a proteolytic digestion (*e.g.*, trypsin, chymotrypsin, Glu-C, Lys-C) can be selected. Ideally, two to five peptides are chosen per target protein[82,86,89]. Peptides that deliver strong MS signatures and uniquely identify the target protein - or a specific isoform thereof - have to be identified empirically. Such peptides are termed proteotypic peptides[90] to ensure accurate quantification and exclude artifacts that can originate from unknown modifications of the endogenous protein. In any case, it is essential that the chosen peptide/peptides should be unique to the protein of interest and have a high response in the MS system

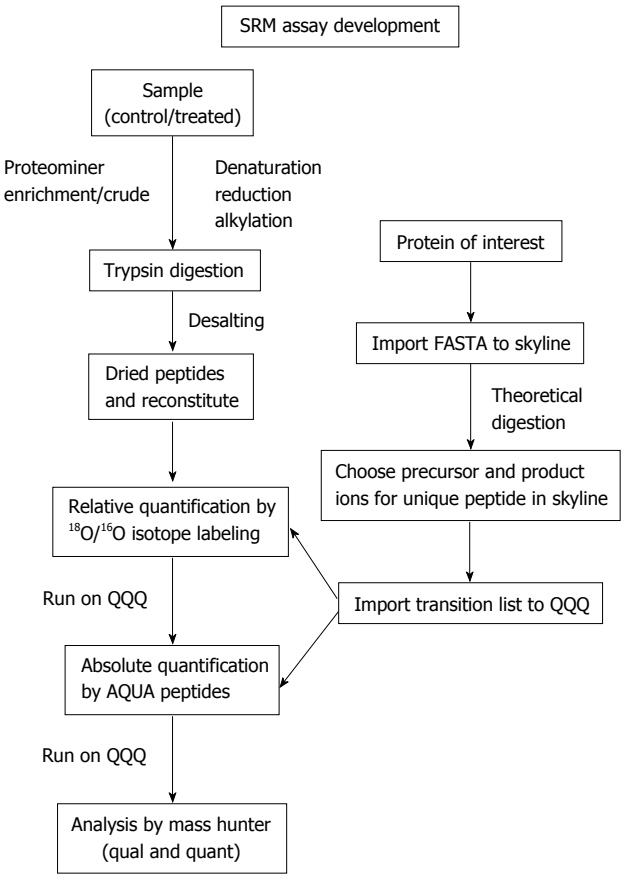

Figure 2 Targeted proteomics work flow for relative and absolute quantification. SRM: Selected reaction monitoring.

to afford the greatest sensitivity ("signature peptide")[91]. Ideally, peptides that have been previously sequenced in a shotgun experiment are chosen to ensure optimal chromatographic behavior, ionization, fragmentation and SRM transitions. Information on sequenced peptides can be found in published proteomics data sets and repositories such as Peptide Atlas[92] (http://www.peptideatlas.org/), Human Proteinpedia[93] (http://www.humanproteinpedia.org/) or PRIDE[94] (http://www.ebi.ac.uk/pride/). These databases can be queried by protein ID, accession number, or peptide sequence. If a peptide is in these databases, the search results return detailed information characterizing the peptide, previously recorded mass spectra of modified and unmodified versions, and information on the samples in which it was identified. To develop methods for targeted SRM analysis on a triple quadrupole mass spectrometer, a key resource exists in academic open source, software called Skyline (http://proteome.gs.washington.edu/software/skyline). The Skyline user interface facilitates the development of mass spectrometer methods and the analysis of data from targeted proteomics experiments performed using SRM. It enables access to MS/MS spectral libraries from a wide variety of sources, to choose SRM filters and verify results based on previously observed ion trap data[95]. The spectrum library can be used to classify fragment ions ranked by intensity, and enable the user to define how many product ions are required to provide a specific and selective measurement given the target sample. The transition lists can be easily exported to triple quadrupole instruments. The Skyline files are in a fast and compact format and are easily shared, even for experiments requiring many sample injections. For a peptide that has not yet been sequenced, an unlabeled peptide can be generated by peptide synthesis and inspected for the ability to function as a reliable AQUA peptide or bioinformatics prediction programs like Skyline can be used to choose potential peptides. In these cases, it is important to take into consideration that certain amino acid sequences or modifications can change the cleavage pattern of the selected protease. For instance, the protease trypsin normally cleaves at the carboxylic side of arginine and lysine. However, if proline is at the amino side of these residues, the bond is resistant to trypsin cleavage. Similarly, if the amino acid on the amino-terminal side is phosphorylated, trypsin cleavage may be inhibited. If the protein contains a series of arginines and lysines, trypsin might cleave after the first arginine or lysine, or after any one following those, creating "missed cleavages" or "ragged ends". So these theoretical software programs provide a starting point. After using Skyline in preliminary verification, using $^{18}O/^{16}O$ labeling to check the ratios (relative quantitation) and method development between particular samples is useful because AQUA peptide used for absolute quantitation are very expensive and it is better to be sure which peptides are best suited for absolute quantitation once the method is developed.

Peptide synthesis

AQUA peptides with a peptide sequence corresponding to that generated during digestion of the endogenous protein are synthesized. During peptide synthesis, amino acids containing stable isotopes (^{18}O, ^{13}C, ^{2}H or ^{15}N) are incorporated into the peptide, leading to a peptide with the same chemical and physical characteristics as the endogenous target, but with a defined mass difference. Most commonly, ^{13}C or ^{15}N are used as stable isotopes because they do not lead to chromatographic retention shifts seen in deuterated peptides. Usually, one heavy isotope-labeled leucine, proline, valine, phenylalanine or tyrosine is incorporated into an AQUA peptide leading to a mass shift of 6-8 Da. For tryptic peptides, the C-terminal arginine or lysine is often heavy isotope-labeled such that the resulting *y*-ion series can be used for monitoring. The peptide is purified, and the exact amount of peptide is determined by amino acid analysis or total nitrogen content. Many commercial vendors synthesize AQUA peptides (including incorporation of a stable isotope-labeled amino acid; *e.g.*, Sigma-Aldrich, Thermo Fisher Scientific or Cell Signaling Technologies).

Peptide validation

After synthesis, the AQUA peptide is analyzed by LC-

MS/MS to verify its chromatographic behavior and fragmentation spectra. If they correspond to the previously detected or predicted characteristics, the peptide is ready for use. Quadrupole-based instruments are much better suited to SRM methods by virtue of their ability to generate a continuous ion beam in the SRM transition. In general, trapping instruments are easier to set up and operate, whereas quadrupole-based instruments often require more expertise to optimize fully. However, well-designed targeted SRM methods on a triple quadrupole MS instrument are capable of significantly lower limits of quantification in mixtures of very high complexity, such as whole-cell lysate or unfractionated serum.

Method optimization

An MS spectrum of the peptide (or peptides) is first collected, either by infusion or by LC-MS or by theoretical digestion (using Skyline). Typically, the initial charge-state distribution is interrogated to establish the most sensitive charge state for further monitoring. Note that the actual charge state distribution in a complex mixture may be different than that observed from purified peptides. When an AQUA method is first deployed in a real biological matrix, it is advisable to test multiple charge states to ensure that the most sensitive form of the analyte is ultimately used. A SIM method with a narrow *m/z* scan range for the charge state with the highest intensity that covers both the AQUA peptide and the endogenous peptide is established from this MS spectrum. For SRM-based methods, the MS/MS spectra of the most intense precursor ions of the AQUA peptide are collected and inspected. Fragment ions at *m/z* ratios higher than the precursor ion are often more suitable for monitoring because of reduction of noise compared with the lower *m/z* space. Ion intensity and retention time can be optimized by varying the amount of organic solvent in the peptide loading buffer and in the column equilibration phase of an LC-MS method. In addition, software tools are now available to assist in developing scheduled SRM methods and interpreting their data.

Sample preparation

The biological samples are collected and digested with trypsin. The sample can be directly protease digested, or, to reduce complexity, the sample can be fractionated or enriched before digestion. Measurement of protein abundance by AQUA is indirect and based on the abundance of the resulting peptides; therefore, complete proteolysis is essential, and care should be taken to digest the target mixture with increasing amounts of protease and/or for longer time periods. Before digestion, a denaturation step is important for optimal trypsinization followed by reduction and alkylation. To remove any salts, desalting of sample is also very important.

MS analysis

The peptide mixture containing the endogenous and the AQUA peptide is analyzed on the mass spectrometer by a SIM or a SRM method, and the amount of endogenous peptide is determined. In contrast to a full MS scan, in a SIM experiment only a very narrow mass range is scanned, often by selectively injecting or trapping ions from the narrow scan range to increase the target ion signal-to-noise ratio. In an SRM experiment, a fragment ion or set of fragment ions is monitored. Typically, triplicate measurements for well-designed AQUA experiments produce coefficients of variation between 8%-15%. Using this workflow of proteomics technologies with other novel biostatistical tools, along with the inclusion of clinical factors such as age and gender shown to improve predictive performance[96], will increase the probability of early diagnosis.

These results can be analyzed using machine learning statistical approaches such as a Multivariate Adaptive Regression Splines (MARS) model, which has the ability to search through a large number of candidate predictor variables to determine those most relevant to the classification model. It is a nonparametric regression procedure that seeks to create a classification model based on piecewise linear regressions. MARS are able to reliably track the very complex data structures that are often present in multi-dimensional data[97-100]. In this way, MARS effectively reveals important data patterns and relationships that other models are typically unable to detect.

CONCLUDING REMARKS: THE FUTURE OF CLINICAL PROTEOMICS

Proteomics is a fast maturing discipline that brings the great promise for extending our understanding of the molecular basis of human diseases, and to identify novel biomarkers suitable for patient diagnosis, prognosis and treatment. Hopefully, this improved understanding will inform precision medicine and its application to patient care in the clinical setting. Several recent advances in proteomics have substantially simplified the analysis of serum proteins. Powerful workflows open up new possibilities for biomarker research that may lead to improved clinical assays and faster, more robust drug discovery and development. However there are still some considerations that must be addressed to meet the conditions that satisfy clinical applications. One key issue concerns sample collection, which varies from site to site, but this can be evaluated using the reference sample set from the National Cancer Institute's Early Detection Research Network, and will serve as a quality control during validation studies. Finally, the power of a panel of biomarkers is exemplified by AFP which requires huge sample numbers compared to statistical approaches. By contrast, quantifiable biomarker panel greatly reduces the requirements for large sample sets during validation[101].

Drugs are launched to market after the lengthy process of development. Despite careful preclinical

assessment to identify the most promising candidates, drug development is a lengthy and expensive process. The risk that a drug candidate is withdrawn from testing during clinical trials, especially in the latter stages, is a real concern that comes at considerable cost. There is an enormous impetus within the pharmaceutical industry to adopt new technologies that will hasten drug development and reduce the costs associated with bringing new drugs to market. Biomarkers are emerging as valuable tools in identifying potential drug failures at an early stage that can inform go/no-go decisions. Omics technologies serve an increasingly important role in biomarker discovery and the latter stages of drug development (*e.g.*, target discovery, mechanism of action or predicting toxicity). Recent advances in mass spectrometry including SRM and novel high-resolution capabilities have catalyzed the advent of proteomics and metabolomics as a central component in biomarker discovery, quantification, validation, and clinical verification.

Clinical proteomics is poised to bring important direct "bedside" applications. We foresee a future in which the physician rely upon targeted proteomic analyses during various aspects of disease management. The paradigm shift will directly affect medicine from the development of new therapeutics to clinical practice during the treatment of patients.

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REVIEW

Oxidative stress: New insights on the association of non-alcoholic fatty liver disease and atherosclerosis

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Abstract

Non-alcoholic fatty liver disease (NAFLD) represents the most common and emerging chronic liver disease worldwide. It includes a wide spectrum of liver diseases ranging from simple fatty liver to non-alcoholic steatohepatitis (NASH), which may progress to fibrosis and more severe liver complications such as cirrhosis, hepatocellular carcinoma and liver mortality. NAFLD is strongly associated with obesity, insulin resistance, hypertension, and dyslipidaemia, and is now regarded as the liver manifestation of the metabolic syndrome. The increased mortality of patients with NAFLD is primarily a result of cardiovascular disease and, to a lesser extent, to liver related diseases. Increased oxidative stress has been reported in both patients with NAFLD and patient with cardiovascular risk factors. Thus, oxidative stress represents a shared pathophysiological disorder between the two conditions. Several therapeutic strategies targeting oxidative stress reduction in patients with NAFLD have been proposed, with conflicting results. In particular, vitamin E supplementation has been suggested for the treatment of non-diabetic, non-cirrhotic adults with active NASH, although this recommendation is based only on the results of a single randomized controlled trial. Other antioxidant treatments suggested are resveratrol, silybin, L-carnitine and pentoxiphylline. No trial so far, has evaluated the cardiovascular effects of antioxidant treatment in patients with NAFLD. New, large-scale studies including as end-point also the assessment of the atherosclerosis markers are needed.

Key words: Cardiovascular disease; Oxidative stress; Non-alcoholic fatty liver disease; Atherosclerosis; Non-alcoholic steatohepatitis

Core tip: Non-alcoholic fatty liver disease (NAFLD) represents the most common chronic liver disease, including a wide spectrum of conditions ranging from simple fatty liver to non-alcoholic steatohepatitis, cirrhosis, hepatocellular carcinoma and liver mortality.

NAFLD is considered the liver manifestation of the metabolic syndrome. The increased mortality of patients with NAFLD is primarily a result of cardiovascular disease (CVD). Oxidative stress represents a shared pathophysiological disorder between NAFLD and CVD. Several antioxidant treatments have been proposed in patients with NAFLD, with conflicting results, but no trial has evaluated their cardiovascular effects in this setting. Further studies are needed.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD)[1] is a very frequent condition rising striking proportions in recent years, with prevalence of 20%-30%[1] in the general population, and 70%-90% in obese or diabetic patients. Non-alcoholic steatohepatitis (NASH) represents one of the most frequent conditions leading to liver transplantation and is projected to eventually become the first one in the next years[2].

NAFLD comprises different conditions, including simple steatosis and NASH. It is noteworthy that in some cases NAFLD patients may develop cirrhosis and hepatocellular carcinoma[3]. Moreover, patients with NAFLD show a higher risk for cardiovascular disease (CVD)[4], and cardiovascular mortality. Actually, patients with NAFLD have a greater probability to experience CVD rather than liver related complications.

NAFLD shows a strong association with many metabolic disorders such as insulin resistance, obesity, dyslipidaemia and hypertension and for this reason is considered the hepatic expression of the metabolic syndrome (MetS)[5]. MetS is a cluster of metabolic and CVD risk factors[6], characterized by low grade chronic inflammation and systemic oxidative stress. However, NAFLD and NASH have a complex pathogenesis and the fatty liver infiltration may be caused by several different mechanisms[3,7].

According to the "two-hit" theory[8], insulin resistance (IR) is believed to play an essential role in the early stages of steatosis. However, it is under discussion whether IR and hyperinsulinemia cause liver steatosis or NAFLD itself promotes hyperinsulinemia because of an inadequate degradation of insulin[5,9]. By contrast, oxidative stress seems to be one of the most important mechanisms leading to hepatic injury in NAFLD, playing a fundamental role in the progression from simple steatosis to NASH. It has been demonstrated that the augmented generation of reactive oxygen species (ROS) can induce lipid peroxidation leading to inflammation and fibrogenesis through the activation of stellate cells[10]. Moreover, ROS inhibit hepatocytes secretion of very low density lipoprotein (VLDL), inducing liver fat accumulation. ROS can also promote hepatic insulin resistance and necro-inflammation and activate several intracellular pathways that can lead to hepatocyte apoptosis[11]. At the same time, sound evidence has been generated that oxidative stress centrally contributes to atherothrombosis and is involved at all stages of atherosclerotic plaque evolution. Therefore, we speculate that increased oxidative stress may represent the missing link between NAFLD and CVD (Table 1).

NAFLD AND CVD

NAFLD and CVD morbidity and mortality

Recent published data showed an increased risk for CVD associated to NAFLD. Söderberg *et al*[12] reported in a 28-year follow-up study of subjects with an elevation of liver enzymes an higher risk of mortality in patients with NAFLD than in the general population. Moreover, in this study, the first cause of mortality was represented by CVD while liver disease was only the third one[13].

Two major studies, based on ultrasonography detection of steatosis, investigated the association between NAFLD and CVD. The first, carried out in a large North American database, reported an higher prevalence of CVD risk factors and events in patient with NAFLD (n = 2492) in comparison with those without. Nonetheless, the rate of CVD mortality during a follow up period of 14 years was not increased in subjects with NAFLD[14]. In the second, a Japanese 5-year prospective study of 1221 healthy subjects, patients with NAFLD (n = 231) showed an increased incidence of CVD events (1.0% *vs* 5.2%; $P < 0.001$) and NAFLD emerged as an independent predictor of CVD[15].

Few prospective studies used liver biopsy based diagnosis, and investigated the correlation between hepatic inflammation and atherosclerosis[16,17]. In a Swedish study, subjects with NAFLD at liver biopsy have been followed-up for about 14 years. Patients with steatohepatitis showed an higher mortality rate than those with simple steatosis[18]. In a further study performed in Japan, ultrasound screening for steatosis was performed in 625 subjects who underwent coronary angiography. Significant stenosis ($\geqslant$ 50%) was more prevalent in subjects with steatosis (84.6%), than in those without (64.1%). However, after 87 wk of follow-up there were no differences in the incidence of fatal CVD, non-fatal myocardial infarction, and coronary revascularization[19].

Cardiovascular risk stratification in patients with NAFLD

The above reported survival data suggest that a correct CV risk stratification is a fundamental step in the management of patients with NAFLD. This will include an accurate anamnesis, physical examination, laboratory and instrumental analyses (Figure 1).

Indeed, the recognition of the presence of early signs

Table 1 Oxidative stress, non-alcoholic fatty liver disease and atherosclerosis: Highlights and open issues

NAFLD is the most common and emerging chronic liver disease worldwide
NAFLD is considered the hepatic manifestation of the metabolic syndrome
Patients with NAFLD are at increased risk of cardiovascular morbidity and mortality and cardiovascular disease is the major cause of death
Chronic oxidative stress is considered one of the key mechanisms responsible for both liver damage progression in NAFLD and atherosclerotic disease
Therapeutic strategies targeting oxidative stress reduction in patients with NAFLD have been proposed, although based on only one RCT
No trial so far, has evaluated the cardiovascular effects of antioxidant treatment in patients with NAFLD

NAFLD: Non-alcoholic fatty liver disease; RCT: Randomized controlled trial.

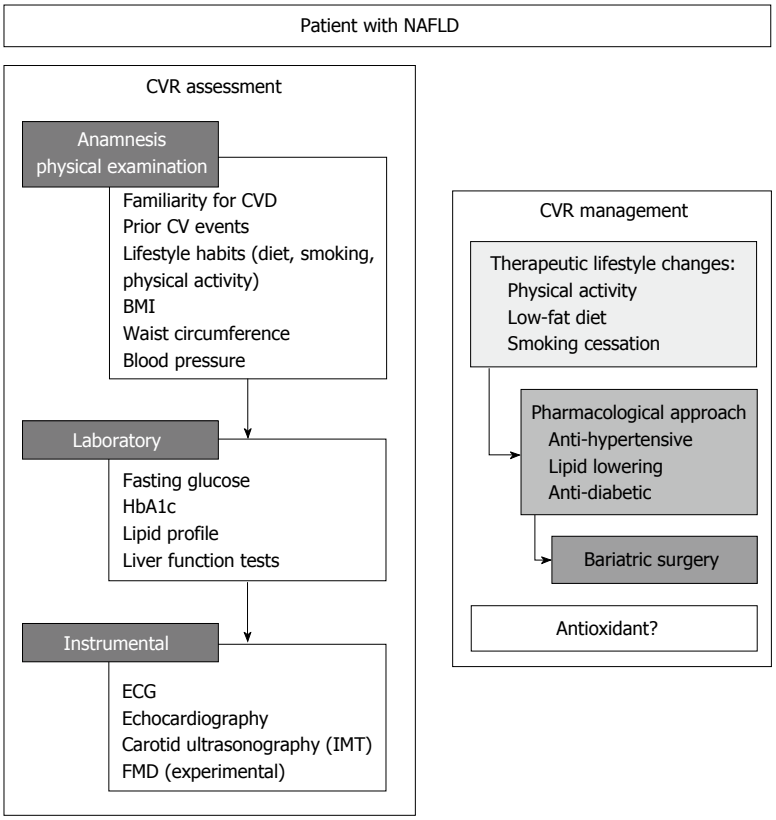

Figure 1 Algorithm for the assessment and management of cardiovascular risk in patients with non-alcoholic fatty liver disease. NAFLD: Non-alcoholic fatty liver disease; CVD: Cardiovascular disease; BMI: Body mass index; IMT: Intima-media thickness; FMD: Brachial artery flow-mediated dilation; CVR: Cardiovascular risk; ECG: Electrocardiogram.

of atherosclerosis is crucial for an effective prevention strategy. Two surrogate markers of atherosclerosis have been studied so far in patients with NAFLD: the carotid intima-media thickness (IMT) and the brachial artery flow-mediated dilation (FMD).

IMT: The use of ultrasound to assess the presence of carotid plaques, or to measure the common carotid IMT is a common screening tool to evaluate the presence of early systemic atherosclerosis. Several studies investigated IMT and carotid atherosclerosis in subjects with NAFLD[20]. In a meta-analysis, Sookoian *et al*[20] observed in 3497 subjects a significantly higher IMT (+13%) in subjects with steatosis (n = 1427), as compared with patients without fatty liver (n = 2070). Moreover, also in pediatric population, available data showed an association between an increased IMT and NAFLD[21]. However, there are conflicting data supporting an independent role of NAFLD for increased IMT[22]. In a small study, Mohammadi *et al*[23] found an independent correlation between NAFLD and IMT. Instead, in a German study, after adjustment for CVD risk factors, NAFLD did not independently predict increased IMT[24]. Similarly, in Kim's study, authors observed an increased IMT only in metabolic patients and speculated that NAFLD could be a marker of more severe MetS[25]. The correlation between NAFLD severity and IMT is unclear and the three major liver biopsy-based studies showed conflicting data. In a Greek study, NAFLD subjects had significantly higher cIMT (0.79 ± 0.18 mm *vs* 0.67 ± 0.13 mm, P = 0.01), compared to controls and there were no differences observed between NAFLD and NASH[26]. Conversely, Brea *et al*[27] and Targher *et al*[28] studies reported a close association between histology of NAFLD and IMT. Several studies investigated also carotid plaques prevalence in patients with NAFLD reporting conflicting data. In the Sookoian *et al*[20] systematic meta-analysis, the relative risk for

carotid plaques in patients with NAFLD is about twice as compared to control subjects.

FMD: Brachial artery FMD is a non-invasive test to evaluate endothelial dysfunction, a clinical marker of early CVD abnormalities[29]. So far, few studies evaluated the relationship between FMD and NAFLD[23,26,30]. Vlachopoulos *et al*[26] found a reduced FMD in patients with NAFLD (1.9% ± 2.1% *vs* 4.8% ± 2.4% in controls, $P < 0.001$); in Mohammadi's study FMD was 6.4% in patients and 15.7% in controls ($P < 0.001$); Thakur *et al*[30] reported a significantly greater degree of FMD impairment in NAFLD patients than controls (OR = 11.7; 95%CI: 1.4-96.5). Villanova's[31] study described a correlation between NAFLD severity and impaired FMD; in fact, lower FMD value were observed in patients with NAFLD/NASH. Finally, among 250 obese children, those with NAFLD and transaminase elevation had significantly impaired FMD[32]. Despite FMD has been shown to be able to predict CV events in some settings[33], it is not commonly used in clinical practice mostly due to its variability. Thus, its utility is now limited to experimental clinical trials.

OXIDATIVE STRESS, CVD AND NAFLD

As mentioned above, in subjects with NAFLD the main cause of death is represented by CVD complications[4]. However, It is still under debate if NAFLD is associated with CVD as a result of the coexistence of multiple CVD risk factors, or if NAFLD independently confers a higher CVD risk, acting as a pro-atherogenic stimulus[34-36]. In fact, most patients with NAFLD are obese or overweight, and many of them have arterial hypertension, diabetes and atherogenic dyslipidemia, thus outlining the clinical features of the MetS. Therefore, NAFLD is usually considered an hepatic expression of MetS[37,38].

NAFLD prevalence is significantly higher in obese patients than in individuals with normal magnetic resonance imaging (BMI) and without metabolic risk factors (80% and 16%, respectively)[39,40] and a significant correlation between NAFLD and BMI has been reported[41]. Many evidences suggest that the distribution of body fat plays a more important role in obesity-associated comorbidities than the total body fat mass. Visceral adipose tissue (VAT) compared with subcutaneous fat (SCF) is a better predictor of hepatic steatosis and is associated with histological severity in NAFLD independent of IR and hepatic steatosis[42]. VAT is more lipolitically active on a per unit weight basis and exhibits greater IR than SCF[43-45], causing enhanced peripheral lipolysis resulting in surplus-free fatty acid turn over into the liver[46]. Furthermore, increased production of pro-inflammatory adipokines and a converse decrease in protective adipokines is more evident in VAT compared with SCF[47]. Waist circumference and waist-to-hip ratio are anthropometric measurements of central adiposity, which correlate well with VAT[48]. On the basis of recent findings, central adiposity could be an independent predictor of hepatic steatosis, as it predicts increased levels of hepatic enzymes independently from BMI[49,50].

Among mechanisms linking CVD risk with hepatic steatosis, the most prominent factors seem to be insulin resistance, low-grade chronic inflammation and atherogenic dyslipidemia[34,51]. In addition, increased oxidative stress may also represent a shared pathophysiological condition between the two conditions (Figure 2). In fact, we have previously described increased oxidative stress in a number of chronic diseases, such as MetS, hypercholesterolemia, obesity, peripheral artery disease and obstructive sleep apnoea syndrome, all associated to increased CVD risk.

Oxidative stress plays a key role in the initiation and progression of both NAFLD and atherosclerosis. An excessive ROS production is responsible for the oxidation of LDL[52], which may promote the transformation of macrophages into foam cells, which represent the first step in the formation of the atherosclerotic lesion. On the other hand, in patients with NAFLD, ROS may also cause lipid peroxidation which may be followed by inflammation, and activation of stellate cells leading to fibrogenesis[8].

In fact, in many clinical studies, elevated systemic markers of oxidative stress and lipid peroxidation have been found in patients with NAFLD[53-59].

Recently, in two studies carried out in patients with NAFLD[60,61], we found increased oxidative stress *in vivo*, by measuring urinary 8-iso-prostaglandin F2α (8-iso-PGF2α), which derives from the non-enzymatic oxidation of arachidonic acid[62], and serum levels of soluble NOX2-derived peptide (sNOX2-dp), which is an indicator of NOX2 activation, a NADPH oxidase isoform involved in ROS generation[63,64]. *In vivo* measurement of 8-iso-PGF2α in urine is a validated and widely accepted reliable biomarker of oxidative stress in health and diseases[65]; a recent study from our group demonstrated that 8-iso-PGF2α production is partly a result of activation of NOX2[65]. Accordingly, we demonstrated that patients with genetically determined low oxidative stress, disclosed impaired formation of urinary 8-iso-PGF2α[63].

Increased values of urinary 8-iso-PGF2α and serum sNOX2-dp levels have been detected also in subjects with cardiovascular risk factors such as hypertension, diabetes, dyslipidemia, obesity, sleep apnoea syndrome, atrial fibrillation and MetS[66-71], which are all commonly present in patients with NAFLD.

Moreover, we also have demonstrated changes of systemic markers of oxidative stress after modulation of risk factors. For example, in patients with familial or polygenic hypercholesterolemia, statin treatment was associated with a parallel decrease of serum cholesterol and urinary 8-iso-PGF2α values[72,73]. Accordingly, in patients with MetS who had lost at least 5% of their initial weight, a decrease in serum NOX2-dp and urinary 8-iso-PGF2α levels was found, with concomitant increase in the levels of antioxidant molecules such as vitamin E (α-tocopherol) and adiponectin[74]. Finally,

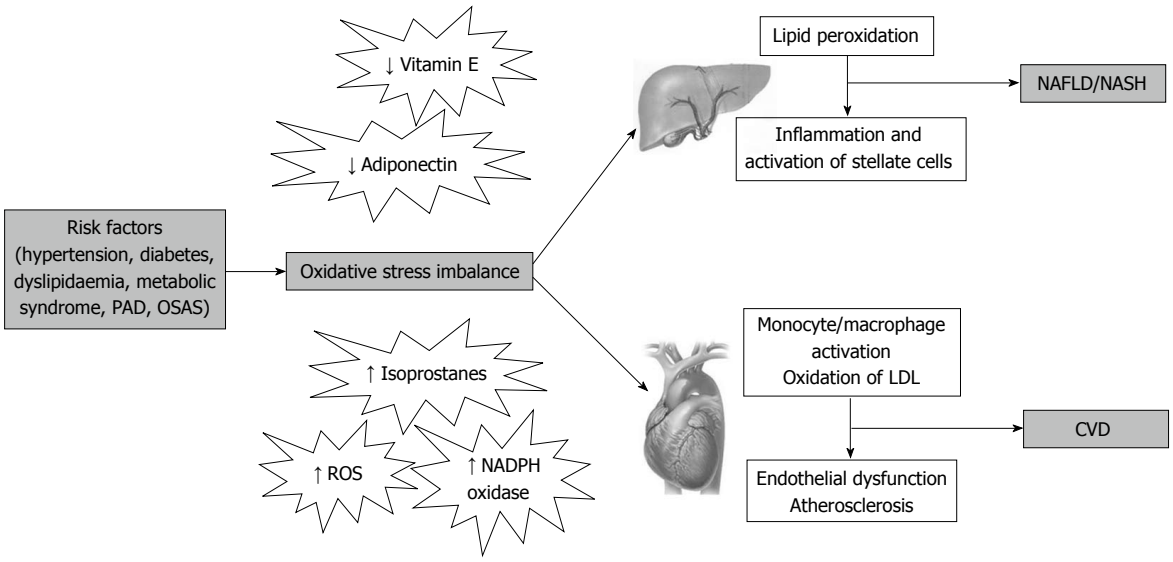

Figure 2 Possible pathophysiological mechanisms linking non-alcoholic fatty liver disease/non-alcoholic steatohepatitis to cardiovascular disease. PAD: Peripheral artery disease; OSAS: Obstructive sleep apnoea syndrome; NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; CVD: Cardiovascular disease; ROS: Reactive oxidant species; LDL: Low density lipoprotein.

in subjects with sleep breathing disorders, treatment with nasal continuous positive air pressure significantly decreased oxidative stress[75].

Oxidative stress may initiate the atherosclerotic process as it has a negative influence on endothelial cells[76-78]. Endothelial dysfunction predisposes patients to experience a CV event[79,80].

FMD is the most often non-invasive test used for assessing endothelial function, regulated by nitric oxide release[29]. Impaired FMD has been found in several CV and metabolic diseases related to chronic low-grade inflammation and oxidative stress[67,69,75,81-84]. Improvement of FMD with a coexistent decrease of NOX2 activation has been observed in patients with MetS after moderate weight loss[69]. Impaired endothelial function was suggested also in NAFLD subjects[31,85].

In conclusion, oxidative stress may increase CVD risk in patients with NAFLD, both by contributing to the pathogenesis of single CVD risk factors, or by inducing endothelial dysfunction. Therefore, oxidative stress may represent an attractive target for improving CVD prevention in these patients, as it may represent a common mechanism underlying multiple CVD risk factors[86]. In particular, targeting platelet NOX may represent a useful complementary anti-thrombotic approach for these patients[87]. Moreover, antioxidant therapy may also be useful to decrease lipid peroxidation and liver disease progression in NAFLD.

ANTI-OXIDANT THERAPY IN NAFLD

Based on the above evidences, several therapeutic strategies targeting oxidative stress reduction in patients with NAFLD have been proposed. Notably, none of these studies took into consideration cardiovascular implications. Moreover, a meta-analysis evaluating the benefits and harms of antioxidant supplements in this clinical subset has reported no enough data to express a conclusive and widely accepted opinion on antioxidant therapy use in NAFLD patients[88]. Note that, only a minority of the randomized controlled trials (RCTs) report pre-treatment and post-treatment histological data to support therapeutic efficacy of antioxidant therapy in biopsy-proven NAFLD or NASH.

Vitamin E in NAFLD

Vitamin E is a lipophilic molecule with antioxidant activity that prevents membrane damage by ROS. Low levels of antioxidants including vitamin E have been observed in subjects with NASH compared to healthy individuals[89]. We confirmed this finding, demonstrating reduced blood values of α-tocopherol/cholesterol in 254 patients with NAFLD compared with 56 patients without NAFLD. Notably, similarly reduced vitamin E/chol values were obtained in a subgroup of 20 patients with biopsy-proven NASH (unpublished data).

The effects of vitamin E have been investigated in several experimental murine models of NAFLD showing an improvement of NASH and a reduction in oxidative stress markers, hepatic stellate cell activation, and histologic fibrosis in mice supplemented with vitamin E[90-92].

The effects of vitamin E or vitamin E plus other drugs on the liver damage, in patients with biopsy-proven NASH, have been investigated in few small studies showing conflicting results[93-95].

Recently, two large multicenter RCTs investigated the efficacy of vitamin E in subjects with NAFLD. In the PIVENS trial, adult patients with aggressive NASH and without diabetes or cirrhosis, high-dose vitamin E supplementation (800 UI q.d.) significantly improved NASH histology compared to pioglitazone or placebo

treatment groups; however, increased insulin resistance and plasma triglyceride levels were reported[93]. By contrast, the TONIC trial found no differences between vitamin E (400 IU bid), metformin (500 mg bid), or placebo in inducing sustained decrease of ALT level in children with NAFLD, even if α-tocopherol showed a significant improvement of NASH[96].

Based only on the results of the above single positive RCT[93], recent guidelines suggest supplementation with high-dose vitamin E (800 UI q.d.) to treat NASH in patients without diabetes[97]. However, over the last few years, concerns about the safety of vitamin E supplementation have been raised. Indeed, a systematic review indicated that α-tocopherol might increase hemorrhagic stroke risk[98] and in another study it has been reported an increased prostate cancer incidence in healthy men taking vitamin E (400 IU q.d.) over 7 years[99]. Moreover, no benefits in-cardiovascular mortality or cerebrovascular events have been demonstrated for vitamin E supplementation[100]. Finally, recent publications reported no benefits by α-tocopherol supplementation for cardiovascular prevention and no evidence for an association with increased all-cause mortality[101,102].

Therefore, even though there are some evidences about the efficacy of vitamin E supplementation in NAFLD/NASH, there are concerns about its safety. Further studies taking in to consideration cardiovascular implications and long-term safety of vitamin E supplementations are needed.

A possible alternative could be the simultaneous supplementation using smaller doses of several small molecule antioxidants in association, such as vitamin A, vitamin E, vitamin C, glutathione, *etc*. A further possibility could be the simultaneous supplementation using a small molecule antioxidant with a trace element (zinc, copper, or selenium) that may increase expression of an enzymatic antioxidant. However, further studies are needed to support the potential roles of these therapeutic approaches.

Other anti-oxidant drugs

Several studies have been conducted investigating the effect of other antioxidant drugs in NASH, but with inconclusive results.

In recent years, many studies have found promising properties of Resveratrol (trans-3,4′,5-trihydroxystilbene) for NAFLD treatment. Resveratrol is a stilbene naturally occurring in several plants and extracted from red grapes. Resveratrol has a well-documented strong capacity against oxidation and inflammation, improves insulin sensitivity and glucose tolerance and reduces plasma lipids[103,104]. Some *in vitro* studies performed in different cell models of steatosis demonstrated that resveratrol shows anti-lipidogenic effects at doses between 10 and 50 μmol/L. The mechanism of action underlying this effect may be mediated by a decrease of *de novo* lipogenesis[105-108].

Many *in vivo* studies demonstrated positive effects on liver steatosis in animal models, showing efficacy both for fatty liver prevention and treatment[108,109]. The doses used in these studies have been generally very high, ranging from 0.5 to 450 mg/kg per day. Data deriving from these studies suggest that the reduction of oxidative stress also contributes to this positive effect.

So far, only one human study investigating the effects of resveratrol supplementation on the liver has been conducted. In this small-randomized double-blind crossover design study, 11 healthy obese male volunteers received 150 mg resveratrol per day or placebo for 1 mo. Plasma alanine aminotransferases (ALT) concentration and intrahepatic lipid content were significantly lower after 30 d of resveratrol supplementation in comparison to placebo[110].

Based on its potent effects on oxidative stress and inflammation, as well as its wide availability, resveratrol has become one of the most interesting candidate for the prevention of fatty liver diseases. Interestingly, a role of resveratrol in CVD protection has been demonstrated in primary and secondary prevention with an improvement of CVD risk markers, such as endothelial function, echocardiographic parameters and cytokines expression in different settings[111]. Nevertheless, there is a lack of long-term randomized clinical trials using resveratrol and improvement of markers of cardiovascular risk does not necessarily coincide with clinical benefits in patients management.

Recently, there has been a renewed interest for silybin, a natural product deriving from - Silybum marianum. Even if its therapeutic efficacy has been questioned for years, silybin is commonly used as hepato-protective agent. Silybin therapeutic efficacy has been demonstrated in several studies performed in different types of experimental liver injury[112-114]. Notably, in a recent study, silybin administration decreased oxidative stress and improved both liver and myocardial injury in an experimental murine model of NAFLD[115]. Moreover, some studies showed a positive effect of silybin on fibrosis and oxidation[116,117]. In a recent large multicenter RCT, a combination of silybin, vitamin E and phosphatidylcholine reduced hepatic damage and IR[118].

L-carnitine, an endogenous substance precursor of carnitine-palmitoyltransferase 1, is involved in the mitochondrial β-oxidation that affects mitochondrial function. L-carnitine has potent antioxidant properties, being a free radical scavenger, and thus may protect tissues from oxidative damage[119,120].

In a recent study, l-carnitine supplementation reduced tumor necrosis factor (TNF)-α, liver function parameters, plasma glucose levels and histological scores in NASH[121]. However, even if a majority of studies have shown that l-carnitine supplementation can improve factors associated with MetS and CVD[122-125], recent evidences suggested that dietary l-carnitine may accelerate atherosclerosis *via* gut microbiota metabolites[126]. Thus, further research is necessary to investigate the effect of chronic l-carnitine supplementation on both atherosclerosis and chronic fatty liver disease.

Pentoxiphylline (PTX) is a methylxanthine derivative with anti-oxidant and anti-inflammatory properties. In fact, pentoxiphylline suppresses *TNF-α* gene transcription and acts as a hydroxyl and peroxyl radical scavenger[127]. Moreover, it has been proven that it increases red blood cell flexibility, reduces blood viscosity and decreases platelet aggregation. PTX it is commonly used for the treatment of intermittent claudication in Western countries and several small clinical trials have reported beneficial effects of PTX supplementation on multiple surrogate clinical markers in subjects with chronic heart failure[128-131].

Indeed, recent evidences showed that PTX can decrease free-radical-mediated lipid oxidation and can improve histological features of NASH[132,133], such as steatosis, lobular inflammation and fibrosis. Thus, PTX may represent a new strategy for treating NAFLD. However, more studies are suggested to confirm its efficacy and the cardiovascular effects in this setting.

CONCLUSION

Today, a large body of clinical and epidemiological data support an association between NAFLD and increased CVD risk, which seems independent of traditional risk factors and the features of the MetS[134]. Indeed, NAFLD mortality is mainly due to CVD rather than liver related diseases. Chronic oxidative stress is considered a major pathogenic factor for hepatic damage in NAFLD/NASH and for atherosclerosis evolution. The increase of ROS production and the decrease of antioxidant factors produce oxidative stress.

In our recent studies, we found increased markers of oxidative stress in subjects with liver–steatosis. In fact, patients with NAFLD had an higher concentration 8-iso-PGF2α in urines, widely considered as a valid test to evaluate oxidative stress *in vivo* and of serum sNOX2-dp which plays a major role in the production of ROS. Together, we found that both simple steatosis and NASH are related with decreased serum levels of vitamin E/chol when compared to controls, suggesting that oxidative stress imbalance could also occur in initial stages of fatty liver disease. Taken together, these results suggest that patients with NAFLD may have a chronic systemic oxidative stress, which may lead to a reduction in natural antioxidant pool including vitamin E.

Moreover, oxidative stress is implicated in cardiovascular diseases and in all stages of atherosclerotic plaque evolution. Increased ROS play a role in endothelial dysfunction and in high-CVD risk diseases, such as MetS, hypercholesterolemia, overweight/obesity, peripheral artery disease, sleep apnoea syndrome. Therefore, increased oxidative stress may well represent a possible link between NAFLD and CVD, and constitute an attractive target for antioxidant therapy. In fact, antioxidant therapy may have a favourable effect both on cardiovascular risk factors and liver histology.

Currently, vitamin E supplementation is recommended to treat non-diabetic, non-cirrhotic adults with active NASH, although this indication is supported only by the findings of a single RCT[93] and a meta-analysis has reported insufficient evidence supporting or refusing a favourable role of antioxidants for the treatment of NAFLD[88]. Moreover, no trial so far, has assessed the cardiovascular benefits of antioxidant treatment in individuals with fatty liver. New, large-scale RCTs including as end-point also the assessment of the atherosclerosis markers are needed.

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REVIEW

Liver steatosis in hepatitis C patients

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Abstract

There is controversy regarding some aspects of hepatitis C virus (HCV) infection-associated liver steatosis, and their relationship with body fat stores. It has classically been found that HCV, especially genotype 3, exerts direct metabolic effects which lead to liver steatosis. This supports the existence of a so called viral steatosis and a metabolic steatosis, which would affect HCV patients who are also obese or diabetics. In fact, several genotypes exert metabolic effects which overlap with some of those observed in the metabolic syndrome. In this review we will analyse the pathogenic pathways involved in the development of steatosis in HCV patients. Several cytokines and adipokines also become activated and are involved in "pure" steatosic effects, in addition to inflammation. They are probably responsible for the evolution of simple steatosis to steatohepatitis, making it difficult to explain why such alterations only affect a proportion of steatosic patients.

Key words: Hepatitis C virus steatosis adiponectin; Leptin; Insulin resistance; Proinflammatory cytokines; Triglyceride synthesis; Fatty acid oxidation

Core tip: Chronic hepatitis C virus (HCV) infection can lead to steatosis and steatohepatitis. Increased liver triglyceride synthesis is mediated by several transcription factors such as sterol regulatory element-binding protein (SREBP) whose expression is enhanced, in turn, by HCV core protein. Chronic HCV infection is also associated with insulin resistance that seems to be selective because although it activates systemic lipolysis, it increases triglyceride synthesis within the liver. This is due to the stimulatory effect of insulin on SREBP. It remains to be answered why not all patients with HCV infection and steatosis develop steatohepatitis despite early cytokine activation and metabolic derangements.

González-Reimers E, Quintero-Platt G, Rodríguez-Gaspar M, Alemán-Valls R, Pérez-Hernández O, Santolaria-Fernández F. Liver steatosis in hepatitis C patients. *World J Hepatol* 2015; 7(10): 1337-1346 Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i10/1337.htm DOI: http://dx.doi.org/10.4254/wjh.v7.i10.1337

INTRODUCTION

Non-alcoholic steatohepatitis is an increasingly common situation, in which fat-laden hepatocytes trigger an inflammatory response which may evolve to liver cirrhosis[1] and hepatocarcinoma[2]. Diabetes and obesity are the most important causes, since insulin deficiency and/or resistance alter the mobilization of fatty acids from adipose tissue to liver, the oxidative pathways, and lipid trafficking between liver and peripheral tissues. Steatosis and steatohepatitis are also observed in chronic hepatitis C virus (HCV) infection. Although HCV by itself-especially genotype 3a- may lead to liver steatosis, obesity and concomitant alcohol abuse are main factors involved[3]. The "two hit theory" sustains that cytokine activation and increased lipid peroxidation contribute to the evolution of liver steatosis to more advanced stages of steatohepatitis[4]. Throughout this manuscript we will show that cytokine activation may already exist, at least theoretically, in early stages of the disease (simple steatosis), but not all the patients showing simple steatosis develop steatohepatitis.

The outstanding role played by some genotype specific HCV viral proteins, which either have a direct steatogenic effect or induce insulin resistance, explains why some HCV infected individuals show liver steatosis in the absence of obesity and has led to the concept that there are two main pathogenetic mechanisms of steatosis in these patients: the so-called "metabolic" steatosis and "viral" steatosis. In the present paper we will revise the main pathways leading to steatosis in these HCV patients. However, viral and non-viral dependent pathways are intermingled, so we will not treat them separately. Also, although the objective of this review is only to revise mechanisms leading to steatosis, and not steatohepatitis, many pathways involved in simple steatosis are already able to trigger inflammation, which is the hallmark of steatohepatitis, so a precise limit between both clinicopathological stages is lacking. We will comment only those aspects of proinflammatory cytokines involved in the pathogenesis of "pure" steatosis.

As mentioned above, patients infected by genotype 3a HCV develop liver steatosis even in the absence of obesity[5], a finding which supports a direct cytopathic and steatogenic effect of this precise genotype[6]. Recent research has shown that this viral effect depends on several mechanisms which will be commented in this review. HCV genotype 3a up-regulates the expression of fatty acid synthase[7]. There are also data which suggest that in chronic hepatitis secondary to HCV there is decreased mitochondrial β-oxidation, possibly due to mitochondrial damage[8]. In addition, HCV impairs export of very low density lipoprotein (VLDL) particles from the liver to peripheral tissues, by several mechanisms. Hepatocyte release of HCV particles utilises the same pathway used in VLDL export, and HCV mediates inhibition of the microsomal triglyceride transfer protein (MTP), a molecule involved in export of intrahepatocytary triglycerides[9].

In addition, several viral proteins of diverse genotypes interfere with insulin signalling, leading to insulin resistance. Insulin resistance is the hallmark of obesity, but in HCV infection, patients do not necessarily have to be overweight for them to develop insulin resistance: despite the principal role of obesity and associated insulin resistance on liver steatosis, this lesion may develop in the face of a normal body mass index (BMI). Therefore, at any given load of fatty acids, HCV infected hepatocytes up-regulate synthesis of more fatty acids, impair β-oxidation of the available fatty acids, and impede the export of triglycerides (Figure 1).

Increased triglyceride synthesis

Fat mobilization is a necessary condition to develop liver steatosis, and liver steatosis is more intense the greater the BMI[10], also in HCV patients (Figure 2). During fasting-a situation characterized by low insulin levels-, fatty acids are released by the adipocyte and reach the liver, where they are taken up by liver cells and are destined to be used either as fuel, as a source of ketone bodies or they can be combined again with glycerol to be re-esterified as triglycerides. Triglycerides coupled with apoproteins and cholesterol form the so called VLDL which are then exported to peripheral tissues.

A situation with some features similar to those observed during fasting may take place in conditions accompanied by insulin resistance: in adipose tissue insulin fails to suppress lipolysis, so that an increased amount of free fatty acids reaches the liver. But in fasting, insulin levels are low, whereas in situations of insulin resistance, insulin levels are usually high. High insulin levels, even in a situation of insulin resistance and not-supressed lipolysis, still enhance liver triglyceride synthesis, but not adipocyte synthesis of triglyceride. Liver triglyceride synthesis implies esterification of glycerol with fatty acids. These fatty acids may derive from adipose tissue, from ingested fat, and also from ingested carbohydrates, the latter constituting the amount synthesized "*de novo*" by the liver. In insulin-resistant patients with non-alcoholic fatty liver disease the rate of *de novo* lipid synthesis is increased. Donnelly *et al*[11] showed that 26% of the triglycerides stored in the liver of 9 obese subjects with non-alcoholic fatty liver disease derived from *de novo* lipogenesis, in contrast with the 5% contribution (in the fasted state) observed among normal individuals[12]. This increased *de novo* liver lipogenesis in insulin-resistance situations is accompanied by a reduced triglyceride synthesis within the adipocyte, due to decreased availability of glycerol 3 phosphate, which is in turn due to an insulin-resistance-mediated decrease in glucose uptake[13].

Several transcription factors are involved in increased liver lipid synthesis[14]. These are:

(1) Sterol regulatory element-binding proteins (SREBP), especially the SREBP-1c. SREBP-1c enhances transcription of genes required for fatty acid synthesis and predominates in the liver[15]. When cells become

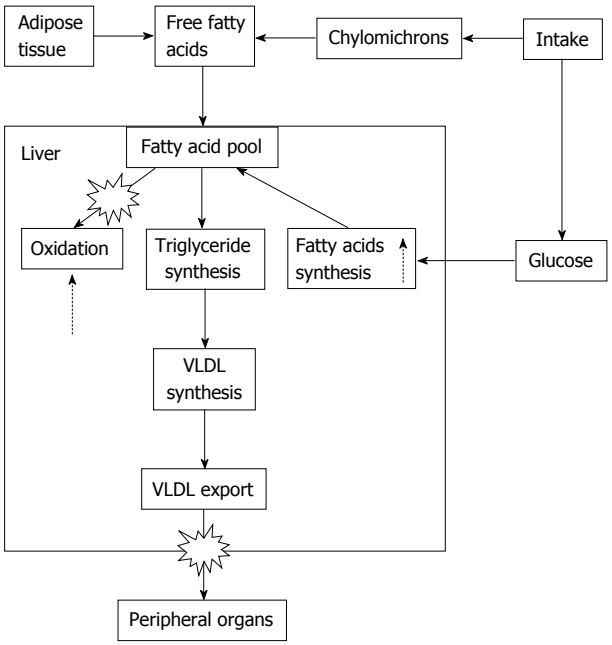

Figure 1 Simplified schematic pathway of lipid metabolism. The symbol corresponds to the points at which hepatitis C virus (HCV) blocks lipid metabolism, and the dotted arrows the point at which HCV enhances it. VLDL: Very low density lipoprotein.

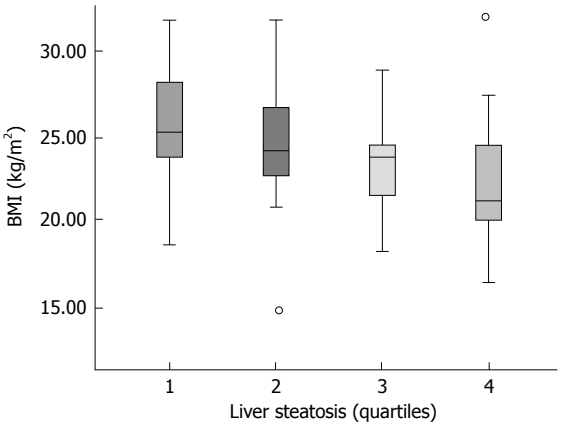

Figure 2 Preliminary data on 83 patients with hepatitis C virus infection. Liver steatosis was histomorphometrically assessed. In this figure, it is shown that the amount of liver steatosis (in quartiles) is related to body mass index.

depleted in cholesterol, a protein called SREBP cleavage-activating protein (SCAP) binds to SREBP and transports it from the endoplasmic reticulum to the Golgi apparatus. In the Golgi apparatus there are two proteases (site 1 protease or S1P, and site 2 protease or S2P) which act sequentially to release the N-terminal active form of SREBP, which enters the nucleus and binds to a sterol responsive element in the enhancer/promoter region of the target genes (for instance, fatty acid synthase), activating transcription. The movement of the SREBP-SCAP complex from the endoplasmic reticulum to the Golgi apparatus is supressed by high intracellular cholesterol levels; therefore, the SRBEP-SCAP system can be viewed as a sensor of

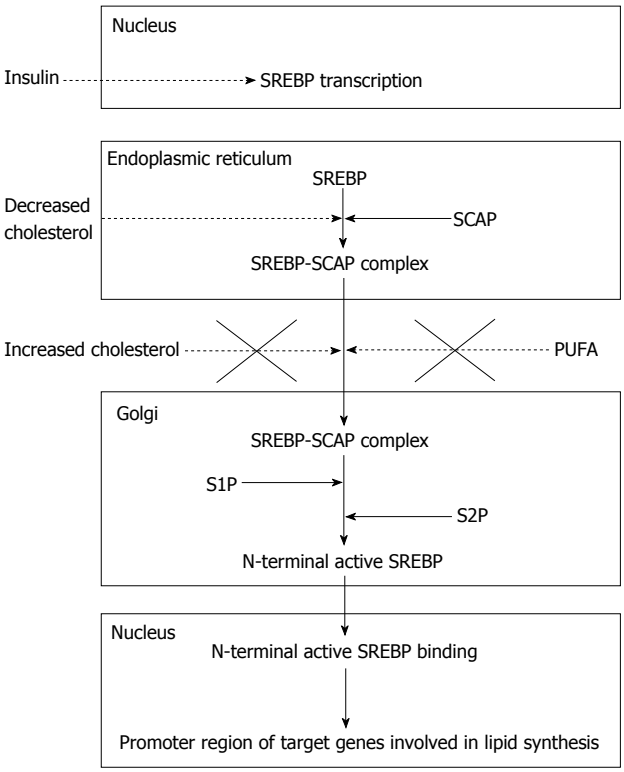

Figure 3 Simplified representation of the effect of steroid response element binding protein on lipid synthesis. Insulin promotes transcription of steroid response element binding protein (SREBP). In the endoplasmic reticulum, SREBP binds to SREBP cleavage-activating protein, a complex which moves to the Golgi apparatus, where the active N-terminal form of SREBP is formed by the sequential action of two proteases (S1P and S2P), and is finally translocated to the nucleus. SCAP: SREBP cleavage-activating protein; PUFA: Polyunsaturated fatty acid.

cholesterol levels in the hepatocyte[16], although this inhibitory action affects SREBP-2. Inhibition of SREBP-1 processing requires the presence of polyunsaturated fatty acids in addition to cholesterol[17] (Figure 3).

SREBP-2 is also present in liver and other organs, and is more specifically involved in cholesterol synthesis. However, when expressed at higher than normal levels, each of the three SREBP isoforms can activate both cholesterol and fatty acid synthesis[18].

SREBP-1a is expressed only at low levels in liver, but in studies performed on genetically engineered mice, overexpression of SRBEP 1a led to a 26-fold increase in fatty liver synthesis and a massive liver steatosis[19].

SREBP transcription is strongly stimulated by insulin[20], whereas glucagon exerts an inhibitory effect. Over-expression of SREBP-1c may lead to an excessive synthesis of fatty acids (and cholesterol and triglycerides) within the liver cell, ultimately leading to liver steatosis[14,21]. Liver X-activated receptors (LXR) α and β are involved in SREBP-1c transcription. These are nuclear receptors that heterodimerize with retinoid X receptors after binding to a ligand. In the case of SREBP, they bind to a LXR response element in the promoter region of the *SREBP-1* gene and activate SRBP-1c transcription[22]. Thus, glucagon, insulin, and

LXR are classical modulators of SREBP-1c transcription.

The activity of SREBP-1c is increased in several situations in which liver steatosis ensues, such as alcoholism (acetaldehyde enhances its transcription[23]), high tumor necrosis factor alpha (TNF-α) levels[24], or HCV infection[25]. HCV core protein enhances both gene expression of SRBP and transcriptional activity of this molecule[26].

On the other hand, SRBP-1 is inactivated by sirtuin-1 (SIRT-1), a molecule whose activity is modulated by several variables, including ethanol and HCV core protein, among others[27]. Sirtuins are involved in the modulation of transcription factor activity by deacetylation of proteins. Biological activity of sirtuins depends on nicotinamide adenine dinucleotide (NAD) availability[28]. Specifically, sirtuin1 deacetylates and inhibitis SREBP-1c activity, therefore decreasing fat synthesis. Ethanol inhibitis sirtuin-1 activity, therefore increasing the lipogenic effect of SREBP 1-c[26]. HCV exerts a similar effect[29].

Sirtuin activity is coupled to that of AMP-activated kinase (AMPK), an enzyme that phosphorylates and, thus, inhibits, acetyl-CoA carboxylase, interrupting the formation of malonyl-CoA, a key step for fatty acids synthesis, and preserving cellular content in NAD[30]. Sirtuin activates AMPK acting on serine/threonine kinase 11, also known as liver kinase B1, a process which leads to an increase in cellular NAD availability, which favours SIRT-1 activity[31]. This reciprocally regulated circuit leads to inhibition of SREBP activity and fatty acids synthesis. It is important to keep in mind that ethanol metabolism consumes NAD, theoretically opposing to sirtuin activation.

Endocannabinoids are also involved in enhanced expression of SREBP-1c[32]. Cannabinoid agonists are orexigenic, and animal models support a role of endocannabinoids on diet-induced liver steatosis[33]. Daily cannabis consumption aggravates steatosis in HCV patients[34]. Conversely, HCV infection may up-regulate cannabinoid receptor 1 expression[35].

(2) However, although cholesterol synthesis seems to depend almost entirely on SREBP activity, suppression of the SREBPs machinery reduces fatty acid synthesis by only 30%[36]. Another transcription factor involved in liver steatosis is carbohydrate response element binding protein (ChREBP), whose activity is induced by a high carbohydrate diet, insulin[37] and ethanol[38]. It increases the expression of both lipogenic enzymes (such as fatty acid synthase) and glycolytic ones[39]. The effect of HCV on ChREBP is not known, to our knowledge.

(3) Peroxisome proliferator-activated receptor (PPAR)-γ is another master transcription factor involved in fat metabolism. Increased activity of PPAR-γ is associated with an increase in lipid synthesis[40] and is seen in patients with liver steatosis. It upregulates genes involved in lipid synthesis, increasing the activity of mediators such as SREBP-1, fatty acid synthase and acetyl coenzyme a carboxylase, all of them leading to increased hepatocyte lipid content.

Interestingly, PPAR-γ is related to increased expression of genes that regulate the synthesis of adipose differentiation related protein[41], which functions to coat lipid droplets within liver cells[42]. It has been shown that HCV core protein increases the transcriptional activity of PPARgamma, although it exerted no effect on PPARgamma gene expression[43].

Synthesis of triglycerides is a complex process, in which several enzymes participate. During fasting, the increased flux of fatty acids to the liver increases the translocation of lipin-1, a protein with dual activity on fatty acid metabolism[44]. Lipin proteins translocate from the cytosol to the endoplasmic reticulum where they show phosphatidate phosphatase (PAP 1) activity. This enzymatic activity transforms diacylglycerol 3 phosphate into diacylglycerol (DAG), which serves as substrate for triglyceride and phopholipid synthesis. Fatty acids are added to the DAG molecule through the action of acyl coenzyme A:diacylglycerol acyltransferase to form triglycerides[45].

In addition to its PAP 1 activity, lipin also translocates to the nucleus, where it enhances expression of genes involved in fatty acid oxidation[46]. This requires interaction with PPAR-α and PPAR-γ coactivator 1α, forming a physical complex. This leads to decreased intracellular levels of fatty acids which defends the cell from the damaging effect of these molecules[47,48].

Consistent with its effect on free fatty acids, insulin stimulates the activity of lipin-1 by unknown mechanisms, and obesity-related insulin resistance down-regulates lipin gene expression[49]. PAP 1 activity is enhanced in ethanol-induced liver steatosis[50], but lipin deficiency may exacerbate ethanol-associated liver steatosis-perhaps by impairment of fatty acid oxidation[51]. Concordantly, ethanol up-regulates lipin-1 gene expression[52]. Liver lipin is also regulated by SIRT-1[53], a molecule whose activity is inhibited by ethanol. However, to our knowledge, the effect of HCV on lipin proteins has not been analysed.

Inhibition of fatty acid oxidation

Fatty acid oxidation takes place mainly in the mitochondria, although in a small proportion it also includes microsomal ω- and peroxisomal β-oxidation. Improper fatty acid oxidation may also contribute to liver steatosis. As mentioned earlier, AMPK stimulates hepatic fatty acid oxidation and ketogenesis, since it lowers malonyl-CoA liver content, thereby permitting fatty acid transport to the mitochondria, where they suffer oxidation[30]. Ethanol exerts an inhibitory effect on AMPK[54] and HCV also downregulates AMPK[29]. In a study performed on 30 patients infected with HCV it was found that mitochondrial β-oxidation of fatty acids was impaired and that this impairment was related to serum levels of HCV core protein[8]. Therefore, both in alcoholic and non alcoholic fatty liver disease impaired fatty acid oxidation plays an crucial role, without the need of accompanying mechanisms. However, insulin resistance and proinflammatory cytokines also exert major effects

on this mechanism.

Insulin resistance

Normally, insulin activates acetyl CoA-carboxylase, leading to the formation of malonyl-CoA, which inhibits mitochondrial fatty acid oxidation; it also strongly inhibits gluconeogenesis by blocking key enzymes such as phosphoenolpyruvate carboxykinase and glucose 6 phosphatase. Additionally, it inhibits lipolysis and promotes glycogen synthesis and *de novo* fat synthesis using carbohydrates as substrate. Finally, it favours SREBP and ChREBP transcription, as was mentioned above. Therefore, it exerts lipogenic effects on the liver cell[55]. Insulin action takes place after binding to a specific receptor, which, upon activation, leads to the phosphorylation of a series of inactive kinases called insulin responsive substrates (IRS), transforming them into active ones. Some final effects of this complex cascade of kinases include phosphorylation of transcription factors, such as forkhead box protein (FOX)O1 and FOXA2, among others[56]. Phosphorylated FOXO1 is unable to activate transcription of key enzymes involved in gluconeogenesis, such as phosphenolpyruvate carboxy-kinase or glucose 6 phosphatase, and thus, liver production of glucose is blocked[57]. Another transcription factor-FOXA2- is involved in hepatic fatty acid oxidation[58].

In states of insulin resistance, insulin fails to phosphorylate FOXO1 and therefore, it fails to block gluconeogenesis. Therefore, fasting hyperglycaemia is observed despite hyperinsulinism and lipolysis is also activated, leading to an increase in the fatty acid load to the liver. However, the expected decrease in liver triglyceride synthesis is not observed. This is interpreted as a result of the stimulatory effect of insulin on SREBP-1c, favouring triglyceride synthesis. Therefore, insulin resistance is selective[59]: the lack of inhibition of gluconeogenesis leads to hyperglycaemia and in turn hyperglycaemia leads to increased insulin secretion, but SREBP activity is enhanced, leading to increased triglyceride synthesis. Other factors that are mentioned below may possibly aid in explaining this paradox.

In normal conditions, insulin not only has an adipogenic effect on the hepatocyte but it also limits VLDL secretion[60]. This effect is mainly dependent on an insulin-derived increased rate of degradation of apoprotein (apo) B but it is also due to the inhibition of apo B 100 synthesis. This preserves the triglycerides stored in the hepatocytes from utilization in the postprandrial state, so that they do not compete with the exogenous fatty acids. Apo B synthesis is a necessary step for VLDL formation. Newly synthesized apo B translocates into the endoplasmic reticulum and encounters MTP, among other chaperone proteins[61]. Importantly, FOXO1 enhances MTP expression[62]. This may explain why in conditions associated with insulin resistance the postprandrial decrease in VLDL secretion does not take place, and why hypertriglyceridemia constitutes a feature of the metabolic syndrome. As mentioned earlier, HCV is able to modulate MTP activation, directly promoting steatosis[9,63]. A recently described orphan receptor protein (orphan receptor small heterodimer partner) also plays an important role in the development of liver steatosis, although precise mechanisms are still unknown[64]. It possibly represses transcriptional activation of MTP; HCV increases its expression[65].

HCV also directly provokes insulin resistance. In HCV infection, insulin resistance is more closely related to viral load than to obesity, supporting a direct effect of HCV on insulin metabolism[66]. In fact, diabetes is more frequently observed among HCV patients[67]. The mechanisms involved in insulin resistance seem to be genotype-specific. It has been shown that HCV non-structural protein 5A (NS5A) is able to phosphorylate serine residues of IRS-1, thereby interfering with the post receptor downstream cascade of insulin action[68]. In accordance with this fact, treatment of HCV patients with pegylated interferon and ribavirin reduces insulin resistance assessed by homeostasis model for assessment[69]. Moreover, NS5A protein also exerts direct lipogenic effect through activation of LXRs[70].

In addition to the effect of non-structural proteins, it has been recently shown that HCV 1 and 4 core proteins are able to alter the degradation of IRS-1 and IRS-2 in a pathway dependent on suppressor of cytokine signalling 3 (SOCS3), thus also altering insulin signalling[71,72] by stimulating ubiquitination and subsequent degradation of IRS. Moreover, Pazienza *et al*[73], in 2007, showed that core protein of genotype 3a promoted IRS degradation by down-regulation of PPAR γ and up-regulation of SOCS7, whereas the core protein of genotype 1b activated the mammalian target of rapamycin (mTOR). Activation of mTOR leads to insulin resistance[74], but also exerts a direct effect on SREBP-1, leading to increased lipid synthesis. Some authors believe that the main mediator of increased lipogenesis in conditions characterized by insulin resistance is mTOR, acting on SREBP[75].

Fat as an endocrine organ: Cytokines

We have seen that steatosis ultimately depends upon the fatty acids pool, mainly derived from adipose tissue. It is also important to keep in mind that fat is not only a source of free fatty acids, but also a source of pro-inflammatory and anti-inflammatory cytokines which are able to modulate the circulation of free fatty acids from fat tissue to liver and again from liver to peripheral tissues[76]. These cytokines are also involved in some key steps of the progressive liver damage observed in individuals affected by steatohepatitis. To add more complexity to this scenario, recent research has shown that fat tissue is not homogeneous. Trunk fat is associated with increased insulin resistance and vascular risk[77], whereas leg fat exerts opposite effects[78]. These differences probably reflect the secretion of a different cytokine profile. In general, trunk fat has a

"negative" cytokine profile: it secretes less adiponectin, a "protective" cytokine, but more TNF and interleukin (IL)-6 than in the gynoid profile of fat distribution (fat around the hips and legs which is associated with increased production of adiponectin)[79]. Therefore, it is important to analyse the diverse fat compartments when studying the influence of these cytokines on liver steatosis. The cytokine profile associated with hepatitis C-liver steatosis, and the potential role of these cytokines on liver fat deposition is controversial[80-82] as well as their relationships with histological changes in chronic HCV infection.

Although cytokines are definitely involved in the inflammatory process which marks the evolution from steatosis to steatohepatitis, they also play a role in simple steatosis, both by aggravating it directly, or by affecting the metabolic axis which controls fatty acid trafficking. For instance, TNF-α is involved in SREBP activation[24]. On the contrary adiponectin activates fatty acid oxidation[83,84], but decreases the activity of fatty acid synthase and acetylcoenzyme A carboxylase[85]. Moreover, it inhibits liver production of TNF[86]. By upregulation of AMP activated protein kinase activity it also influences other pathways involved in lipid metabolism decreasing SREBP-1 and up-regulating PPAR-α in several tissues[87], an effect which is shared by IL-6[88]. PPAR-α is a transcription factor for several genes involved in the transport, oxidation, and export of free fatty acids[89,90]. It can be viewed as a sensor of intracellular free fatty acids, since it becomes activated by intracellular free fatty acids. PPAR-α deficiency promotes the development of fatty liver, and its activity is altered by classic factors involved in liver steatosis, such as ethanol consumption and HCV infection. Chronic ethanol feeding inhibits PPAR-α function due to the effect of acetaldehyde, which inhibits transcriptional activation of PPAR-α[91]. HCV causes down-regulation of PPAR-α[92], and is able to inhibit its activity by inducing repression of PPAR-α signaling by micro RNA-27b[93].

Leptin, another fat-derived cytokine, may promote fibrogenesis through up-regulation of transforming growth factor-β[94], but it also protects the liver from fat accumulation by lowering the expression of SREBP-1[95]. These nearly opposite effects may explain, perhaps, disparate findings in relation to leptin levels in chronic HCV infection[96]. Increased leptin levels, but also normal[97] or even decreased ones[98] have been reported in chronic HCV infection and leptin may[99] or may not be related to liver steatosis[96,100] in chronic HCV infection.

Increased trunk fat is not the only factor responsible for increased cytokine secretion in HCV infection. Increased reactive oxygen species (ROS)-which also directly impair mitochondrial oxidation of fatty acids[101] activate nuclear factor kappa B (NFκB), a key transcription factor for the expression of cytokines[102] such as TNF-α or IL-6, among others. In addition to the many proinflammatory effects of TNF-α, it also causes insulin resistance and liver steatosis by inhibiting IRS[103]. Excessive ROS production depends on the intracellular effect of HCV. NS3 and 5A are able to activate mitochondrial ROS production by altering calcium trafficking at the endoplasmic reticulum membrane[104]. This altered calcium influx also triggers increased transcription of STAT-3 and NFκB, leading to increased cytokine production which closes a positive feed-back loop. In addition, NS3 and NS 5A are also able to stimulate toll-like receptor-4, in a way similarly to that caused by the lipopolysaccharide in the initial stages of alcoholic hepatitis[105]. Furthermore, LXR, which can be directly activated by HCV, regulates a set of genes that encode proinflammatory mediators[43].

CONCLUSION

Liver steatosis is a very complex process, in which many proteins and enzymes are involved. As shown, viral proteins may affect several of the metabolic pathways leading to simple steatosis, including cytokine activation. Indeed, cytokine production takes place even at early stages, and, among many other questions outlined in this review, it remains to be answered why, despite early cytokine activation, only some patients evolve to steatohepatitis, a key step in the progression of HCV-induced liver damage.

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REVIEW

Liver transplantation as a management of hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide and has a poor prognosis if untreated. It is ranked the third among the causes of cancer-related death. There are multiple etiologic factors that can lead to HCC. Screening for early HCC is challenging due to the lack of well specific biomarkers. However, early diagnosis through successful screening is very important to provide cure rate. Liver transplantation (LT) did not gain wide acceptance until the mid-1980s, after the effective immunosuppression with cyclosporine became available. Orthotopic LT is the best therapeutic option for early, unresectable HCC. It is limited by both, graft shortage and the need for appropriate patient selection. It provides both, the removal of tumor and the remaining cirrhotic liver. In Milan, a prospective cohort study defined restrictive selection criteria known as Milan criteria (MC) that led to superior survival for transplant patients in comparison with any other previous experience with transplantation or other options for HCC. When transplantation occurs within the established MC, the outcomes are similar to those for nonmalignant liver disease after transplantation. The shortage of organs from deceased donors has led to the problems of long waiting times and dropouts. This has led to the adoption of extended criteria by many centers. Several measures have been taken to solve these problems including prioritization of patients with HCC, use of pretransplant adjuvant treatment, and living donor LT.

Key words: Hepatocellular carcinoma; Management; Liver transplantation; Pretransplant adjuvant therapy; Milan criteria

Core tip: Hepatocellular carcinoma (HCC) has a poor prognosis if untreated. Screening is challenging due to the lack of specific biomarkers. Successful screening is very important as early diagnosis can provide curative opportunities. Orthotopic liver transplantation (LT) is the best therapeutic option for early, unresectable HCC. When transplantation occurs within the established Milan criteria, the outcomes are good. The shortage of organs from deceased donors led to the adoption of extended criteria. Several measures have been taken to solve these problems including prioritization of patients with HCC, use of pretransplant adjuvant treatment, and living donor LT.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a major health problem[1]. It is ranked as the third cause of cancer-related death worldwide[2]. Up 80%-90% of cases are associated with underlying cirrhosis[3]. Liver resection and local ablative procedures are regarded as potentially curative treatments, but their effect on the functional reserve of the liver restricts their application. Also, there is a high chance of recurrence in the liver remnant[4]. Liver transplantation (LT) is the ideal treatment for patients with HCC and cirrhosis. It has the ability to remove the tumor as well as the underlying liver cirrhosis. This gives the chance to restore the liver function and decrease the risk of development of new HCC[5]. Since the first LT done by Starzl *et al*[6] in 1963, the procedure did not gain wide acceptance until the mid-1980s, when immunosuppression with cyclosporine became available[7]. The goal of LT is to provide liver recipients with the maximum benefit possible in a fair, ethical, and cost-effective manner. Mazzaferro *et al*[8] in Milan established criteria for orthotopic LT (OLT) as a variable treatment for HCC in a study published in 1996. By adoption of these criteria, LT can achieve a good prognosis in the recipients who fulfilled them and avoid the poor prognosis in those who exceeded them.

SELECTION CRITERIA

Initial results of OLT in patients with HCC were poor[2,9,10]. In 1996, a study from Milan found that restrictive selection criteria (single tumor up to 5 cm or up to three tumors, each no larger than 3 cm, without macrovascular invasion or extrahepatic spread) led to similar outcomes when compared with OLT performed in patients without HCC[11]. These Milan criteria (MC) were used by the United Network for Organ Sharing (UNOS) to arrange the listing priority of patients presenting with HCC. The aim of these criteria was to achieve satisfactory results in patients who fulfilled the criteria and avoid poor outcomes in recipients who exceeded them. This study concluded that the 10-year overall survival was 70% in 300 liver transplants for HCC that fulfilled the MC. The same results have been confirmed worldwide[12,13]. Other groups have showed favorable outcomes in patients with early HCC[11,14-18].

The good outcome of OLT depending on the MC has encouraged putting more patients with HCC on the list of transplantation. Some studies found that the MC may be too restrictive and prevent some recipients from their chance in LT. Extending the selection criteria showed relatively good results. This extension was applied to both transplantation of patients with tumors outside the MC and the use of pre-LT treatment to downstage the tumor stage to fulfill the MC[19-21].

A multicenter study performed in multiple European centers to assess how to expand the MC. Data were collected from 466 patients who were transplanted for HCC with tumors exceeded the MC diagnosed by posttransplant pathologic assessment[13,21]. One of the proposed expanded criteria was the UCSF criteria which include single tumor nodule up to 6.5 cm; or three or fewer tumors, the largest of which is 4.5 cm with the sum of the tumor diameters 8 cm[20]. The 1 and 5-year survival rates in 60 patients included in the study who met these criteria were 90% and 75.2%, significantly higher than patients with HCC exceeded these limits (50% at 1 year, $P < 0.0005$) and comparable with the survival rates in the Milan study (75% at 4 years)[11]. Some groups have studied the expanded criteria and had results in favor of the Milan study[22-28]. A study performed by the same group in Milan. They gathered retrospective data regarding outcome in 1112 patients exceeding the original MC[29]. A 71.2% 5-year survival could be achieved using recipients with HCC up to 7 cm of the largest tumor and number of tumors up to 7. This is known as the "up-to-7" criteria. There is a direct association between the larger tumor size and increased number, with the worse outcome. Preoperative imaging understaging tumors has been one of the major concerns for expanding the MC[30]. This understaging occurs in 20% of patients[13]. Up till now, the MC remains the only universally accepted criteria. Currently, by increasing demand and organ shortage, multiple studies have suggested a 50% 5-year patient survival to be the minimum acceptable to approve the expansion of MC[13]. This point was studied by the UCSF group, who has applied expanded criteria to benefit an additional 10% of patients with HCC regarding posttransplant survival and tumor recurrence. In living donor LT (LDLT), the recipients with larger and/or multiple tumors with no vascular invasion, are not excluded from transplantation as the graft donation here not public but depends on the donor's intention[31].

Prioritization of liver transplant candidates on waiting list, decreasing dropout rates and shorting the waiting time for LT.

After the selection of patients with HCC for transplantation and putting them on a waiting list, the problem of the progression on waiting list arise. This progression will lead to exceeding the MC and dropout from the list. Dropout rates become an increasing problem with the prolonged waiting times. One study concluded that, with a short waiting time (mean 62 d) there are minimal or no dropouts resulting in 85% 2-year survival, while a longer waiting time (mean 162 d) lead to 23% dropout rate and less than 60% 2-year survival[32]. The available liver grafts have to be allocated to the sickest patients.

In February 2002, UNOS adopted a modified form of scoring system as the basis of its liver allocation

policy. This system aims to arrange the recipients on the waiting list for LT based on statistical formulas to predict who is most likely to die soon from liver disease. The model for end stage liver disease (MELD) is used for adult and the pediatric end stage liver disease model is used for pediatric patients[33-35]. The MELD scoring system was initially developed to detect the risk and mortality in patients undergoing transjugular intrahepatic portal systems shunt[36]. Wiesner *et al*[34] and Wiesner *et al*[35] used the MELD score to patients with end-stage liver disease not undergoing transplantation and proved its relevance in UNOS status 2A or 2B patients listed for transplant between November 1999 and December 2001[37]. MELD score is a numerical scale, ranging from 6 (less ill) to 40 (gravely ill). It gives each individual a "score" which denotes how urgently the patient needs a liver transplant within the next three months. MELD score can be calculated from three laboratory values: creatinine, total bilirubin, and international normalized ratio of the prothrombin time[35]. It's ability to predict 3-mo mortality was not affected by other complications of cirrhosis as ascites, encephalopathy, variceal bleeding, and spontaneous bacterial peritonitis[35]. The application of MELD scoring system is an important step in moving from a retrospectively derived allocation system to a prospective evidence-based approach[38]. By prioritization organ donation on the basis of mortality risk, the MELD scoring system had achieved improvements in waiting list mortality and fairness of access to LT[39,40].

Patients with early HCC usually have near normal synthetic liver function which gives them a low MELD score, and lower chance for liver transplant. Most of these patients have the risk of progression of their HCC leading to dropout form the waitlist rather than by deterioration of their liver disease. This problem was solved by the invention of MELD score specially for HCC patients attempting to reflect the risk of dropout, and consider the equivalent in outcome to death[37]. Patients with T1 lesions which mean a single tumor up to 2 cm, were given a MELD score of 24 points and patients with T2 lesions which are beyond T1 but within MC, were allocated a score of 29 points. By applying this MELD score for patients with HCC, there was an increase in the LT for HCC patients, relative to non-HCC patients. LT for HCC increased from 7% to 22% of all deceased donor transplants, with improvements in the waiting list times and dropout rates for HCC patients relative to the pre-MELD era[41]. Also, the outcomes for patients with HCC exceeded those of non-HCC patients with equivalent MELD scores[42]. Some investigators found that more than 33% of patients diagnosed preoperatively to have T1 lesions, found not to have HCC in the explant[43]. Since the start of use of HCC MELD score in 2002, three changes have occurred, aimed at addressing fairness between HCC and non-HCC patients. Under the current system, the native MELD score is used for patients with T1 lesions. For patients with T2 lesions, they receive an HCC score of 22 points and also receive point upgrades, for every three month period on the waiting list[41]. The update HCC MELD score now provides a fair way to access LT for HCC patients[44].

Bridging therapy and managing liver transplant candidates on the waiting list: It is very important to continuously evaluate the patients with HCC on the waiting lists to make sure that they are still within the inclusive criteria for LT. Multiple procedures have been developed to manage the patients whose HCC is at risk or shows signs of progression while on the waiting list for LT. There is no ideal imaging method nor proper specific timing to follow up these patients, although a 3-mo interval is common[45]. Some reports showed that an increase in α-fetoprotein level is associated with poorer outcome after LT and the use of locoregional therapy (LRT) is effective in the reduction of that risk. Periodic evaluation of the patients on the waiting list should be performed by imaging (dynamic computed tomography (CT), dynamic magnetic resonance imaging (MRI), or contrast-enhanced ultrasonography) and serum α-fetoprotein measurements[45]. Cancer progression in patients with HCC on waiting list is common and leads to dropout from the waiting list which is reported to be at least 20%[46].

Pre-transplant LRT are used on patients with HCC on the waiting list aiming at decrease the tumor progression and lowering the dropout rate[47]. They are not of benefit in patients with UNOS T1 tumors less than 2 cm[45]. Llovet *et al*[15] found good survival results of OLT in early HCC with no adjuvant therapy with waiting time 58.9 ± 45.1 d. There is no randomized control data to prove any benefit of pre-LT LRT for the prevention of dropout or reduction in post-LT recurrence[5,48]. Most data that showed benefits of pre-LT LRT came from small single centre, non-controlled studies. As a result, there is no universal consensus as to the optimum bridging therapy prior to transplantation[49]. The bridging strategies might be of benefit for patients with UNOS T2 tumors which means one nodule 2-5 cm or three or fewer nodules each ⩽ 3 cm, who may wait for 6 mo or more on the waiting list. High α-fetoprotein levels and large sized tumors seem to have a higher risk for dropout[50-52]. The most commonly used procedures include transarterial chemoembolization (TACE), radiofrequency ablation (RFA), multimodality therapy using combinations of TACE and RFA[48], Percutaneous ethanol injection (PEI) and combination of TACE with PEI[53,54]. Multiple studies showed no statistically significant survival improvement[11,17], but Harnois *et al*[16] showed that TACE was well tolerated and associated with promising results in selected HCC patients with some functional hepatic reserve with average waiting time of 167 d. In a retrospective study, it was found that pre-LT TACE can produce downstaging or total necrosis in tumors less than 3 cm[55]. Other retrospective studies have shown that multimodality local treatment is associated with an average survival benefit[56,57]. Explants of patients received pre-LT RFA and TACE were analyzed. The results showed complete tumor necrosis rates of 47%-66% and 16%-27%, respectively[52,58,59]. Other studies showed

that combination of TACE with PEI led to improved survival and complete tumour necrosis compared with no pre-LT treatment in small numbers HCC patients[53,54]. Some studies have proved[60] and emphasized[61] the role of TACE in the HCC patients on the waiting list for LT. Another study reported the survival results of arterial embolization or chemoembolization against symptomatic treatment in HCC not suitable for curative treatment and Child-Pugh class A or B and Okuda stage Ⅰ or Ⅱ[62]. This study showed 2-year survival probability 50% for embolization, 63% for chemoembolization and 27% for control (chemoembolization *vs* control $P < 0.009$). A retrospective study assessed tumor necrosis in 61 patients did not find any local ablation therapy (LAT) procedure to be superior[63].

A recent study by Lesurtel *et al*[64] did not show any benefit for pre-LT TACE regarding the dropout rate or post LT survival. Similarly, several small, single centre, uncontrolled studies reported the results of pre-LT RFA on dropout rates, without giving any conclusion[58,59]. Major complication and mortality rates for TACE as pre-LT LRT are amounted to 5% and 2.5%, respectively[65]. Regarding RFA as pre-LT LRT, major complications were observed in about 8% of cases[59]. One study showed an increased rate of post-LT tumor recurrence following incomplete tumor necrosis induced by LRT[66]. Other compilations of LRT include, needle track seeding and extra-hepatic metastases[67].

Other new pre-LT modalities are likely to show up aiming to improve post-LT results[68,69]. Recent randomized controlled trials showed the ability of the oral multikinase inhibitor sorafenib to delay HCC progression and improve survival. A randomized control trial examining the utility of this agent in combination with pre-LT TACE is already in progress[70]. External beam radiotherapy also showed promising results as a new pre-LT LRT[71].

Hepatic resection has been used as a bridge to LT[72]. Unlike LAT, resection should achieve the best tumor control. It gives the chance for intra-operative assessment of liver status and tumor burden. All the data needed about the nature of the tumor, natural history and microvascular invasion can be issued from the analysis of the surgical specimen. On the other side, resection, is associated with complications and should only be offered to well-compensated cirrhotic patients[49].

So currently, no recommendation can be made on bridging therapy in patients with UNOS T1 lesion (⩽ 2 cm). In patients with UNOS T2 lesions with one nodule 2-5 cm or three or fewer nodules each ⩽ 3 cm that fulfill MC and a likely waiting time > 6 mo, LRT may be appropriate. No superiority of one LRT to others. Patients who progressed on the waiting list and exceeded the criteria for listing should considered for understaging by LRT. Patients with progressive disease, in whom LRT intervention is not considered of benefit or effective should be dropout from the waiting list[45].

The role of LDLT in HCC: LDLT has evolved, mainly due to the scarcity of donor livers and in some cases, totally unavailable[73-75]. It is the main source of grafts for recipients on the list for LT in Japan and much of Asia because the lack of societal acceptance of organ retrieval from brain dead donors[76,77]. Moreover, in most Asian countries, HCC is the most common cancer and a common indication for OLT. Also, due to organ shortage, long waiting times associated with deaths on the waiting list, drop-out due to medical reasons, or progression of tumors beyond acceptable criteria, LDLT has been used in countries with well established programmes for organ donation from brain dead or non-heart-beating donors[45]. The main principle in LDLT is the safety of donor[78,79]. Although there are concerns of donor morbidity and mortality, LDLT has opened up the possibility of living donation to the adult patients with end-stage liver disease. Some studies compared deceased-donor LT (DDLT) and LDLT for HCC[80-85]. No significant difference in outcome could be identified between both types of grafts. The results from LDLT appear to show good long term survival rates with retrospective studies showing comparable rates to OLT[82,86,87]. Some other retrospective studies showed a higher rate of tumor recurrence with LDLT than with conventional OLT[81,83,84]. This may be due to the short waiting time for patients in LDLT and hence patients with aggressive tumors are transplanted without declaring themselves while with OLT these patients are not transplanted because of their tumor progression whilst on waiting list due to longer waiting time so they will be removed from the outcome analysis. To minimise donor risk and maximise recipient outcome, LDLT must be performed only in centers of excellence in liver surgery and LT[45]. Psychosocial considerations for both the donor and recipient are very important. Unlike deceased-donor donation, it is ethically acceptable for LDLT to be offered to patients with tumour exceeding the MC since, other listed patients will not by this process[88]. Deceased-donor grafts can be offered for failed grafts after LDLT, even if extended criteria were used. Other centers offer retransplantation to recipients who received a living graft, within the accepted criteria, however, it is not recommended for patients following LDLT for HCC outside the accepted regional criteria for DDLT[89]. Rates of retransplantation due of graft failure after LDLT are low and their outcomes are favorable.

Post LT management: The risk of tumour recurrence is the main concern after LT. It occurs in 8%-20% of recipients[90]. It is usually seen within the first 2 years after LT, and is associated with a median survival of less than 1 year from the time of diagnosis[91]. Early recurrence can be diagnosed by routine imaging and α-fetoprotein monitoring[92]. Post-LT monitoring may include 6-12-mo contrast-enhanced CT or MRI imaging for the first 3-5 years after LT and α-fetoprotein levels[45].

Surgery can be offered for resectable for resectable recurrent HCC lesions[45]. LRT as RFA, or TACE, has been successfully used in selected patients when

technically feasible and with limited disease[93]. Some studies showed that sirolimus, an mechanistic target of rapamycin (mTOR) inhibitor, was associated with lower tumour recurrence and improved survival after LT[94-96]. However, no recommendation can be made on the use of mTOR inhibitors to reduce the risk of HCC recurrence outside clinical trials[45]. A study showed that, sorafenib had an antitumour effect in patients with advanced HCC[68]. It is currently studied in phase 3 trial (STORM trial) as an adjuvant therapy after resection or ablation of HCC. It has been used with limited side effects after LT[97]. Licartin, a 131I-radiolabelled murine monoclonal antibody was shown to have a positive effect on prevention of tumour recurrence and on survival[98]. Retransplantation is not appropriate treatment for recurrent HCC, as most recurrences are associated with systemic tumour dissemination. Development of *de-novo* HCC in the transplanted graft should be treated as having new tumors and retransplantation might be considered[99].

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REVIEW

Review on immunosuppression in liver transplantation

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Abstract

The optimal level of immunosuppression in solid organ transplantation, in particular for the liver, is a delicate balance between the benefit of preventing rejection and the adverse side effects of immunosuppression. There is uncertainty about when this level is achieved in any individual recipient. Immunosuppression regimens vary between individual centers and changes with time as new agents and data are available. Presently concerns about the adverse side effects of calcineurin inhibitor, the main class of immunosuppressive agents used in liver transplantation (LT), has led to consideration of the use of antibody induction therapies for patients at higher risk of developing adverse side effects. The longevity of the transplanted organ is potentially improved by better management of rejection episodes and special consideration for tailoring of immunosuppression to the individual with viral hepatitis C, hepatocellular carcinoma or pregnancy. This review provides an overview of the current strategies for post LT immunosuppression and discusses modifications to consider for special patient populations.

Key words: Liver transplantation; Immunosuppression; Immunosuppression induction; Immunosuppression maintenance

Core tip: This manuscript is a review on common aspects and principles of immunosuppression in liver transplantation (LT) including new advents. It covers the sections of induction, maintenance and monitoring of immunosuppression and also discusses on immunosuppression in special populations. In this review, it has been tried to be connected with last updates in the field of immunosuppression in LT.

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INTRODUCTION

Liver transplantation (LT) is a life saving procedure for patients with end stage liver disease and its

complications, and for liver failure. LT is also curative for some hereditary metabolic disorders like familial hypercholesterolemia and for selected cases of malignancies involving the liver, such as hepatocellular carcinoma (HCC) and hepatoblastoma. Recipients of orthotopic LT have excellent survival rate (83% for 1 year and 75% for 5 years) that has improved markedly over the past three decades[1].

Development of new agents and changes in post transplant immunosuppression regimens are major contributing factors for this improvement. However, while long term post transplant immunosuppression decreases rejection episodes in LT recipients, it also puts the patients at increased risk of infection, malignancies and specific adverse side effects unique to each agent. There are different immunosuppression protocols used by transplant centers worldwide; however, any LT recipient may need an individually tailored immunosuppression regimen to balance the benefits and potential harm of therapy while decreasing the risk of recurrence of their primary disease.

The basis of solid-organ post transplant immunosuppression has commonalities between the different organs, but the liver itself is unique in its immunologic response to provocation. This privilege is called "liver tolerance" which is mostly attributed to the role of the regulatory T-cell[2,3].

LT does not require human leukocyte antigen (HLA) matching between donor and recipient[4], though there is increased interest in understanding HLA and humoral rejection and graft survival. Simultaneous transplantation of liver with another solid organ, for example, a kidney decreases the incidence of rejection episodes for the second organ[5-7] and facilitates minimization of the immunosuppression to a lower level than typically allowed thereby, reducing adverse side effects and cost of therapy. Given these considerations as a general principle, LT recipients are maintained on lower levels of immunosuppression than other solid organ transplant recipients. Moreover, in some selected LT recipients, the allograft may achieve long-term survival even after immunosuppression withdrawal[8].

Living donor LT recipients experience less rejection episodes comparing with deceased donor LT[9,10]. However, the immunological benefit as the only explanation for this observation is in doubt by some researchers[11]. Shorter cold ischemia time and operation in non-emergency conditions are among the factors affecting transplantation outcome from living donors. On the other hand, although, the beneficial effect of HLA similarity between the living donor and recipient has not been proven in LT[11-13], high success rate for weaning from immunosuppression has been reported in pediatric patients receiving liver from their parents[14].

The aim of this review is to provide an overview of strategies for post liver transplant immunosuppression, with special attention to specific populations where modification of standard regimens may be beneficial.

IMMUNOSUPPRESSIVE AGENTS IN LT

Immunosuppressive agents are required in solid organ transplantation for induction of immunosuppression in the early phase, maintenance of immunosuppression in the late phase or for the treatment of organ rejection. Table 1 summarizes the immunosuppressive agents commonly used in LT and their specific applications. The site of action of these agents is briefly shown in Figure 1.

Induction immunosuppression

The definition of induction therapy is intensive perioperative prophylactic immunosuppression used to prevent acute cellular rejection in the first months following transplantation[15]. Specific agents are discussed individually below.

Corticosteroids

Corticosteroids have been the mainstay for induction of immunosuppression since the first successful cases of solid organ transplantation[13,16].

Intravenous (*iv*) injection of corticosteroid are administered in high doses during the transplant operation and in the first few post operation days (usually up to 3 d) in combination with at least one other immunosuppressant agent.

The dose and duration of *iv* administration of drugs differ according to the local practice among different centers; however a typical dosage is 500 or 1000 mg of methylprednisolone. Corticosteroids are rapidly tapered over the first week to relatively low doses, 10 to 20 mg daily, and are usually maintained in immunosuppression regimen at least for the first 3 to 6 mo post transplant. The major concerns with corticosteroids, especially with high doses, are their adverse side effects. Delirium is a common early problem, and infections and metabolic derangements such as hypertension, hyperlipidemia, diabetes, and obesity may cause significant short and long-term morbidity among liver recipients. In these individuals, steroid reduction or elimination may be indicated. There is also concern that higher doses of steroids increase the risk of disease recurrence in LT patients with chronic viral hepatitis. However, the risk of organ rejection may increase following early corticosteroid dose reduction or withdrawal[17].

Usually, a calcineurin inhibitors (CNI), alone or with an anti-proliferative agent mycophenolic acid (MPA) or azathioprine is started early post transplantation in combination with a corticosteroid to help maintain immunosuppression[18]. More recently, antibody therapies have been combined with corticosteroids or used to facilitate "steroid-free" regimens.

Antibodies

Use of antibodies that are designed specifically to inhibit or deplete recipient T-cells has been reported to decrease acute rejection episodes in the liver allograft[19,20].

Use of antibody induction also provides an oppor-

Table 1 Commonly used immunosuppressive agents in liver transplantation

Agent	Classification	Indications	Dose
Methyl prednisolone (Medrol®), Prednisone or prednisolone[13,16,111]	Corticosteroids	Induction of immunosuppression, treatment of acute cellular rejection, Maintenance of immunosuppression	Variable according to the centers, the etiology of liver disease and history of rejections
Tacrolimus (Prograf®, Astagraf®)[53]	CNI	Maintenance of immunosuppression	Starting 0.1-0.15 mg/kg per day divided every 12 h and adjust to the desired trough level
Cyclosporine (Neoral®, Sandimmune®, Gengraf®)[52,55]	CNI	Maintenance of immunosuppression	Starting 10-15 mg/kg per day divided every 12 h and adjust to the desired (C2) level
Mycophenolate mofetil (Cellcept®, Myfortic®)[60]	Anti-metabolite	Maintenance of immunosuppression, treatment of rejection	Variable doses may be desired in any individual case
Azathioprine (Imuran®)[65]	Anti-metabolite	Maintenance of immunosuppression	Variable, maintenance dose may be 1.5-2.5 mg/kg per day, needs to be adjusted for adverse side effects
Sirolimus (Rapamune®)[48,68,71]	mTORI	Maintenance of immunosuppression, treatment of rejection, special interests for use in malignancies	Usual dosing is a 6 mg (or 3 mg/m^2) oral loading, followed by 2 mg/d (or 1 mg/m^2 per day) single dose, higher doses may be administered for individual cases[1]
Everolimus (Afinitor®)[48,69,72]	mTORI	Maintenance of immunosuppression, treatment of rejection, special interests for use in malignancies	Starting at 1 mg oral every twice a day and adjust to a trough level of 3-8 ng/mL[1]
[2]Muromonab-CD3 (OKT3)	T cell depleting monoclonal antibody	Induction of immunosuppression, treatment of steroid resistant rejection	Withdrawn from the market because of reduced use, no longer available since 2010
Alemtuzumab (campath-1H®)[44-46]	T cell depleting monoclonal antibody	Induction of immunosuppression	Variable between centers, a single dose of 30 mg may be used in operating room
ATG (Thymoglobulin®, ATGAM®)[27-30]	T cell depleting polyclonal antibody	Induction of immunosuppression, treatment of steroid resistant rejection	Variable between centers, For induction 1.5 mg/kg per day *iv* for 3 d and for treatment of rejection 1.5 mg/kg per day *iv* for 5-7 d of thymoglobulin may be used. For ATGAM a higher dose of 15 mg/kg per day is usually used
[2]Daclizumab (Zenapax®)[23,115]	IL-2Ra, monoclonal antibody	Induction of immunosuppression, treatment of steroid resistant rejection	For induction the first dose of 1 mg/kg is given within 24 h before Tx and 4 more doses are given after Tx with 2 wk intervals Withdrawn from the market because of reduced use, no longer available
Basiliximab (Simulect®)[23,113,114]	IL-2Ra, monoclonal antibody	Induction of immunosuppression, treatment of steroid resistant rejection	For induction a 20 mg *iv* dose is administered within 2 h prior to reperfusion and another 20 mg on days 4 post Tx

[1]Best to be started at least 30 d after transplantation; [2]Not manufacturing anymore. CNI: Calcineurin inhibitor; mTORI: Mammalian target of rapamycin inhibitor; *iv*: Intravenous; IL-2Ra: Interleukin-2 receptor antagonists; Tx: Transplantation.

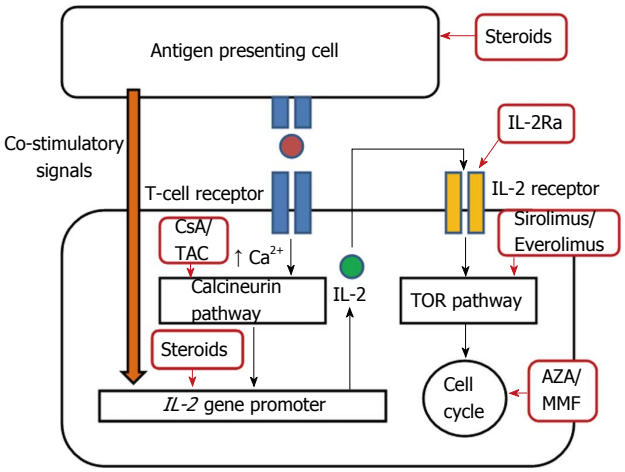

Figure 1 The cellular site of action of the immunosuppressive agents commonly used in solid organ transplantation. AZA: Azathioprine; CsA: Cyclosporine; IL-2: Interleukin-2; IL-2Ra: Interleukin-2 receptor antagonist; MMF: Mycophenolate mofetil; TAC: Tacrolimus; TOR: Target of rapamycin.

tunity to decrease the dose of other concomitant immunosuppressive agents such as corticosteroids and CNIs[17] thus minimizing the adverse side effects related to these agents. Antibody administration has been used for induction therapy in "steroid-free" protocols where there is elimination of corticosteroid in the induction of immunosuppression in LT patients[21,22].

Compared with corticosteroid induction, less hyperglycemia and diabetes and less cytomegalovirus (CMV) infections are found with antibody induction[17,23].

This "steroid-free" strategy may be especially beneficial for patients with hepatitis C patients and for those with diabetes and hypertension. Antibody induction along with delayed CNI introduction can be used to preserve renal function in LT recipients and reduce renal dysfunction in those with impairment[24].

Overall, no significant increase in adverse side effects was observed in solid transplant recipients receiving antibody induction[23,25]. However, their use

adds to the cost of perioperative care.

Antibodies used for induction of immunosuppression in LT are classified into two groups; T-cell depleting and non-depleting [interleukin 2 receptor antagonists (IL-2Ra)][26].

T-cell depleting antibodies

This group includes:

Polyclonal antibodies: Anti-thymocyte globulins (ATG)s are polyclonal animal antibodies against multiple T-cells receptors that are used to achieve circulating lymphocyte depletion. There are two preparations of antithymocyte globulin (ATG) available for clinical use in the United States. Equine ATG (eATG, ATGAM®) is of equine origin and rabbit ATG (rATG, Thymoglobulin®) is generated in rabbits. ATG has been widely used for the treatment of steroid resistance rejections[27,28] as well as induction of immunosuppression in LT[29,30].

rATG is one of most commonly used agents for antibody induction therapy in organ transplantation in the United States. Much of the initial experience with polyclonal antibody induction therapy was learned from kidney transplantation. rATG is superior to the equine originated ATG in prevention of episodes of acute renal rejection[31]. Less severe rejections, fewer serious adverse side effects and even less CMV infection occur, but more profound leucopenia have been observed in renal allograft recipients receiving rATG compared with those who received eATG as induction therapy[32].

The protocol for ATG induction therapy differs between centers. A 10 d course of single infusion of 2.5 mg/kg of rATG was the standard induction in the earlier cyclosporine era, while shorter courses (a 3-d course) was shown to have the same protective effect but less severe life threatening infections[33]. The efficacy of the 3-d induction protocol has been supported even when the first dose is delayed for 48 h post-transplant[34,35]. Intermittent dosing of eATG based on CD3 counting using flow cytometry has been shown to be effective and less costly in induction of immunosuppression for kidney and kidney/pancreas transplantation. In this regimen, the second dose of eATG after transplantation was not given until the CD3 count is above 20 cells/mm^3[30].

Administration of rATG may infrequently cause infusion related reactions. Pre-medication with antihistamines and acetaminophen is recommended as rare severe cardiovascular reactions and anaphylaxis have been reported[36]. Serum sickness may develop following rATG administration[37]. More common adverse side effects of AGT are the result of severe cytokine release syndrome induced by the agent. The risk of infectious complications following induction therapy with rATG was comparable to the regimens without antibody induction[38,39]. Although a major concern, a more severe recurrence of hepatitis C virus (HCV) infection has not been observed with rATG induction therapy, and there is even some limited evidence for a decreased risk[40,41]. There are insufficient data regarding the possible increased risk of post transplantation lymphoproliferative disorders (PTLD) following rATG induction in LT patients. However, the overall incidence of PTLD in kidney and heart transplant recipients receiving rATG induction therapy was low and the effect of prophylactic antiviral therapy on the risk reduction has been supported[42].

Monoclonal antibodies: Alemtuzumab (campath-1H) is a humanized rat monoclonal antibody against CD52 receptors on peripheral mononuclear cells. It has a significant depleting effect on peripheral as well as lymph node lymphocytes[43].

As a potent immunosuppressant agent alemtuzumab has its own potential benefits for induction therapy in LT; however, the increased risk for infectious complications may limit its use to special subgroups, in particular for those who need renal sparing regimens[44-46].

The safety of alemtuzumab induction is most in doubt in HCV positive recipients as increased complications and a rapidly progressive recurrence of HCV have been reported[47].

Further studies are required to address the risk benefit issues on use of this agent as induction immunosuppression for LT.

Muromonab-CD3 (OKT3) was a monoclonal antibody directed against CD3 receptors on peripheral T-cells that was successfully used for the treatment of steroid unresponsive acute liver rejection and also for immunosuppression prophylaxis. However, the side-effect profile was considerable and with the availability of newer agents, the manufacturer discontinued its production in 2010.

Non-depleting antibodies: IL-2Ras are humanized monoclonal antibodies that bind to IL-2 receptor on T-cells and thus suppress the proliferative response of T-cells to circulating IL-2. These agents are less immunogenic than other antibodies such as OKT3[48]. For LT, IL-2Ras have a role for patients who need to avoid or to decrease dosages of an accompanied immunosuppressant agent, such as corticosteroids or CNIs. Less frequent diabetes mellitus, less CMV infections and higher glomerular filtration rate were observed among patients receiving IL-2Ra *vs* those who received corticosteroids as induction therapy. The two IL-2Ra agents, basiliximab and daclizumab did not differ in the mentioned advantages when analyzed by Penninga *et al*[23] and may be used interchangeably. Although, daclizumab has been off the market since about 2010, the results of few studies using this agent as an IL-2Ra will be reviewed here.

In an analysis of the United Network for Organ Sharing (UNOS) database, Uemura *et al*[49] compared ATG, daclizumab and steroid alone and ATG plus steroid. They showed a satisfactory short and long-term outcome for daclizumab in LT patients with all etiologies of liver disease including HCV[49]. In a randomized multicenter study, Klintmalm *et al*[50] concluded that

corticosteroid free induction therapy using IL-2Ra (daclizumab) does not increase the risk of fibrosis progression in HCV LT recipients. Basiliximab induction was not associated with increased risk of PTLD, CMV infection or HCV recurrence in another study by Ramirez *et al*[51], while the rate of acute rejection was decreased and rejection free survival increased.

Maintenance immunosuppression

CNI: CNIs function as immunosuppressants by blocking the signal 2 of T-cell activation by binding to specific receptors and blocking calcineurin, a calcium dependent phosphatase within T-cells. The introduction of the two CNIs, cyclosporine (in 1970s and early 1980s) and tacrolimus (in 1990s) as immunosuppressant agents, greatly improved the outcome of LT. Overall survival of patients undergoing LT on cyclosporine immunosuppressive therapy was 3 times higher than those on azathioprine alone[52].

With the advent of the newer agent, tacrolimus, the outcome of LT improved further. Tacrolimus is superior to cyclosporine in increasing patient and graft survival. Less acute cellular rejection and steroid-resistant rejection episodes are seen with tacrolimus use in the first post-transplant year compared with cyclosporine[53].

Because of their excellent organ protective effects, CNIs, especially tacrolimus, have been included as the main agents in maintenance immunosuppression protocols in most LT centers, worldwide. Tacrolimus is preferred over cyclosporine in most centers because of its greater potency and improved cardiovascular adverse side effect profile. Tacrolimus is usually started with a low (0.1-0.15 mg/kg per day divided in 2 doses every 12 h) oral (or sublingual, if the patient could not tolerate oral medication) dose on the first day post transplant, though some centers delay its start for 2-4 d, and the dose is gradually increased to achieve the desired trough level. An *iv* formulation of tacrolimus is available, but seizures are a significant risk with use of this form of the medication.

There is controversy, thus center variability, about the optimal trough (C0) level for tacrolimus. Usually levels of 10-15 ng/mL are targeted for the first 4-6 wk, and 5-10 ng/mL thereafter are accepted by many centers; however, it is possible to target lower levels (*i.e.*, 6-10 ng/mL for early post transplantation) without increasing the risk of acute rejections with a benefit toward kidney protection[54].

Cyclosporine is not the CNI of choice for LT recipients; however, in special cases there might be a need to switch from tacrolimus to cyclosporine. When it has to be used the recommended dose is 10-15 mg/kg per day divided in 2 doses, but it may be started at lower doses with gradual increase to achieve the target level. For C0 levels, in the early post transplant period the target level is 250 ng/mL, and 150 ng/mL later on. For cyclosporine, C2 (2 h after dose) monitoring has also been implemented at some centers. The C2 level of cyclosporine may be appropriate in the range of 800-1400 ng/mL for the first 3 mo, 600-1000 ng/mL after 6 mo and 500-700 ng/mL after 1-year post transplantation[55].

The mostly commonly concerning adverse side effect of CNIs is their nephrotoxic effect at high levels. CNIs produce afferent renal arteriolar vasoconstriction that could induce renal dysfunction and also tubular injury. This vasoconstrictor effect is dose dependent and reversible. However, these agents may also have a role in inducing chronic renal injury or at least being a contributing factor along with other factors in non-renal solid organ transplant recipients[56].

Renal insufficiency has a special significance for LT in applying the model for end-stage liver disease as the major driver for organ allocation; more patients with renal dysfunction get priority for LT. These patients with higher risk of post transplant renal dysfunction may need immunosuppression regimens with less risk for nephrotoxicity, such as delayed CNI introduction, use of anti-metabolites for minimization or even elimination of CNI use.

Hypertension, neurotoxicity, metabolic abnormalities including hyperglycemia, electrolyte imbalance and hyperlipidemia are among the common adverse side effects of CNIs. The diabetogenic effect of tacrolimus is greater than cyclosporine[53]. Cyclosporine can cause hirsutism and gingival hyperplasia. Tacrolimus and cyclosporine could be associated with an increased risk of infection, although bone marrow suppression is rarely seen with CNIs in contrast with MPA and azathioprine. The risk of malignancy may be increased after chronic use of CNIs[57,58]. Another important issue while using CNIs is that their metabolism by cytochrome P450 predisposes them to an array of drug interactions that may raise or lower levels. In addition, certain foods (for example grapefruit) that can alter levels of p-glycoprotein can affect the absorption of CNIs and cause significant changes in CNI drug level exposure.

Mycophenolate mofetil: Mycophenolate mofetil (MMF, Cellcept®) and its active compound MPA is a reversible purine synthase inhibitor with anti-proliferative activity against T-cells and B-cells. It blocks the signal 3 of cell activation. There are other presumed mechanisms for immunomodulator effects of this agent. MPA can inhibit monocyte chemo-attraction, destroy activated lymphocytes and induce immune tolerance by affecting regulatory T-cell/helper T-cell balance[59].

As an immunosuppressive agent, MMF was introduced to the field of LT in the 1990s. Its lack of potential nephrotoxicity made it useful for patients with renal dysfunction who need to decrease CNIs doses. MMF is also a useful agent in combination with CNIs in immunosuppressive regimens where corticosteroid withdrawal is desirable. In the United States, the use of MMF for adult LT has been increased to over 80% of transplantations in 2012 usually as an adjunct with CNIs[60]. MMF and an enteric coated formulation mycophenolate sodium (EC-MPS Myfortic®) are the two preparations of MPA available. The bioavailability of MMF is high and

monitoring of drug level is not usually recommended, though it may be useful in rare individuals. The average dose for MMF is 1 g every 12 h (720 mg every 12 h for EC-MPS), but it is better tolerated when is started with lower doses and gradually increased. Higher doses may be required but are not usually recommended. There are also reports on the non-linear pharmacokinetics of MPA with decreasing the bioavailability of drug by increasing the dose[59]. The major adverse side effects of MPA are hematologic and gastrointestinal (GI). Bone marrow suppression is usually dose dependent and responds to dose reduction. Nausea, vomiting, abdominal discomfort and diarrhea are common complaints in patients taking MPA derivatives. Dividing the dose to a four times a day schedule may be helpful; however, more serious adverse GI effects as inflammatory bowel disease-like colitis and graft *vs* host disease-like enteropathy related to MPA have been reported[61,62].

MPA seems to have a protective effect against post transplant *de novo* malignancies and when included in immunosuppression regimens may increase the time to develop malignancies[63], however, MPA was not shown to be effective in prevention of post liver transplant recurrence of HCC[64].

Azathioprine: Azathioprine is a purine synthase inhibitor and one of the first immunosuppressive agents used in the field of solid organ transplantation. For many years, azathioprine was included in the post organ transplant immunosuppressive maintenance as the only immunomodulatory agent and then later was used as an adjunct with CNIs. However, with the introduction of newer and more potent agents such as tacrolimus, the need for azathioprine was reduced and later it was replaced by MPA when a second agent was needed. Today, azathioprine is less commonly used for LT but may be helpful when there is a need for intensifying immunosuppression and when other agents are not tolerated due to their adverse side effects. In addition, as azathioprine is less costly, in some instances where finances are limited, it is preferred over MPA.

The major adverse side effects of azathioprine are related to bone marrow suppression and its hematologic consequences and hepatotoxicity. A minority of patients are at risk of developing severe bone marrow suppression due to genetically reduced or deficient thiopurine methyl transferase (TPMT) activity, the enzyme responsible for metabolizing 6-mercaptopurine, leading to over-accumulation of 6-thioguanine nucleotides (TGNs). TGNs are the active metabolites of azathioprine. Laboratory genotype or phenotype testing for TPMT may help to recognize these patients. In some other patients TGNs may fail to reach their therapeutic levels despite increasing drug dosage. In these patients who are at increased risk of hepatotoxicity due to accumulation of 6-methy-mercaptopurine (6-MMP), TGN level monitoring during treatment and the ratio of 6-MMP/TGN may be useful in helping to recognize this condition. The complexity, availability and cost of these tests should also be considered[65].

Mammalian target of rapamycin inhibitors: Sirolimus is a macrolide antibiotic which is structurally similar to tacrolimus and binds to FK binding protein but inhibits the mammalian target of rapamycin inhibitors (mTORI), the molecules with kinase activity, instead of inhibiting calcineurin. It acts through blocking signal 3 of cell activation from IL-2 receptors in T-cells and B-cells. Interestingly, despite binding to the same cell receptor, sirolimus and tacrolimus do not compete with each other and act synergistically[48]. Sirolimus was approved for use in renal transplantation by the Food and Drug Administration (FDA) in 1999. It has been considered a non-nephrotoxic immunosuppressant agent that might be replaced by CNIs in liver recipients with renal dysfunction; however, the benefit of this strategy has been questioned by some studies. In a randomized trial, Shenoy *et al*[66] demonstrated that although the early result of CNI replacing with sirolimus was promising, there was no significant renal function improvement after one year in liver recipients with renal dysfunction on sirolimus. No specific renal improvement benefit by CNI to sirolimus conversion was also demonstrated in another study by Abdelmalek *et al*[67], while higher biopsy proven rejections and treatment associated adverse side effects were seen in the sirolimus group. Even *de novo* use of sirolimus with low dose tacrolimus resulted in a high rate of graft loss, death and sepsis when compared with conventional tacrolimus dose, leading to the premature termination of a prospective randomized trial by Asrani *et al*[68]. Everolimus is another mTORI that when introduced early post LT and combined with low dose tacrolimus, showed promising results with respect to rejection rates and significant beneficial effects on renal function after 2 years[69]. However when used as a single agent without CNI, rejection rates were higher in the everolimus only group.

The other clinical utility of mTORIs in the field of LT is based on their anti-tumor effect. mTOR signaling plays a role in tumor angiogenesis and proliferation that is important in carcinogenesis of HCC[70]. In LT for HCC, sirolimus based immunosuppression was reported to be associated with lower tumor recurrence rate, longer recurrence-free survival and overall survival, and lower recurrence-related mortality compared with CNIs[71]. Everolimus has been used for cancer treatment in HCC. Its protective effect on post liver transplant HCC has not been sufficiently investigated in clinical trials, although reduced recurrence rate has been reported[72].

mTORIs are also possibly beneficial when included in immunosuppression regimen in recipients with malignancies other than HCC, but its use for this purpose needs to be explored by further studies[64].

The common adverse side effects related to mTORIs are edema, hyperlipidemia and oral ulcers. Another less common adverse side effect of mTORIs is proteinuria

and glomerular injury that when present may be associated with further impairment of renal function than was present in LT recipients after conversion of CNIs to mTORIs[73]. Acute respiratory distress syndrome has been reported as a rare but lethal complication associated with sirolimus[74,75]. Pleural and pericardial effusion are among other rare complications of sirolimus[76].

mTORIs are also capable of impairing the surgical wound healing and thus may contribute to more wound complications when introduced very early after solid organ transplantation[77].

Preliminary reports of high incidence of complications led to adding a black box warning to sirolimus by the FDA for and increased risk in mortality, graft loss and hepatic artery thrombosis (HAT) following its use in LT recipients. Most cases of HAT in patients who received sirolimus early on occurred within 30 d of LT. The incidence of HAT related to sirolimus use varies in different reports. Molinari *et al*[78] reported a higher incidence of HAT in sirolimus group with CNI group but no HAT was reported in LT recipients on sirolimus in another study by Harper *et al*[79]. Similar results were reported by Fischer *et al*[80], and they did notice no HAT following conversion of CNIs to everolimus.

If use of one of the mTORIs is considered for an individual LT patient, it is preferred that it be started at least 1 mo post-transplant to avoid the risk of HAT and allow enough time for wound healing. Concomitant use of CNIs and mTORIs is suggested in order to decrease the risk of rejection with mTORIs alone.

Immunosuppression in specific LT populations

Recipients with hepatitis C infection: HCV cirrhosis is one of the leading causes of LT in the world and the most common indication for LT in United States and Europe. Post transplantation recurrence of HCV and its complications are major causes of mortality and morbidity in this group of patients. HCV recurs in almost all LT recipients if the viral load is detectable at the time of transplant. In about 10% of LT recipients, recurrence of HCV is associated with rapidly progressive fibrosis leading to early graft loss. HCV recurrence in the remaining patients results in rapidly progressive fibrosis and cirrhosis in approximately 30% of patients within 5 years after LT[81].

Factors influencing the complications resulting from HCV recurrence are multiple and include host, donor, viral and immunosuppression related factors[82,83]. Recipient's immune response, donor age, pre-transplant viral load and genotype are among implicated important factors.

The role of immunosuppression and tailoring the regimen for HCV recipients are challenging. There is evidence for association of early and severe recurrence of disease following treatment of steroid-responsive acute rejection episodes in liver recipients[83]. Numbers and severity of rejection episodes (both steroid responsive and steroid resistant) are also associated with early recurrence[84].

The beneficial effect of antibody induction in order to avoid steroids in HCV patients has not been documented, although its use has been thought to lower rates of acute cellular rejection. Several studies have shown the safety of ATG and daclizumab as induction agents in HCV patients who undergo LT[49,50].

In the trial by Klintmalm *et al*[50], severe fibrosis was less frequently observed in HCV recipients receiving steroid free immunosuppression with daclizumab induction compared with a steroid containing regimen at 1 year, but the difference between the groups was not statistically significant. In the same trial, patients without HCV recurrence in the daclizumab group at 1 year had a significantly reduced frequency of severe fibrosis at 2 years after LT[50].

It can be concluded that antibody induction is safe in HCV liver recipients and even may be associated with some benfits in this group of patients, but this needs to be documented in future studies. However, it should be considered that the safety of alemtuzumab in HCV patients is in doubt (as mentioned above).

The role of CNIs on the course of post transplantation HCV recurrence is challenging. There is evidence of *in vitro* anti replicative effect of cyclosporine on HCV RNA[85,86]. Cyclosporine has also been reported to show less interferrence with the anti-viral effect of interferon-α in HCV infected human hepatocytes when compared with tacrolimus[87]. The results of studies comparing the effect of cyclosporine and tacrolimus on post transplant HCV recurrence, fibrosis progression and sustained virologic response after treatment are conflicting[88].

However, improved long term patient survival was reported with tacrolimus compared to cyclosporine in HCV patients[89] and also, in a UNOS database analysis of 8809 HCV liver recipients by Irish *et al*[90] where patients on cyclosporine were reported to be at increased risks for patient death, graft loss and biopsy proven acute rejection compared to those on tacrolimus.

Use of mTORI, sirolimus in post LT immunosuppression regimens has raised some interest for its potential benefit in HCV patients. Sirolimus has proven anti-fibrotic effects in animal models[91,92] and has been shown to be associated with a reduced risk of significant fibrosis in post liver transplant HCV[93,94]. However, despite promising results for sirolimus on fibrosis progression in HCV patients post LT, use of sirolimus has been reported to be associated with increased mortality and graft loss both in HCV and non-HCV liver recipients and its use solely for this purpose could not be recommended[95].

Regarding the conflicting results of choice of immunosuppression on outcomes for HCV liver recipients, the advent of new and potent direct acting antiviral agents for the treatment of HCV and their utilization in pre and post-transplant HCV therapy may eliminate the need to focus on the effect of the immunosuppression regimen on HCV recurrence and progression in LT recipients.

Post LT pregnancy: With growing number of LTs as

the curative treatment for end-stage liver diseases, more female candidates in child-bearing ages receive organs. There are many issues regarding the management and outcome of pregnancy after solid organ transplantation. Safe contraception, fertility, maternal risks, risk of miscarriage, fetal and neonatal outcome and risk of immunosuppression are among the most important ones.

Post liver transplant pregnancies are classified as high-risk pregnancies due to the increased rate of complications that include hypertension, preeclampsia and pre-term delivery[96].

Fewer complications may be expected when the pregnancy is planned. The time of conception is advised to be at least one year after transplantation, and some suggest waiting 2 years for a better outcome[96].

Overview and management of immunosuppression in liver transplanted female candidates for pregnancy need expertise to balance the risk of rejection and maternal and fetal complications.

Corticosteroids: If the patient is maintained on a low dose of corticosteroids due to the underlying liver disease etiology, like autoimmune disease, or because she has experienced episodes of rejection, there might be a need for an increased dose of steroid during pregnancy[97].

The risks related to use of steroids are gestational diabetes and hypertension that warrant special attention and management.

CNIs: CNIs are the main agents as maintenance immunosuppression in most LT recipients. Potential complications related to CNIs that may adversely affect the maternal outcome are hypertension, diabetes, renal insufficiency and neurotoxicity. Rate of these complications could be minimized by careful drug level monitoring during pregnancy. Reports on comparing the complications resulting from cyclosporine based *vs* tacrolimus based immunosuppression during pregnancy are conflicting. However, both agents seem to be safe during pregnancy and the complication rates may not be significantly different[98].

Azathioprine: Azathioprine is now less frequently used as an immunosuppressant agent in LT. When patients are on this medication, it seems to be reasonable to continue this agent during pregnancy as there is a low rate of reported risk[97].

MMF: MMF is teratogenic and is reported to be associated with multiple fetal defects and increased risk for miscarriage. It is advised to stop MMF in patients on this medication who wish to get pregnant at least 6 wk before conception.

For female liver recipients on MMF who have a plan for pregnancy, switching of MMF to another immunosuppressant agent rather than abrupt discontinuation may be considered in order to decrease the risk of acute rejection. Although the experience in the field of solid organ transplantation is limited, switching of MMF to azathioprine was reported to be associated with a favorable obstetric outcome in pregnant patients with lupus nephritis[99].

Sirolimus: Data on the safety of sirolimus during pregnancy and its teratogenicity is limited, although no significant fetal malformation has been reported[100].

Recipients with HCC

Prevention and management of post LT recurrence of HCC is of great concern as more patients with HCC are receiving LT. Many factors can affect the risk of tumor recurrence. The role of immunosuppressive therapy in HCC recurrence is an important and challenging issue.

CNIs possess pro-oncogenic effects as documented in experimental models and also in clinical trials both retrospective and prospective[64]. Both tacrolimus and cyclosporine are associated with increased risk of *de novo* malignancies in solid organ transplantation. The risk of post liver transplant HCC recurrence has been shown to be related to the high blood levels of CNIs, particularly in the early post transplant period rather than the type of CNI[64,101].

The results of studies comparing the effect of the type of CNI (cyclosporine *vs* tacrolimus) are conflicting and it seems that the role of dosage and blood levels of CNIs is more important than the choice of agent[64,101,102].

Anti-lymphocyte antibodies which are increasingly used for induction of immunosuppression or treatment of steroid resistant rejections are also of concern for increasing the risk of post transplant HCC recurrence. Use of muromonab (although not available anymore) and ATG has been shown to be associated with increased risk of HCC recurrence[103].

Also, Basiliximab has been shown to have a negative impact on tumor recurrence when used as an induction therapy in HCC patients receiving LT[104].

MMF possesses anti-proliferative properties but has not been documented to play a role in prevention of HCC recurrence[64,102].

mTORIs (sirolimus and everolimus) possess anti-tumor properties (refer to above section of Maintenance of Immunosuppression) and are of particular interest for use in LT patients with HCC. The results of preliminary studies on both sirolimus and everolimus and their beneficial effect on post transplantation recurrence of HCC are promising[70-72,105].

Although many centers are already using mTORIs in recipients with HCC, the result of prospective, randomized control trials are required for recommendation on their use for this purpose.

Pediatric patients

In children both pharmacokinetic and pharmacodynamic of drugs are different from adults. All stages of drug

absorption, distribution, metabolism and excretion are affected by a child's age and will change when they reach adolescence. The major issue for post transplant immunosuppressive therapy in pediatrics is the lack of adequate control trials in this population of patients. Most of the immunosuppressive drugs used for pediatric liver recipients are used based on adult data. However, generally in younger children the clearance of most drugs is increased and most children need higher weight based doses of immunosuppressive medication.

CNIs, tacrolimus and cyclosporine are approved for use in pediatric liver recipients[106]. Tacrolimus based regimen is superior to cyclosporine based with significant improvement in patient and graft survival, less rejections, less hypertension and decreased dose of required corticosteroids.

Other immunosuppressant agents as MMF or mTORIs may be used off-label in pediatric LT for specific indications. Confronting the adverse side effects of CNIs, need for an auxiliary agent for management and further prevention of acute rejections or withdrawal from corticosteroids are among these indications.

T-cell depleting antibodies as ATG and IL-2Ra are increasingly used in children by some LT centers for indications similar to adults, though the experience with their use in this population is limited[106,107].

There are major issues specific for pediatric patients receiving post transplant immunosuppression that need special consideration.

Linear growth may be affected by immunosuppression therapy especially with corticosteroids. Thus, there is a desire for steroid-free immunosuppression or rapid withdrawal in pediatrics if possible[108].

Children are at increased risk of developing PTLD in comparison with adult due to higher risk of primary exposure to Epstein-Barr virus (EBV). Prolonged high-dose immunosuppressive therapy is also a major risk factor[106]. Special attention is needed for prevention of this complication (EBV screening) and early diagnosis in pediatric patients.

Monitoring of the immune status

Treatment of rejection: With the advent of high efficacy immunosuppression regimen, the rate of acute cellular rejection (ACR) has been decreasing but still complicates up to about 25% of liver transplants, with less frequent rejections rates following living liver donation[9,10].

The diagnosis of ACR should be considered when there is evidence of abnormal liver tests and confirmed by liver biopsy. The other possible causes of abnormal liver test in a liver transplant patient should always be considered in the differential diagnoses and excluded. Among these, the most important are HAT, primary graft non-function, biliary leakage and sepsis in the early post transplant phase. Primary disease recurrence, particularly for HCV, viral infections such as CMV and EBV, recurrence of HBV, biliary strictures and drug toxicity occur later on post transplantation. An important challenge exists in differentiating HCV recurrence from acute cellular rejection, though acutely higher viral loads and presence of apoptotic bodies on biopsy favor HCV over rejection.

The histologic grading of acute cellular rejection is not always correlated with clinical outcomes[109]. It also may not precisely predict the response to medical management[110].

High dose *iv* corticosteroid is the first line of treatment for moderate to severe ACR in most centers. About 60%-80% of episodes of ACR are responsive to the first course of high dose corticosteroids, and the remainder may need more than one course or might be resistant to steroids. The method of corticosteroid pulse therapy (dosage and duration) varies between centers. Usually a 3 d course of 500-1000 mg of methylprednisolone per day is given to the patient. Volpin *et al*[111] have suggested a single dose of 1000 mg methylprednisolone followed by a 6 d tapering from 200 to 20 mg/d is more effective and associated with less infectious complications.

About 5%-15% of episodes of liver allograft ACRs are unresponsive to more than one course of dose steroids and thus called steroid resistant rejections (SRR). SRR episodes although rare are associated with poor outcomes[112].

Muromonab was among the first agents used in liver SRR with acceptable success rate; however, with withdrawal of muromonab from the market, this agent is no longer available. Following the promising results from renal transplantation, the polyclonal antibody, ATG was used for the treatment of SRR in most LT centers. The results have been promising with most cases of SRR responding to ATG[27,28]. For treatment of rejection, ATG is usually started with a dose of 1.5 mg/kg per day intravenously. The duration of treatment is variable between centers and also for any individual case ranging from 5 to 14 d. The absolute lymphocyte count may be monitored to achieve a goal of 200/mm^3 or less.

The important issue in the treatment of SRR is monitoring for the treatment associated adverse side effects. Patients who have received treatment for SRR are at increased risk of infectious complications and malignancy.

IL-2Ras have been used for the treatment of SRR in LT. There are reports of successful treatment of SRR using basiliximab both in pediatric and adult LT groups[113,114]. Daclizumab has been also used as the rescue therapy for SRR with good results[115]. The promising results of using IL-2Ra for the treatment of SRR in LT and the limited related complication rates has been resulted in increasing use of these agents and their preference over anti-lymphocyte antibodies for this purpose[107].

Increasing the individual base level of immuno-suppression is usually required in cases of ACR. This usually includes one or more of the following strategies:

(1) Adjusting the CNIs to higher trough levels, if there is no contraindication; (2) Changing CNI to tacrolimus if the patient is already on cyclosporine; (3) Adding MMF (or azathioprine) or increasing their dose; and (4) Adding mTORI.

Withdrawal of immunosuppression: Withdrawal of immunosuppression is of particular interest with the aim of minimizing the adverse side effects and improving the quality of life in LT patients and the unique immune tolerant character of the liver makes this potentially possible. According to the published data, weaning of immunosuppression may be possible in up to 40% of liver recipients[116,117].

The success rate of weaning immune suppression may be even higher in pediatric patients receiving parental organs[14]. However, it is important to select the right candidate for weaning and perform the weaning at the best time.

The most important factor that correlates with successful immunosuppression weaning is the time from transplantation[8,118]. Other factors that have been implicated to have a role in a successful weaning in other studies are male gender, older age at the time of transplantation, and some have suggested the significance of biochemical indices as lower HLA mismatch between donor and recipient[117] and indexes of lymphocytes stimulation[8]. It should be considered that candidates for immunosuppression weaning have been carefully selected in most studies among patients with non-immune causes of liver disease, those with stable post transplantation liver function and less rejection episodes.

In general, it could be stated that complete immunosuppression withdrawal is possible in highly selected liver recipients. Further studies are required to identify the important clinical and paraclinical factors for a confident and successful weaning.

CONCLUSION

Transplant immunology is an ever changing science. Further elucidation of interacting cellular mechanisms involved in rejection and graft tolerance would be welcomed by transplant physicians and patients alike. The aim of treatment is to maximize the effectiveness and minimize the adverse side effects of immunosuppressive therapy with the hope for a higher quality and increased longevity of the graft and patient's life. Any individual recipient needs a customized immunosuppression regimen according to: (1) age that will change over time; (2) the co-morbid conditions as renal failure and diabetes with the preference of CNI minimizing or avoiding; (3) transplantation indications as autoimmune hepatitis that need a longer duration of steroid therapy, HCV infection requiring early steroid withdrawal and concomitant HCC with possible benefit from mTORIs; (4) the behavior of the graft over time; intensifying the immunosuppression with repeated rejection episodes and lowering in the absence of rejection; (5) occurrence of immunosuppression related complications with the need for lowering the dose or changing the specific agents to another one; and (6) physiologic conditions after transplantation as pregnancy, weight loss or gain requiring changes in dosage or agent choice.

Future studies would enable the art of individually designed immunosuppression for individual transplant recipients.

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REVIEW

Non-alcoholic fatty liver disease - the heart of the matter

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is one of the most common forms of chronic liver disease in the Western world. There is a close association with the metabolic syndrome and NAFLD is considered to be the hepatic manifestation of the metabolic syndrome. The components of the metabolic syndrome include hypertension, obesity and insulin resistance which are well established cardiovascular risk factors. The mortality rate of NAFLD patients from myocardial infarction is higher than that in the general United States population and there is also an increased risk of non-fatal cardiovascular events. This article reviews the cardiovascular complications associated with NAFLD. In order to provide comprehensive care of NAFLD patients, physicians need to be aware of, and search for, the cardiac morbidity associated with NAFLD.

Key words: Cardiovascular; Diastolic dysfunction; Sleep apnea; Palatin-like phospholipase domain containing 3 gene; Non-alcoholic fatty liver disease

Core tip: Non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of the metabolic syndrome. Due to the overlapping cardiovascular risk factors in the metabolic syndrome, there are cardiovascular consequences linked to the presence of NAFLD in a patient. We review these complications and also a less well appreciated complication of diastolic dysfunction that is intimately associated with NAFLD. Physicians looking after NAFLD patients need to be aware of these complications and actively search for and treat them.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is now considered to be one of the most common forms of chronic liver disease in the Western world. NAFLD refers to a clinicopathologic spectrum of conditions ranging from simple steatosis (simple fatty liver) to non-alcoholic steatohepatitis (NASH), involving inflammation and some evidence of liver cell damage, and in some cases, cirrhosis. It occurs in an estimated 25% to 30% of the United States general population, whereas its potentially progressive form, NASH is reported in 2%-3% of the population[1].

Table 1 Definitions of the metabolic syndrome

	NCEP ATP Ⅲ	IDF
Absolutely required	None	Central obesity (waist circumference) ⩾ 94 cm in males or ⩾ 80 cm in females European origin > 90 cm (men), ⩾ 80 cm in females
Criteria	Any three of the five criteria below	Central obesity plus two of the four criteria below
Obesity	Waist circumference > 40 inches in males, or > 35 inches in females	
Hyperglycemia	Fasting glucose ⩾ 100 mg/dL or treated for DM	Fasting glucose ⩾ 100 mg/dL
Dyslipidemia	TG ⩾ 150 mg/dL or treated for dyslipidemia Or HDL cholesterol < 40 mg/dL in males, or < 50 mg/dL in females or under treatment	TG ⩾ 150 mg/dL or treated for dyslipidemia Or HDL cholesterol < 40 mg/dL in males, or < 50 mg/dL in females or under treatment
Hypertension	> 130 mmHg systolic or > 85 mmHg diastolic or treated for HTN	> 130 mmHg systolic or > 85 mmHg diastolic or treated for HTN

NCEP ATP Ⅲ: National cholesterol and education program-adult treatment panel Ⅲ; IDF: International diabetes federation; TG: Triglycerides; HDL: High density lipoprotein; HTN: Hypertension.

Natural history studies of NAFLD showed that 1%-5% of patients with simple steatosis developed cirrhosis[1,2], while patients with NASH showed pathological progression of fibrosis in 15%-39% within 10 years[3,4]. NASH can potentially progress to cirrhosis and its complications including decompensated liver disease and hepatocellular carcinoma. The mortality rate of NAFLD patients in the community was higher than that in the general ultrasound (US) population[5]. Death occurred ranging from 13% to 45% with mean follow up of 8-11 years and coronary artery disease (CAD) was the leading cause of death (25%-28% of mortality)[5,6].

It is the purpose of this article to review the cardiovascular consequences of NAFLD and to suggest a diagnostic approach.

GENETICS

Advances in genome analysis, including the development of informative genetic markers, improved physical mapping methods and improvements in high throughput genotyping technologies, have contributed to the understanding of the pathogenesis of complex diseases. The Dallas Heart Study carried out a genome-wide association study of liver fat content in 2111 people from different ancestry groups[7]. They found a connection between NAFLD evaluated by proton magnetic resonance spectroscopy, with the rs738409 G allele of the palatin-like phospholipase domain containing 3 gene (PNPLA3), also known as adiponutrin. The sequence variation is a C > G single nucleotide change which encodes for the 148 isoleucine to methionine protein variant (Il48M) of PNPLA3.

The PNPLA3 GG genotype has been associated with a higher severity of carotid atherosclerosis in young patients with NAFLD[8]. However, recently a variant of the *TM6SF2* gene (E167K0) has been shown to be linked to fatty liver due to reduced secretion of very low-density lipoprotein lipoproteins. This variant has also been shown to provide protection against cardiovascular disease[9].

This suggests that there is likely to be an effect of multiple genetic polymorphisms in the development of NAFLD, which require further study in diverse populations.

METABOLIC SYNDROME

The metabolic syndrome (MS) is defined by differing criteria in the United States, Europe and Asia (Table 1). The essential components of the MS include visceral obesity, insulin resistance, dyslipidemia and hypertension. Along with the epidemic of obesity, the prevalence of MS is increasing worldwide, both in the developing and developed countries. MS is associated with a risk of cardiovascular disease and is a common early abnormality in the development of type 2 diabetes.

In patients with NAFLD, metabolic abnormalities have been reported in 33% to 100% of cases, depending on study methods and selection criteria of NAFLD patients[5,10,11]. MS components including central obesity, hypertension, hypertriglyceridemia, decreased high density lipoprotein cholesterol and impaired glucose test or type 2 diabetes mellitus are commonly found in NAFLD[10,12]. Diabetes and hypertension are present up to 15-fold in patients with NASH compared to those with steatosis alone independent of age or body mass index (BMI)[13].

Thus the metabolic syndrome is strongly linked to NAFLD. Patients presenting with NAFLD need to be examined for the presence of the components of the metabolic syndrome and their complications.

NAFLD AND CARDIOVASCULAR ABNORMALITIES

Since the MS is linked to many well recognized cardiovascular risk factors, it is to be expected that there will be a high prevalence of cardiovascular morbidity in patients with NAFLD (Figure 1). Retrospective studies of cohorts of NAFLD patients have shown myocardial

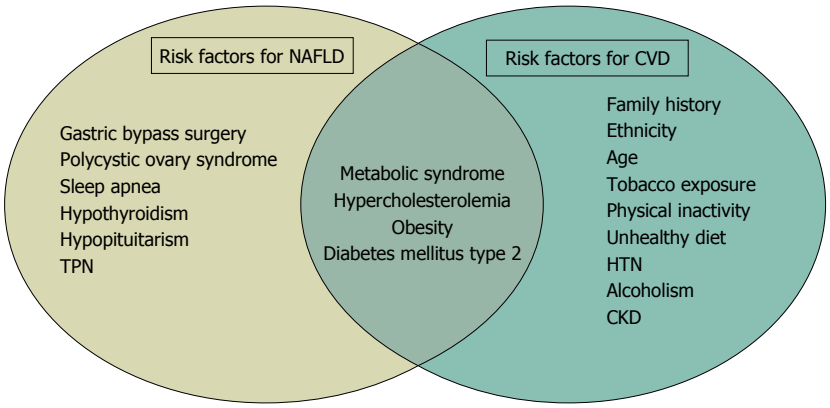

Figure 1 Confluence of the risk factors for both non-alcoholic fatty liver disease and cardiovascular disease. NAFLD: Non-alcoholic fatty liver disease; TPN: Total parenteral nutrition; HTN: Hypertension; CKD: Chronic kidney disease; CVD: Cardiovascular disease.

infarction to be the cause of death in 25% compared to 13% in patients with other liver diseases[5]. Another study with biopsy-proven NAFLD found an increased mortality in patients with NASH but not with simple steatosis and that this was primarily due to cardiovascular disease and not liver-related causes[11]. Similar results have been reported from other groups[6,11,14]. These studies had small cohort sizes. In a study with a larger cohort size, cardiovascular disease remained the number one cause of death but there was no difference detected between those patients with NASH or simple steatosis.

Prospective studies are usually considered as of higher quality than retrospective studies. Several prospective studies have shown an increased risk of either non-fatal cardiovascular disease events or mortality[15-22].

Coronary artery disease in asymptomatic people can be detected by computed tomography. Several studies have found a connection between coronary artery calcification and NAFLD[23-27], but 2 studies did not find a significant association[28,29].

Patients with NAFLD have a higher prevalence of CAD independent of other risk factors, including glycemic control and MS components[10,26]. The incidence of new CAD events in non-cirrhotic patients with NAFLD varied from 2% to 11% with the overall mortality of 12%-13%[6,10,16,19,30,31]. The CAD related mortality ranged from 1% to 3% in NAFLD[19,31], and from 12% to 16% in patients with NASH[6,32].

The mechanism for the increase in atherogenesis in NAFLD is multi-factorial including genetic predisposition, insulin resistance and atherogenic dyslipidemia, oxidative stress, chronic inflammation, reduced levels of adiponectin, and altered production of pro and anticoagulant factors[33].

NAFLD AND LEFT VENTRICULAR FUNCTION

Our group was the first to report an association between NAFLD and impaired left ventricular diastolic function[34]. We examined 38 patients with NAFLD diagnosed by ultrasound less than 55 years of age, with a normal exercise test and no diabetes or hypertension. They were compared to an age and gender matched control group. The NAFLD patients had altered left ventricular (LV) geometry and early features of LV diastolic dysfunction. On multivariate analysis only early diastolic velocity, assessed on tissue Doppler imaging was found to be associated with NAFLD. This has also been found by other centers[35-41]. Furthermore this may be an early consequence of NAFLD.

Fallo *et al*[35] reported diastolic dysfunction in a group of 48 never-treated hypertensive patients with ultrasound diagnosed NAFLD compared to 38 with no NAFLD, who just had LV hypertrophy (LVH). This was independently linked to NAFLD and HOMA on multivariate analysis.

Fotbolcu *et al*[36] examined 35 NAFLD patients by tissue Doppler imaging that did not have either hypertension or diabetes mellitus. They found a lower early diastolic velocity and also a higher systolic velocity. Another group has found a higher incidence of LVH in hypertensive NAFLD patients (diagnosed by ultrasound) compared to the hypertensive patients without NAFLD[42]. The finding of early diastolic dysfunction in patients with NAFLD has also been found in a study comparing 38 diabetic patients with US diagnosed NAFLD to 18 diabetic patients without NAFLD. The patients with NAFLD had early features of diastolic dysfunction on tissue Doppler echocardiography, which was significant after adjusting for hypertension and other cardiometabolic factors[37]. Furthermore, in obese adolescents, the presence of NAFLD has been shown to be an early marker of cardiac dysfunction[43]. A larger study of 180 obese adolescents compared to 68 healthy controls employing pulsed-wave Doppler echocardiography and pulsed-wave tissue Doppler imaging showed, the NAFLD group had normal LV systolic function, impaired diastolic function, and altered global systolic and diastolic myocardial performance compared to 68 healthy controls[44].

Another study on obese children and adolescents with NAFLD included 108 obese children, 54 with hepatic fat fraction over 5% on magnetic resonance imaging and 18 lean and healthy subjects. Forty one of the NAFLD patients also underwent liver biopsy and 26

Table 2 Non-alcoholic fatty liver disease and cardiac structure and function

Ref.	Methods	Results
Goland *et al*[34]	38 patients with NAFLD, < 55 years of age and normal exercise test, were compared with an age and sex-matched control group TT echo study including TDI	Patients with NAFLD have mildly altered LV geometry (Increased thickness of the intraventricular septum, posterior wall, and larger LV mass), and early features of left ventricular diastolic dysfunction. Early diastolic velocity on TDI is the only index identifying the patients with NAFLD and metabolic syndrome
Fallo *et al*[35]	Left ventricular morphology/function, metabolic parameters and NAFLD in 86 never-treated essential hypertensive patients subdivided into two subgroups according to the presence (n = 48) or absence (n = 38) of NAFLD at ultrasonography	Patients with NAFLD had similar prevalence of LVH compared to patients without NAFLD, but a higher prevalence of diastolic dysfunction
Fotbolcu *et al*[36]	35 non-diabetic, normotensive NAFLD patients and 30 controls. TT echo and TDI performed	Patients with NAFLD have impaired LV systolic and diastolic function and lower E' (early diastolic velocity on TDI)s. TDI systolic velocity (S' on TDI) values were lower in NAFLD
Bonapace *et al*[37]	50 patients with type 2 DM, US diagnosed NAFLD. 32 patients (64%) with NAFLD, compared to other 18 patients. TT echo and TDI performed	Early features of LV diastolic dysfunction may be detected in patients with type 2 diabetes and NAFLD
Kim *et al*[38]	1886 participants without CVS disease. Stratified by the presence or absence of CT-diagnosed NAFLD, MetS. Assessed by TDI, carotid ultrasound and baPWV	Subjects with both NAFLD and MetS had a higher E/Ea ratio and baPWV, and lower TDI Ea velocity (P < 0.001). Subjects with either NAFLD or MetS also showed significant differences in TDI Ea velocity and baPWV (P < 0.05). No significant differences of CIMT values
Ozveren *et al*[39]	59 patients with NAFLD and 22 healthy subjects as controls. Basal electrocardiography, echocardiography, and treadmill exercise testing were performed on all patients and controls	The heart rate recovery index is deteriorated in patients with NAFLD
Petta *et al*[41]	Anthropometric, biochemical and metabolic of 147 consecutive biopsy-proven NAFLD cases	Diastolic posterior-wall thickness, left ventricular mass, relative wall thickness, left atrial volume, as well as ejection fraction, lower lateral TDI e', E/A ratio and epicardial fat linked to severe liver fibrosis
Mantovani *et al*[42]	116 consecutive patients with hypertension and type 2 diabetes. US diagnosed NAFLD, LVH diagnosed by TT echo	LVH higher among diabetic patients with NAFLD. NAFLD is associated with LVH independently of classical CVS risk factors
Singh *et al*[43]	IHTG content (magnetic resonance spectroscopy), insulin sensitivity and β-cell function, and left ventricular function (speckle tracking echocardiography) among 3 groups adolescents: (1) lean-BMI = 20 ± 2 kg/m^2); (2) obese with normal IHTG content, BMI = 35 ± 3 kg/m^2); and (3) obese with increased IHTG content, BMI = 37 ± 6 kg/m^2	The disposition index (β-cell function) and insulin sensitivity index were approximately 45% and about 70% lower, respectively, and whole body insulin resistance, was about 60% greater, in obese than in lean subjects, and about 30% and about 50% lower and about 150% greater, respectively, in obese subjects with NAFLD than those without NAFLD (P < 0.05 for all)
Sert *et al*[44]	80 obese adolescents and 37 lean subjects. NAFLD based on elevated transaminases	LV mass and CIMT higher in both NAFLD and non-NAFLD obese patients compared to lean children
Pacifico *et al*[45]	TDI, and MRI for measurement of HFF and abdominal fat mass distribution in 108 obese children, 54 with (HFF ⩾ 5%) and 54 without NAFLD, and 18 lean healthy subjects. 41 of the children with NAFLD underwent liver biopsy	Asymptomatic obese children with NAFLD exhibit features of early LV diastolic and systolic dysfunction, and are more severe in those with NASH
Kocabay *et al*[46]	55 biopsy-proven NAFLD patients and 21 healthy controls. Categorized as simple steatosis, borderline NASH, definitive NASH	LA-Res, LA-Pump and LA-SR(A) were lower in the NAFLD *vs* control. LA-Res and LA-pump significantly lower in NAFLD subgroups. There were significant differences in LA-SR(A) between healthy controls compared with simple steatosis and borderline
Karabay *et al*[47]	55 NAFLD patients and 21 healthy controls. Biopsy-proven NAFLD. Categorized as simple steatosis, borderline NASH, definitive NASH. All had echocardiography	Patients with NAFLD and its subgroups have evidence of subclinical myocardial dysfunction in relation to the presence of insulin resistance
Gianotti *et al*[48]	171 subjects aged > than 65 yr. US diagnosed NAFLD and TT echo	NAFLD had borderline significant association with higher end-diastolic thicknesses of left-ventricle edPW and right-ventricle wall
Perseghin *et al*[49]	21 nondiabetic men with or without fatty liver matched anthropometrically features assessed by (1) cardiac MRI; (2) cardiac P-MRS; and (3) hepatic H-MRS to assess quantitatively the IHF content	In newly diagnosed patients with fatty liver, fat accumulated in the epicardial area and despite normal LV morphological features, systolic and diastolic functions, there was abnormal LV energy metabolism
Hallsworth *et al*[50]	19 adults with NAFLD were age-, sex-, and BMI-matched to healthy controls. Cardiac structure and function assessed by high-resolution cardiac MRI. High-energy phosphate metabolism was assessed using[31] P-MRS	Adults with NAFLD had significantly thicker left ventricular walls at systole and diastole than those without fatty liver and showed decreased longitudinal shortening. The eccentricity ratio was significantly higher in the NAFLD group indicating concentric remodelling. Peak whole wall strain was higher in the NAFLD, as was peak endocardial strain. Cardiac metabolism, measured by PCr/ATP ratio, was not altered in NAFLD

NAFLD: Non-alcoholic fatty liver disease; TDI: Tissue Doppler imaging; DM: Diabetes mellitus; CT: Computed tomography; MetS: Metabolic syndrome; baPWV: Brachial-ankle pulse wave velocity; BMI: Body mass index; IHTG: Intrahepatic triglyceride; HFF: Hepatic fat fraction; NASH: Non-alcoholic steatohepatitis; MRI: Magnetic resonance imaging; MRS: Magnetic resonance spectroscopy; CIMT: Carotid intima-media thickness; TT: Transthoracic; US: Ultrasound; CVS: Cardiovascular; LV: Left ventricular; LVH: Left ventricular hypertrophy.

were shown to have NASH. Diastolic dysfunction was again found in the patients with NAFLD and also the Tei index which reflects combined systolic and diastolic LV dysfunction was significantly higher in those children with NAFLD. Those patients with biopsy-proven NASH had a significantly lower e' velocity and higher E-to-e' and Tei index than those with only NAFLD[45].

The use of 2D speckle tracking echocardiography to determine left atrial deformation parameters was not found to be helpful in a recent study on 55 NAFLD patients and 21 controls from Turkey[46,47]. These studies are summarized in Table 2.

A finding of NAFLD on ultrasound in the population aged more than 65 years of age may be valuable to alert for the coexistence of multiple cardiovascular risk factors and changes in cardiac morphology and diastolic dysfunction[48].

In newly diagnosed individuals with fatty liver, both systolic and diastolic LV functions were normal but there was abnormal LV energy metabolism[49]. A cohort of 19 NAFLD adults compared to age, gender and BMI-matched controls were shown to have a thicker left ventricular wall in both systole and diastole as well as decreased longitudinal shortening than those without fatty liver[50].

One of the consequences of diastolic dysfunction is atrial fibrillation and NAFLD has been shown to have an increased risk (OR = 4.49) of atrial fibrillation which was independent of age, gender, hypertension, and left ventricular hypertrophy and pr interval[51].

Thus adults with NAFLD have changes in cardiac structure and function that may predate overt cardiac artery disease. We suggest that NAFLD patients undergo a routine transthoracic echocardiogram examination as part of their assessment.

NAFLD AND OBSTRUCTIVE SLEEP APNEA SYNDROME

Obstructive sleep apnea (OSAS) syndrome is a common condition with prevalence estimates of 2% to 4% in the general population. Amongst obese patients the prevalence is as high as 35%[52]. OSAS is accompanied by proinflammatory cytokine production, platelet aggregation, endothelial dysfunction and metabolic dysregulation which can increase the risk of cardiovascular disease[53].

OSA has been shown to be a risk factor for insulin resistance[54-60] and carotid intima media thickening[61], and in addition there are reports of ischemic hepatitis accompanying severe OSAS[62,63].

A study of 163 non-drinking OSAS patients found that the more severe the OSAS, sufferers had more insulin resistance and more steatosis and fibrosis[64]. This was independent of weight, It has also been shown that continuous positive airway pressure (CPAP) therapy for OSAS improves cardiac function, decreases cardiovascular risk and improves insulin resistance[7].

Furthermore, OSAS is linked to NAFLD. This association is more than just a convergence of risk factors. There is evidence for a derangement of the immune system in such patients resulting in a low-grade chronic inflammation[65]. Uncontrolled treatment studies of adults and children with OSAS receiving CPAP treatment and tonsillectomy have shown a decrease in liver enzymes[66]. In addition a proportion of NAFLD patients without severe obesity are at risk for OSAS and this is associated with the severity of the liver damage independently of BMI and other co-factors[67].

Thus the finding of NFALD should prompt physicians to inquire regarding symptoms of OSAS. In addition patients with OSAS need to be assessed for the presence of NAFLD.

CONCLUSION

NAFLD is considered to be the hepatic manifestation of the metabolic syndrome, although there are cases that do not fulfill the metabolic syndrome criteria. Patients with NAFLD have an increased risk for cardiovascular disease, which is in part due to the overlapping of the risk factors of the metabolic syndrome with the risk factors for cardiovascular disease. In addition, however, there are changes in left ventricular function both systolic and diastolic, endothelial function, arterial stiffness and a link with sleep apnea syndrome.

Physicians treating NAFLD patients need to be aware of these cardiovascular complications and actively search for them in order to provide the comprehensive care that patients deserve. We suggest that all patients with NAFLD have an echocardiogram performed in order to detect LVH, systolic and diastolic dysfunction which may increase the risk for cardiac mortality. The benefit of treatment of the metabolic syndrome and associated NAFLD likely extends to many aspects of the cardiovascular system as well. Thus an integrative approach considering all the possible organ complications of the metabolic syndrome is necessary for NAFLD patients.

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REVIEW

Hepatitis C virus: Virology, diagnosis and treatment

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Abstract

More than twenty years of study has provided a better understanding of hepatitis C virus (HCV) life cycle, including the general properties of viral RNA and proteins. This effort facilitates the development of sensitive diagnostic tools and effective antiviral treatments. At present, serologic screening test is recommended to perform on individuals in the high risk groups and nucleic acid tests are recommended to confirm the active HCV infections. Quantization and genotyping of HCV RNAs are important to determine the optimal duration of anti-viral therapy and predict the likelihood of response. In the early 2000s, pegylated interferon plus ribavirin became the standard anti-HCV treatment. However, this therapy is not ideal. To 2014, boceprevir, telaprevir, simeprevir, sofosbuvir and Harvoni are approved by Food and Drug Administration for the treat of HCV infections. It is likely that the new all-oral, interferon-free, pan-genotyping anti-HCV therapy will be available within the next few years. Majority of HCV infections will be cured by these anti-viral treatments. However, not all patients are expected to be cured due to viral resistance and the high cost of antiviral treatments. Thus, an efficient prophylactic vaccine will be the next challenge in the fight against HCV infection.

Key words: Hepatitis C virus; Diagnosis; Treatment; Hepatocellular carcinoma; Nucleic acid test; Enzyme immunoassay; Interferon; Direct acting antivirals; Host-targeted agents; Sofosbuvir

Core tip: Understanding the general properties of hepatitis C virus (HCV) viral RNA and proteins facilitates the development of sensitive diagnostic tools and effective antiviral treatments. At present, serologic screening test is recommended to perform on individuals in the high risk groups and nucleic acid tests are recommended to confirm the active HCV infections. To 2014, in addition to pegylated interferon and ribavirin, boceprevir, telaprevir, simeprevir, sofosbuvir and Harvoni are approved by Food and Drug Administration to treat HCV infections. The majority of HCV infections can be cured by these anti-viral treatments. An efficient prophylactic vaccine will be the next challenge in the fight against HCV infection.

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INTRODUCTION

Hepatitis C virus (HCV) accounts for approximately 15%-20% cases of acute hepatitis. After acute infection, around 50% to 80% of HCV patients will develop chronic infection. Approximately, HCV infects 170 million individuals worldwide (http://www.who.int). Chronic hepatitis C (CHC) patients are at high risk to develop life-threatening complications, including cirrhosis in 20% of cases and hepatocellular carcinoma (HCC) at an incidence of 4%-5% per year in cirrhotic patients[1-3]. Epidemiological studies also indicate that HCV is associated with a number of extrahepatic manifestations including insulin resistance, type 2 diabetes mellitus, glomerulopathies, oral manifestations, *etc.*[4-7].

Most HCV-infected patients would develop chronic hepatitis, but approximately 15%-40% of them could clear the virus spontaneously. What are the factors responsible for the different outcomes of HCV infection? The viral evolutionary dynamics and host genetic polymorphisms, *e.g.,* the interleukin 28B (*IL28B*) gene, are important in determining the outcomes of HCV infection[8,9].

After the discovery of HCV, great nucleotide diversity among isolates was reported[10,11]. Due to the error-prone viral RNA-dependent RNA polymerase (HCV NS5B protein), a closely related but diverse population of viral variants known as quasispecies is produced within HCV-infected patients[12]. There is 1%-5% variation in HCV nucleotide sequence from a single infected patient. Accumulation of nucleotide substitutions in the virus has resulted in diversification into distinct subtypes and even genotypes. Therefore, the HCV RNA genome sequences are highly heterogeneous. At present, HCV is classified into eleven genotypes (designated as 1-11) differing in their nucleotide sequence by 30%-50%, six of them are the major ones (genotypes 1 to 6)[13,14]. Within HCV genotype, several subtypes (designated as a, b, c, *etc.*) can be defined that differ in their nucleotide sequence by 15%-30%[15,16]. The prevalence of HCV genotypes and subtypes is geographically different[17,18]. At present, genotype 1 is the most prevalent (46%) globally, followed by genotype 3, genotype 2 and genotype 4. Various genotypes have different infectivity and pathogenicity, thereby influencing the rate of progression to cirrhosis and the risk of HCC. HCV heterogeneity would also result in different responses to anti-viral treatments, *e.g*., genotypes 1 and 4 are more resistant to interferon based therapies than genotypes 2 and 3[13,19,20]. Therefore, HCV heterogeneity poses a challenge to the development of pan-genotypic anti-viral treatments. In addition, HCV heterogeneity hinders the development of a successful vaccine to against all HCV genotypes. Of course, HCV heterogeneity could also affect viral diagnosis.

Though heterogeneous, different HCV genotypes preserve the similarity of life cycle in cells. This review briefly describes the HCV life cycle and the general properties of viral RNA and proteins, which are related to the diagnosis of viral infection and the development of anti-viral therapy. This review also summarizes the current methods to detect and treat HCV infections.

VIROLOGY

HCV life cycle[21-23]

HCV is a small enveloped RNA virus belonging to the family *Flaviviridae* and genus *hepacivirus*. HCV genomic RNA was single-stranded with positive polarity, which was packaged by core protein and enveloped by a lipid bilayer containing two viral glycoproteins (E1 and E2) to form the virion[24]. Despite the nucleotide sequence divergence among genotypes, all currently recognized HCV genotypes are hepatotropic and pathogenic.

The HCV lifecycle begins with the attachment of a virion to its specific receptors on hepatocytes[25]. Up to now, the high-density lipoprotein receptor scavenger receptor class B type Ⅰ, tetraspanin CD81, tight junction protein claudin-1, and occludin are the known cellular receptors initiating the attachment step of HCV infection. It is proposed that the virus, after binding with its receptor complex, is internalized, and that the nucleocapsid is released into the cytoplasm. The virus is then uncoating to free its genomic RNA, and the HCV genomic RNA is used both for polyprotein translation and replication in the cytoplasm. HCV replication takes place within the "replication complex" containing the viral non-structural proteins and cellular proteins[26].

HCV replication is catalyzed by the NS5B protein. However, other viral nonstructural proteins are also important. The NTPase/helicase domain of the NS3 protein has several functions important for viral replication, including RNA stimulated NTPase activity, RNA binding, and unwinding of RNA regions of extensive secondary structure. NS4B initiates the formation of replication complex that supports HCV replication. The NS5A protein also plays an important regulatory role in virus replication. New direct acting antivirals (DAAs), specifically designed to inhibit the NS5B RNA dependent RNA polymerase are now becoming available. Several other newer DAAs (*e.g.,* inhibitors to the NS5A protein) have also shown promise in clinical studies[27,28].

A number of cellular factors are involved in HCV replication, such as, cyclophilin A, required for HCV replication through its interacting with NS5A and the NS5B, and microRNA-122, which helps HCV replication through its binding with the 5'un-translated region (5'UTR) of the HCV genome. Therefore, host factors may also become the potential targets for anti-HCV therapies. At present, at least two host-targeted agents (HTAs) have reached clinical development, including

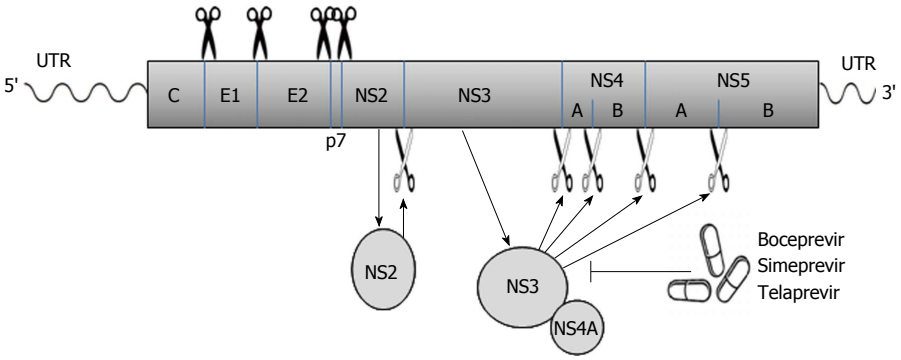

Figure 1 The hepatitis C virus poly-protein is processed co- and posttranslationally by host and viral proteases into at least 10 different proteins, which are arranged in the order of NH2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH. Host signal peptidase is required for the cleavages at C-E1, E1-E2, E2-p7, and p7-NS2 junctions. NS2 cleaves the site between NS2 and NS3; NS3/4A serine protease cleaves the sites at NS3-NS4A, NS4A-NS4B, NS4B-NS5A, and NS5A-NS5B junctions. Several new DAAs (*e.g.*, boceprevir, simeprevir, and telaprevir) specifically designed to inhibit the NS3/4A protease are now becoming available. The wavy lines mark the un-translated regions (UTR) of hepatitis C virus genomic RNA while the rectangle represents the poly-protein derived from the long open reading frame.

specific inhibitors of cyclophilin A and antagonists of microRNA-122[21,28,29].

The very low-density lipoprotein synthesis/ secretion machinery is involved in the production of the infectious HCV particles. HCV uses this lipoprotein biosynthetic pathway to produce mature viral particles and to export them[30,31].

General properties of HCV RNA and proteins

Viral genomic RNA[22,23,32]: The HCV genomic RNA contains three distinct regions[21,32]: (1) a 5′UTR or non-coding region; (2) a long open reading frame (ORF) of more than 9000 nucleotides (nt); and (3) a short 3′ UTR.

The HCV 5′UTR contains 341 nt located upstream of the ORF translation initiation codon. The 5′UTR contains the internal ribosomal entry site which forms a stable pre-initiation complex by direct binding with the 40S ribosomal subunit for the HCV polyprotein translation.

The long ORF encodes a polyprotein of approximately 3000 amino acids, which will be further processed by host and viral proteases.

The 3′UTR is principally involved in minus-strand priming during HCV replication.

The nucleotide sequence variability is distributed throughout the entire viral genome. The 5′UTR is the most conserved region in the genome while the regions encoding envelope proteins (E1, E2) are the most variable ones. Thus, the highly conserved 5′UTR region is usually the target of choice for HCV genome detection across different genotypes (in "Diagnosis" section).

Viral proteins[21-23]: The long ORF encodes a poly-protein which is processed co- and posttranslationally by host and viral proteases into at least 10 different proteins, which are arranged in the order of NH_2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH. Host signal peptidase is required for the cleavages at C-E1, E1-E2, E2-p7, and p7-NS2 junctions. Host signal peptide peptidase is also needed for the further maturation of HCV core proteins. Two viral proteases are involved in the processing of HCV nonstructural proteins: NS2, a zinc-dependent metalloprotease that cleaves between NS2 and NS3; and NS3/4A, a serine protease that cleaves between the NS3-NS4A, NS4A-NS4B, NS4B-NS5A, and NS5A-NS5B junctions (Figure 1). These viral proteins remain associated with intracellular membranes after processing.

Core, a 191-amino acid polypeptide, is a highly conserved basic protein which packages its RNA genome to form the viral nucleocapsid. Core protein may be involved in hepatocarcinogenesis and steatosis[33]. The alternate reading frame protein (ARFP)/core+1/F (frameshift) protein is generated as a result of a -2/+1 ribosomal frameshift in the core-encoding region. The role of ARFP/core+1/F protein in the HCV lifecycle remains unknown[33]. Two envelope glycoproteins, E1 (33-35 kDa) and E2 (70-72 kDa), assemble as non-covalent heterodimers and are necessary for viral entry. Unlike HCV core protein, E2 contains hypervariable regions with amino acid sequences differing up to 80% between different HCV isolates. Therefore, enzyme immuno-assay (EIA) to detect the HCV core antigen rather than the envelope proteins is performed to represent the number of the virions (in "Diagnosis" section).

p7, a 63-amino acid polypeptide, forms ion channels with an essential role in virus infection. NS2, a 21-23 kDa transmembrane protein, is also essential for the completion of the viral replication cycle. The highly hydrophobic N-terminal residues of NS2 protein form three or four transmembrane helices inserting into the endoplasmic reticulum (ER) membrane while the C-terminal domain of NS2 protein plays an important role in NS2/3 auto protease activity together with the N-terminal domain of NS3. NS3, a 67 kDa polypeptide, possesses multifunctional activities. The N-terminus of NS3 has serine protease activity and its C-terminus has

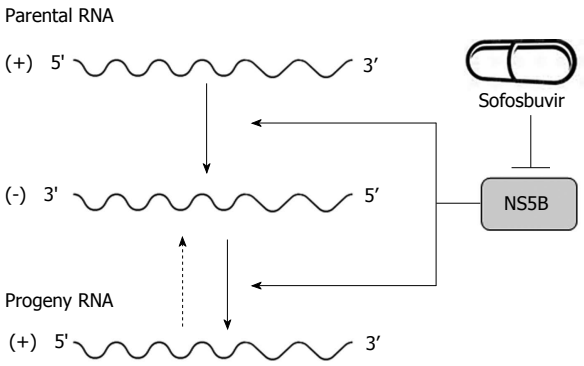

Figure 2 Hepatitis C virus NS5B acts as RNA-dependent RNA polymerase and plays an important role in the synthesis of new RNA genomes. As the central component of the hepatitis C virus replication complex, NS5B has emerged as a major target for antiviral treatment. New direct acting antivirals, specifically designed to inhibit the NS5B are now becoming available (*e.g.*, sofosbuvir).

NTPase/helicase activity. The NS3 proteins are bound with ER membrane through interacting with NS4A proteins. The enzymatic activity of the NS3 NTPase/helicase activity is indispensable for viral RNA replication. NS4A, a 54-amino acid polypeptide, is a cofactor for NS3 protein. The interaction between NS4A and NS3 proteins stabilizes and facilitates its protease activity. The NS3/4A protease is essential for the cleavages of the viral nonstructural proteins. Thus, it is the target of choice for anti-HCV therapy. Several new DAAs specifically designed to inhibit the NS3/4A protease are now available[21,28,34] (Figure 1). NS4A is also required for the phosphorylation of NS5A and can directly interact with NS5A. NS4B, a small hydrophobic 27 kDa protein, recruits other viral non-structural proteins to form the replication complex. NS5A, a 56-58 kDa hydrophilic phosphoprotein, is also important in viral replication though its exact role is not clear. NS5B, a 65 kDa protein, is an RNA-dependent RNA polymerase (RdRP) responsible for the synthesis of new genomic RNAs. As a central component of the HCV replication complex[26], NS5B has become a major target for antiviral therapy[21,28] (Figure 2).

More than twenty years of study has provided a better understanding of HCV life cycle, including the properties of viral RNA and proteins. This effort facilitates the development of sensitive diagnostic tools and effective antiviral treatments toward HCV infections.

DIAGNOSIS

The purpose of diagnosis of viral infection is to allow the infected persons to be identified and treated. Thus, diagnosis of viral infection is important to prevent disease progression and viral spread. Majority of primary HCV-infected patients are asymptomatic, thus, symptoms could not be used as specific indicators for HCV infection. HCV viremia could still exist despite a normal serum alanine aminotransferase (ALT) level. Therefore, virological methods rather than ALT levels are used to diagnose HCV infection[35].

In general, the virological methods for examining viral infections include indirect and direct tests. The indirect tests are to detect antibody induced by viral infection, including IgM for recent infection and IgG for recent or past infection. The direct tests include virus isolation, detection of viral antigens and viral nucleic acids.

At present, it is difficult to isolate and culture HCV using clinical specimens. Furthermore, anti-HCV IgMs could be detected not only in 50%-93% of patients with acute hepatitis C but also in 50%-70% of CHC patients[36]. Therefore, anti-HCV IgM cannot be used as a reliable marker for the acute HCV infection, and IgM assays have not been used in clinical practice[37,38]. At present, diagnostic assays for anti-HCV total antibody, viral core antigen, and viral genomic RNA are used in clinical practice[19,35,39-44] (Figure 3).

Detection of antibody production

In general, serological tests for detecting anti-HCV antibodies include tests for screening and confirmation. Screening tests are used first to screen the antibody positive specimens while confirmatory tests are then used to verify the positive screening specimens.

Screening test: EIA: At present, the third generation test of EIA for the anti-HCV antibody detection is commonly used in the diagnostic laboratory[45]. Conserved antigens from the HCV core, NS3, NS4 and NS5 regions are used in these tests to detect anti-HCV antibodies. The sensitivity of third-generation EIAs was estimated at 98.9% and the specificity was found at 100% in patients with chronic liver disease[46]. EIAs are easy to use and inexpensive. Furthermore, this assay could be fully automated and adapted to large volume testing. Therefore, EIAs to detect anti-HCV antibody are generally recommended for screening the HCV infections[40]. However, this assay should not be used in infants younger than 18 mo due to the possibility of reactivity with maternal antibody[47]. Several Food and Drug Administration (FDA)-approved antibody-based assays are available[47]. However, the time between HCV infection and the appearance of detectable antibodies (serological window period) is generally more than 40 d using the third generation EIAs[48]. In 2008, the fourth generation EIA has become available which could detect the anti-HCV antibody significantly earlier than the other assays (www.microgenbioproducts.com). The antigens utilized in the fourth generation anti-HCV assay are derived from the core (two different epitope clusters), NS3, NS4A, NS4B, as well as the NS5A regions. NS3 and NS4 antigens are derived from genotypes 1a, 1b, 2 and 3.

Screening test: The rapid, point-of-care test[23,49]: Point-of-care tests are used directly at the site of patient care, outside of the diagnostic laboratory. Several point-of-care tests (POCTs) have been developed to detect anti-HCV antibodies with a relatively high sensitivity

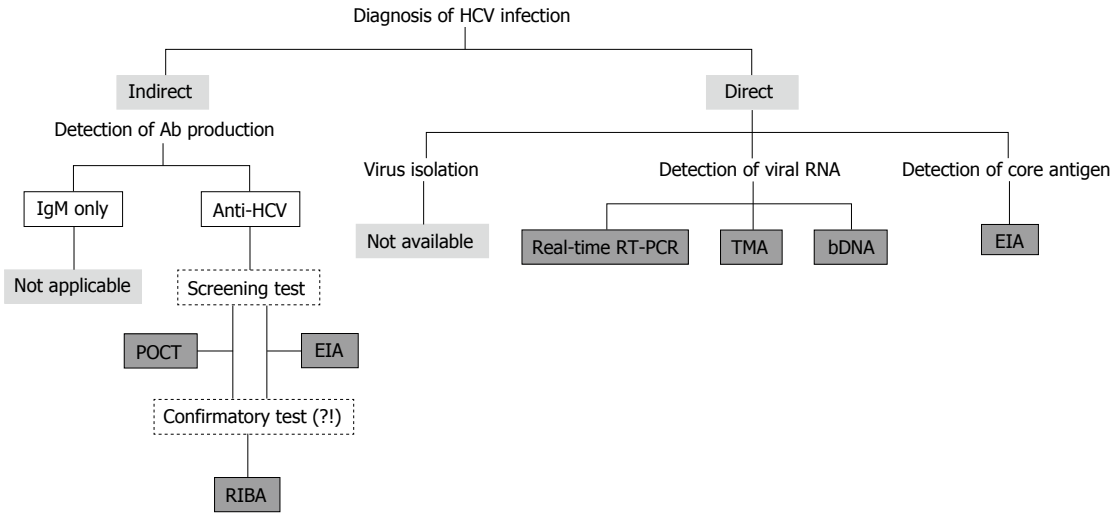

Figure 3 Assays to detect anti-hepatitis C virus antibody, viral core antigen, and viral genomic RNA are used to diagnose HCV infection in clinical practice. HCV: Hepatitis C virus; POCT: Point-of-care test; EIA: Enzyme immuno-assay; RIBA: Recombinant immunoblot assays; RT-PCR: Reverse transcription-polymerase chain reaction; TMA: Transcription-mediated amplification; bDNA: Branched DNA.

and specificity[50,51]. The test currently approved by the FDA in 2010 is the OraQuick HCV Rapid Antibody Test (OraSure Technologies, Bethlehem, PA). It is approved for use in patients over 15 years old, for screening persons who are considered at risk for HCV infection. This test detects anti-HCV antibodies in different specimens, *e.g.,* fingerstick and venipuncture whole blood, serum, plasma, or oral fluid. Recombinant proteins or synthetic peptides of core, NS3 and NS4 antigens are immobilized on a nitrocellulose membrane to perform an indirect lateral flow immunoassay, and the results are directly visualized using colloidal gold labeled protein A, which generates a reddish-purple line within 20 to 40 min in the presence of anti-HCV antibodies in the specimens. These rapid tests are suitable for resource-limited settings because they are cheap, simple to perform and fast[52].

Confirmatory tests: Recombinant immunoblot assays[49]: Recombinant immunoblot assays (RIBA) can be used to confirm the presence of anti-HCV antibodies for individuals who have showed positive reactivity by EIAs. This assay is highly specific, as the presence of antibodies against each of the several HCV proteins is assessed as individual bands on a membrane strip[53]. The INNO-LIA™ HCV Score (Fujirebio Europe, previously Innogenetics) assay can be automated. This assay includes recombinant proteins and synthetic peptides from E2 hypervariable region, NS3 helicase, NS4A, NS4B and NS5A regions.

Due to the high sensitivity and specificity of anti-HCV EIAs, RIBA is no longer needed in the diagnostic laboratories for verification[19,45]. Furthermore, nucleic acid tests for viral RNA rather than RIBA are used as a confirmatory test for HCV infection[19].

HCV infection can be detected easily using currently available third generation EIAs[45]. In addition, the use of POCTs can increase HCV screening opportunities. However, these indirect virological tests to detect anti-HCV antibody cannot distinguish current from past infection[37,38]. Active HCV infection must be confirmed by the direct diagnostic methods.

Detection of viral RNA

Based on the items used for amplification, nucleic acid amplification tests (NAT) are divided into target amplification, signal amplification and probe amplification methods[54]. Target amplification methods [*e.g.,* reverse transcription-polymerase chain reaction (RT-PCR) and transcription-mediated amplification (TMA)] and signal amplification methods [*e.g.,* branched DNA (bDNA)] were commonly used to detect the presence of HCV RNA[19,47,49,55]. The presence of HCV RNA in the serum is a reliable marker of viremia. Universal standardization for HCV RNA titer is important. The World Health Organization (WHO) has established an international standard for HCV RNA quantification units[56], *i.e.,* an HCV RNA international unit (IU), which is currently used in all of the commercial HCV RNA quantitative assays no matter what the techniques used[35,57].

Qualitative HCV RNA detection[47]: Qualitative detection assays are based on the principle of target amplification using either RT-PCR or TMA. Several FDA-approved qualitative assays for HCV RNA are available[47]. HCV RNA is extracted and converted into complementary DNA (cDNA) using reverse transcriptase. The cDNA is subsequently processed *via* cyclic enzymatic reactions leading to the generation of a large number of double-stranded DNAs in PCR-based assays or single-stranded RNAs in TMA. Detection of these amplified products is achieved by hybridizing the produced amplicons onto specific probes. In general, the highly conserved 5'UTR region is the target of choice for HCV

genomic RNA detection across different genotypes[49].

Quantitative HCV RNA detection[47]: HCV RNA can be quantified by means of target amplification techniques (real-time RT-PCR or TMA) or signal amplification techniques (bDNA assay). Several FDA-approved quantitative assays to detect HCV RNA are also available[23,39,47]. Real-time RT-PCR is the method of choice for the quantification of HCV RNA levels in clinical practice. This assay is highly sensitive with wide dynamic range of quantification and can prevent carryover contamination.

Fully automated HCV NAT assays have been available in the United States since 2007, and guidelines regarding the requirements for HCV NAT assays were issued in 2010 (http://www.fda.govQBiologicsBloodVaccinesQGuidance-ComplianceRegulatoryInformation/Guidances/default.htm). However, it is necessary to remember that not all HCV genotypes are detected equally by NAT assays, most likely because of nucleotide mismatches which has occurred before[58,59].

HCV RNA in the serum is probably the earliest detectable marker of acute HCV infection, preceding the appearance of anti-HCV antibody by several weeks[35]. CHC infection is defined as the presence of HCV RNA more than 6 mo. HCV RNA levels remain relatively stable over time in CHC patients. Therefore, after a positive reaction screened by the anti-HCV antibody test, NATs to detect HCV RNA is often used as the confirmatory tool to diagnose CHC infection[60]. Detection of HCV RNA is also used to determine the viral load both prior to and during antiviral treatments (www.who.int). On the other hand, the HCV RNA level has no prognostic value[61]. The level of HCV genomic RNA, reflection of HCV replication, does not correlate with the severity of liver disease, not with the risk of liver disease progression to cirrhosis or HCC.

Detection of viral core antigen[44]

Compared to other diagnostic methods like EIA, the advantages of NATs are having higher specificity and sensitivity. However, the disadvantages of these assays are time-consuming and require sophisticated technical equipment, trained technicians, dedicated laboratory space and expensive reagents. In patients with HCV infection, it has been demonstrated that the HCV core antigen level strongly correlates with the HCV RNA level for various genotypes[62]. Thus, due to cheap and easy-to-perform, the HCV core antigen quantification assay can be used as an alternative method to NATs to detect HCV RNA[44]. Currently, core antigen detection by means of a chemiluminescent microparticle immunoassay can be fully automated in the Architect HCV Core antigen test (Abbott Laboratories)[63]. The Architect HCV Ag assay had a specificity of 100%, with a lower limit of detection of 3 fmol/L corresponds to approximately 1000 IU/mL of HCV RNA[62]. Whereas,current HCV RNA assays have a lower level of detection between 5-15 IU/mL[44]. In general, about 90% of HCV RNA positive samples are positive with a viral load above 10000 IU/mL[64], well in the sensitivity range of the HCV core antigen assay[44]. Therefore, HCV antigen detection might be the next step following a positive antibody screening test. Several combination assays for detection of both anti-HCV antibodies and HCV core antigen have been developed[65].

At present, EIA to detect HCV core antigen is too insensitive to replace the NATs to detect HCV RNA in the blood bank setting[66] and in the treatment monitoring according to the current clinical practice guidelines. However, it could be used as a supplemental test in resource-limited settings[67]. The Architect HCV Ag assay has been suggested as a better monitoring tool in the era of new all-oral, interferon-free antiviral treatments that do not require high analytical sensitivity[62].

Interpretations of diagnostic results[19,41,42,47]

The presence of HCV RNA in the absence of anti-HCV antibodies is strongly indicative of acute hepatitis C (AHC), which can be confirmed by seroconversion (*i.e.,* the appearance of anti-HCV antibodies) a few days or weeks later (Figure 4). However, there are still other possibilities for the presence of HCV RNA in the absence of anti-HCV antibodies, *e.g.,* CHC infection in the immunodepressed patients, hemodialysis patients or agammaglobulinemic subjects.

The presence of both anti-HCV and HCV RNA does not allow one to distinguish AHC from an acute exacerbation of CHC. However, the anti-HCV IgG avidity index within the first 8 d following the onset of clinical symptoms may be useful in identifying actual AHC[68].

If the antibody test is positive and the HCV RNA test is negative, this result indicates a resolution of HCV infection or AHC during a period of low-level viremia. If the HCV RNA assay is negative and remains negative for more than 6 mo, then the individuals are recovered from a past HCV infection.

CHC is defined as the persistence of HCV RNA for more than 6 mo. In patients with clinical signs of chronic liver disease, CHC is certain when both anti-HCV antibodies and HCV RNA are present.

Genotyping

Different HCV genotypes would result in different responses to antiviral treatments[35]. Thus, genotyping is important to predict the likelihood of response and determine the optimal duration of therapy[47].

Serological method: The HCV genotype can be determined by detection of antibodies against HCV genotype-specific epitopes using a competitive EIA[69]. The currently available assay (Murex HCV serotyping 1-6 HC02, Abbott Laboratories, North Chicago, Illinois) could identify the six HCV genotypes (1-6) but not subtypes, and provide interpretable results in approximately 90% of chronically infected immunocompetent patients[7].

Molecular techniques: The reference method for HCV genotyping is genome sequencing of the core/E1 or the

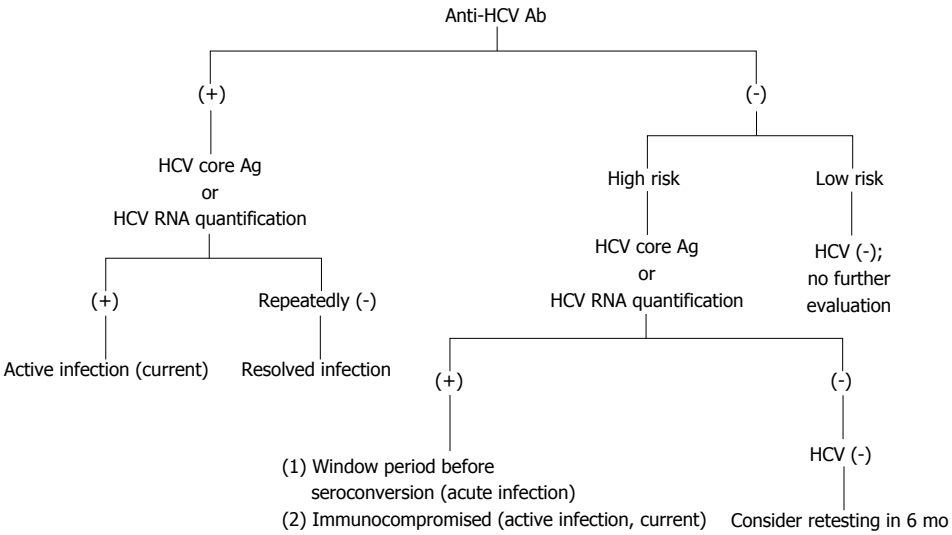

Figure 4 Possible diagnostic results for hepatitis C virus infection. Individuals are in high risk, *e.g.*, persons who have been exposed to HCV; persons with elevated alanine aminotransferase; persons who are immunocompromised. HCV: Hepatitis C virus.

NS5B regions and subsequent phylogenetic analysis[70]. However, this in-house method is restricted to reference centers. HCV genotyping assays approved for *in vitro* diagnostic use are also commercially available[23,49]. The Linear Array HCV Genotyping Test (Roche Molecular Systems) targets the 5'UTR[71]. This assay is based on conventional PCR amplification followed by reverse hybridization onto membrane strips containing specific probes. The obtained band pattern can be either visually interpreted or read by a scanner. Assays targeting other regions in addition to the 5'UTR have been recently developed to better discriminate between subtypes 1a and 1b. The Versant HCV genotype 2.0 assay (Siemens) is also based on reverse hybridization and targets the 5' UTR and core regions[72]. On the other hand, the Abbott RealTime HCV Genotype Ⅱ (Abbott Molecular) targets the 5'UTR and NS5B regions. This assay is based on a single-step real-time RT-PCR with labeled genotype-/subtype-specific probes that minimizes contamination with amplified products[73,74].

Subtyping

HCV subtyping is important for epidemiological studies, especially in the case of outbreaks, but it is not considered to be clinically relevant for the treatment of interferon-α and ribavirin. However, subtyping may be clinically relevant in the era of DAAs. For example, the phase 3 studies of telaprevir, boceprevir, faldaprevir and simeprevir showed lower sustained virologic response (SVR) rates for HCV-subtype 1a than those for subtype 1b[75]. In addition, BILB 1941, a non-nucleoside inhibitor of HCV NS5B, has been shown to have better antiviral efficacy in patients with subtype 1b than in those with subtype 1a[76]. Therefore, methods to determine the HCV subtypes should be important in the era of DAAs. The second-generation line probe assay, a reverse hybridization assay that uses probes targeting both the 5'UTR and core-coding region, correctly identified HCV subtypes 1a and 1b in more than 99% of cases. Thus, this assay could be used to differentiate HCV subtypes 1a and 1b in clinical trials and practice[74].

Screening for HCV-infected patients

According to the WHO (www.who.int), up to 80 percents of HCV-positive patients do not show symptoms. Therefore, most cases of HCV infection are currently undiagnosed. The major way to diagnose HCV infection is to screen high risk groups for anti-HCV antibodies. Humans are the primary HCV reservoir[77]. HCV transmission occurs primarily through direct percutaneous exposure to blood. Therefore, the most common risk factors for HCV infection are persons with history of injection of illicit drugs and with blood transfusion prior to July 1992. The populations with less common risk factors for HCV infection are persons with organ transplant prior to July 1992, receiving clotting factor concentrate prior to 1987, being born to an HCV-infected mother, and with a history of chronic hemodialysis, intranasal use of illicit drugs, acquiring a tattoo, incarceration, having sex with an HCV-infected partner, needlestick or other mucosal exposure, with persistently elevated levels of ALT[40]. Therefore, WHO recommends that anti-HCV EIA be performed on individuals who are part of a population with high HCV seroprevalence or who have a history of HCV risk exposure and/or behavior, rather than at the time of presentation with symptomatic diseases. In addition, it is suggested that NATs for the detection of HCV RNA be performed directly following a seropositive test result to establish a definitive diagnosis of HCV infection (www.who.int). The Center for Disease Control (CDC) has also recommended screening high-risk individuals for HCV since 1998. The CDC further modified the HCV screening guidelines in 2012 to include a one-time HCV test for all US residents born during 1945-1965, independent of risk

factors[40].

TREATMENT

Around 50%-80% of persons with acute hepatitis C will develop CHC infection, and 5%-25% of them reportedly progress to cirrhosis after 20-25 years[78]. Persons with cirrhosis are at risk for developing end-stage liver disease as well as HCC[79]. The goal of antiviral treatment for CHC is to halt disease progression, prevent cirrhosis decompensation and reduce the risk of HCC[80]. However, it is really difficult to design and carry out clinical trials to provide direct evidence related to these outcomes (www.ahrq.gov). SVR is defined as undetectable levels of HCV RNA at least 24 wk after completion of therapy. At present, SVR is the primary endpoint of successful therapy and is associated with durable clearance of virus[81]. CHC patients with a SVR after antiviral therapy had a lower risk of all-cause mortality than patients with no SVR[82]. Therefore, SVR is the standard marker of the successful antiviral treatment in clinical trials.

In the early 2000s, the combination of pegylated interferon plus ribavirin (PR) became the standard anti-HCV treatment[19,28,83]. However, the anti-HCV interferon therapy is not ideal because it requires weekly injections and is associated with numerous systemic side effects (*e.g.,* flu-like symptoms, fatigue, *etc.*). Therefore, other anti-HCV therapies are needed. In principle, every step of the HCV lifecycle, including receptor attachment, endocytosis, uncoating, translation, polyprotein processing, RNA replication, virion assembly, maturation and release, can be a target for new anti-HCV drugs[21]. Advances in understanding of the HCV lifecycle have led to the development of numerous highly effective, well-tolerated oral DAAs[84-86]. In 2011, the United States FDA approved the first DAAs, boceprevir (trade name Victrelis™) (www.fda.gov/ForConsumers/ByAudience/ForPatientAdvocates/ucm255413.htm) and telaprevir (trade name Incivek®) (www.fda.gov/ForConsumers/ByAudience/ForPatientAdvocates/ucm256328.htm), for the treatment of chronic HCV genotype 1 infection (Figure 1). Both drugs are classified as NS3/4A protease inhibitors, with a potential advantage of shorter therapy duration (24 to 28 wk) compared with standard PR treatment for genotype 1 infection (48 wk)[87,88]. Either drug is administered in combination with PR[89]. In 2013, FDA approved another NS3/4A protease inhibitor: simeprevir (http://www.olysio.com/). The HCV NS5B protein is an essential enzyme (RNA-dependent RNA polymerase) in HCV viral replication and has been a prime target in the search for antiviral therapies. In 2013, the FDA approved sofosbuvir (an inhibitor of NS5B) in combination with ribavirin for oral dual therapy of HCV genotypes 2 and 3, and for triple therapy with PR for treatment-naive patients with HCV genotypes 1 and 4 (Figure 2). Sofosbuvir treatment regimens last 12 wk for genotypes 1, 2 and 4, and 24 wk for treatment of genotype 3. This is typically half the time as with prior treatments. Thus, to December 2013, licensed treatments for HCV infection include pegylated and standard interferon alpha, ribavirin, the NS3/4A protease inhibitors boceprevir, telaprevir and simeprevir; and the NS5B nucleotide polymerase inhibitor sofosbuvir. Without taking resource used into consideration, WHO provides the following guidelines (http://www.who.int/hiv/pub/hepatitis/hepatitis-c-guides): (1) Pegylated interferon in combination with ribavirin is recommended for the treatment of CHC rather than standard non-pegylated interferon with ribavirin; (2) Treatment with the DAAs telaprevir or boceprevir, given in combination with PR, is suggested for genotype 1 chronic HCV infection rather than PR alone; (3) Sofosbuvir, given in combination with ribavirin with or without pegylated interferon (depending on the HCV genotype), is recommended in genotypes 1, 2, 3 and 4 HCV infection rather than PR alone (or no treatment for persons who cannot tolerate interferon); and (4) Simeprevir, given in combination with PR, is recommended for persons with subtype 1b HCV infection and for persons with subtype 1a HCV infection without the Q80K polymorphism rather than PR alone.

Although interferon-free anti-HCV therapies will be available in the near future, before then, peginterferon will be still required with either the protease inhibitor simeprevir, or the nucleotide analogue polymerase inhibitor, sofosbuvir, for the treatment of genotype 1 infection. Peginterferon also appears to be a useful adjunct to sofosbuvir and ribavirin for patients with genotype 3 infection, particularly those with cirrhosis. Therefore, pretreatment assessments are needed for the anti-HCV treatments containing interferon, including HCV genotype determination, liver disease staging (*e.g.,* fibrosis), psychiatric assessment (*e.g.,* depression and suicide risk), assessments for alcohol or substance use disorders; adherence, evaluation for HIV co-infection, pregnancy, testing for *IL28B* genotype, and concomitant medical conditions (*e.g.,* autoimmune disorders). Adverse effects will be still checked for the anti-HCV treatments containing interferon, including anemia, neutropenia, rash and skin reactions, anorectal signs and symptoms, elevated uric acid, bilirubin levels, *etc.* (http://www.who.int).

HCV resistance is defined as the selection of viral variants, reducing the susceptibility to the drug's inhibitory activity in the presence of anti-HCV drugs. Resistance-associated variants are naturally produced during the HCV replication. At present, there is no commercially available assay to detect the presence of resistant viruses before or during antiviral treatments[75]. The only way to check if a patient has developed a resistant virus is to monitor for HCV RNA rebound (more than 10 fold increase from the nadir HCV RNA) during anti-HCV treatment.

During treatment, NATs to quantitate HCV RNA should be performed at weeks 4, 8 (with boceprevir-containing regimens), 12, and 24 of treatment, at the end-of-treatment, and 24 wk after treatment to monitor the viral titers. The determination of the viral titers helps

to detect drug-resistant viruses and to adjust the dose and duration of the anti-HCV treatment.

The factors influencing the efficacy of anti-HCV treatments based on interferon are divided into two categories: viral-related and host-related factors[90,91]. The viral-related factors include the HCV genotype, baseline viral load, and virological response during treatment. The host-related factors include age, gender, race-ethnicity, fibrosis stage, obesity, hepatic steatosis, low-density lipoprotein cholesterol, insulin resistance, and *IL28B* gene polymorphisms. In particular, *IL28B* gene polymorphisms are associated with the SVR. Thus, host genetic factors are important to determine the effect of anti-HCV therapy based on interferon. The anti-HCV treatment will change significantly over the next few years as therapeutic regimens based on interferon-free are rapidly evolving. Thus, it is necessary to determine the effects of these viral-related and host-related factors on the efficacy of anti-HCV therapy based on DAAs therapy without interferon.

In early 2014, several reports regarding novel DAAs have been published: (1) Combined simeprevir and sofosbuvir was efficacious and well tolerated for patients with HCV genotype 1[92]; (2) Combined daclatasvir (NS5A replication complex inhibitor) and asunaprevir (NS3/4A protease inhibitor) could be used as an all-oral, PR-free treatment option for patients with HCV subtype 1b infection, including those with cirrhosis[93]; (3) In combinations with other oral DAAs, dasabuvir (a non-nucleoside inhibitor of NS5B) results in very high rates of SVR (about 95%) in patients with HCV genotype 1 infection with a good tolerability and safety[94]; (4) The sustained response rate of ABT-450, a potent inhibitor of NS3/4A protease, plus other direct antiviral drugs reaches 90%-95% in both naïve and treatment-experienced genotype 1 patients, and tolerability is good[95]; (5) Sofosbuvir was also effective in patients co-infected with HCV and HIV[96]; (6) Sofosbuvir plus PR achieved high SVR rates in patients with HCV genotype 1 infection, and also appeared effective in patients with HCV genotype 4, 5 or 6 infection. Oral sofosbuvir was generally well tolerated in CHC patients[97]; (7) In combinations with other oral DAAs, ombitasvir (an inhibitor of the HCV NS5A) achieves very high rates of SVR (about 95%) in patients with HCV genotype 1 infection with a good tolerability[98]; and (8) Combination of daclatasvir and asunaprevir results in a very high rate of viral eradication in both treatment-naïve and treatment-experienced patients, with a SVR rate of 80%-90%[99]. In October of 2014, FDA approved Harvoni (ledipasvir and sofosbuvir) to treat chronic HCV genotype 1 infection (http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm418365.htm). Ledipasvir is an inhibitor of HCV NS5A protein (www.gilead.com). Harvoni is the first combination pill approved to treat chronic HCV genotype 1 infection.

Not only DAAs, HTAs as anti-HCV therapy are also developed. HTAs block HCV production by interacting with cellular factors. Because they target conserved host proteins, not variable viral proteins, HTAs have the potential for pangenotypic antiviral activity and a high barrier to resistance[29]. Until now, only two HTAs have reached clinical trial, including specific inhibitors to cyclophilin A peptidyl-prolyl cis/trans isomerase activity and antagonists of microRNA-122[21,28].

The pace of DAAs and/or HTAs entering clinical trials is breathtaking[84]. The optimal combination of DAAs (and/or HTAs) that maximizes potency, minimizes resistance, and limits toxicity will be available soon[100]. Once combination DAA therapies are available, peginterferon will serve a smaller and smaller role[101]. Indeed, DAAs trump interferon-alpha in their capacity to rescue exhausted T cells upon HCV clearance[102]. From 2015, interferon-free anti-HCV regimens with short treatment duration and fewer side effects will be available[84,85,103]. However, peginterferon may still have a role in resource-limited regions due to high cost of DAAs[104].

CHC patients achieved a SVR after anti-HCV treatments exhibited a reduction in all-cause mortality > 50% compared with those who are non-responders[82]. However, in such a nonrandom clinical trial, an improved outcome could be biased by parameters, such as the good health conditions of the responders. Indeed, it has been reported that some patients who achieve SVRs still go on to develop end-stage liver disease[105]. Thus, the concept which cure rests solely on SVR may not be always correct. Actually, despite improving SVR, there is no evidence that PR beneficially affects patient-relevant outcomes such as mortality and liver morbidity[106,107]. Therefore, it is better to remember that SVR might not work as a surrogate for patient-relevant outcomes[108].

The cure rate for HCV infection is expected to be over 95% with the new all-oral, interferon-free regimens within the next few years. However, due to drug resistance[109,110], suboptimal activity against certain HCV genotypes and the extremely high cost[100,104], not all patients can be cured. Currently, no effective vaccine is available for HCV infection. Therefore, an efficient prophylactic vaccine will be the next challenge in the combat against HCV infection[111-113].

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REVIEW

Chemokines and their receptors play important roles in the development of hepatocellular carcinoma

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Abstract

The chemokine system consists of four different subclasses with over 50 chemokines and 19 receptors. Their functions in the immune system have been well elucidated and research during the last decades unveils their new roles in hepatocellular carcinoma (HCC). The chemokines and their receptors in the microenvironment influence the development of HCC by several aspects including: inflammation, effects on immune cells, angiogenesis, and direct effects on HCC cells. Regarding these aspects, pre-clinical research by targeting the chemokine system has yielded promising data, and these findings bring us new clues in the chemokine-based therapies for HCC.

Key words: Chemokines; Hepatocellular carcinoma; Immune cells; Chemokine receptors; Inflammation; Angiogenesis; Tumor behaviors; Treatments

Core tip: The chemokine system not only serves as the core components in orchestrating the normal immune response but also plays a key role in the microenvironment of hepatocellular carcinoma (HCC). Therefore, the thorough understanding of its role is indispensible for devising effective treatments. During the progress of HCC, the chemokine system boosts aberrant inflammation and angiogenesis through simultaneously affecting different kinds of immune cells and influencing the migration, invasion, growth and survival of tumor cells. Targeting the chemokine system has elicited powerful anti-tumor effects and this indicates an encouraging treatment option in HCC.

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INTRODUCTION

The chemokines are a family of small chemotactic molecules about 8-14 kDa which have been well

described during the past decades. There are now over 50 chemokines and 19 chemokine receptors, and these chemokines can be divided into four subclasses: CX_3C, CXC, CC and (X)C according to the arrangement of the N-terminal two cysteine residues. Corresponding to the four subclasses of chemokines, the chemokine receptors are also subdivided into four families [CX_3CR, CXCR, CCR and (X)CR] which are typical G-protein coupled receptors with seven trans-membrane domains[1,2].

The chemokine system is initially found to be critical for immune cells. They orchestrate the migration and localization of immune cells in both lymph organs and other tissues, exerting the "chemotactic effects" which are necessary for the normal immune response *in vivo*[3]. In addition to chemotactic effects, chemokines can also directly influence the differentiation, survival and functions of immune cells, among which include CCR4, CCR7 and CCR8[4-8]. These observations suggest the chemokine system is not merely the guide signs for the immune cells; instead, they are pleiotropic small molecules with various functions.

The original interest of chemokines in tumor is torched by the observation of immune cells infiltration in tumor tissues. Several groups have speculated that some molecules might be responsible for attracting these immune cells[9]. Although the full spectrum of these molecules is still on the way, some of these important molecules turn out to be chemokines. Since the first chemokine monocyte chemotactic protein 1/CCL2 was detected in the culture media of several different tumor cell lines in 1980s[10,11], more and more chemokines and chemokine receptors have been identified in tumors, including the hepatocellular carcinoma (HCC).

Various studies on the chemokine system have greatly broadened our understanding of its role in HCC, and there are 23 chemokines and 15 chemokine receptors reported in HCC (Table 1). On the one hand, the chemokine system in HCC exerts pleiotropic effects on immune cells and other stroma cells in the microenvironment, and brings both anti- and pro-tumor effects; on the other hand, the HCC cells themselves express chemokine receptors, which allow the chemokines to directly modulate the behaviors of tumor cells including the migration, invasion, growth and survival (Table 2). Data from clinical studies again emphasize the importance of chemokine system in HCC, closely correlating with prognosis. In this review, we will summarize the key roles of the chemokine system in HCC.

THE SIGNIFICANCE OF CHEMOKINES AND CHEMOKINE RECEPTORS

Chemokines in HCC tissues are derived from different sources, including tumor cells, and non-tumor cells such as hepatic stellate cells, T cells, macrophages, neutrophils, *etc*. Similarly, the chemokine receptors that are involved in the progression of HCC are either expressed on tumor cells or non-tumor cells. This complicated network reflects the mutual interaction between HCC cells and other cells in the microenvironment (Figure 1).

Of all the chemokines and their receptors in HCC, the CXC subclass accounts for the largest group. Among them, the CXCL12-CXCR4/CXCR7 axis is the most documented, and abnormal expression of either CXCL12 or CXCR4/CXCR7 is correlated with clinicopathological characteristics[12-15]. CXCL8 is a potent pro-inflammation chemokine widely studied in other tumors and it is also elevated in serum from HCC patients and represents a risk factor for survival[16]. The CXCL9/CXCL10-CXCR3 axis also shows important influences on prognosis of HCC patients[17-19]. The increase of CXCL1, CXCL2 and their common receptor CXCR2 indicates the increased risk for HCC[20,21]. The possible roles of CXCL5 and CXCL14 are unveiled too in HCC patients[22,23].

The CC subclass constitutes another major part of chemokines in HCC. Expression levels and genetic polymorphisms of CCL2 and CCR2 affect the prognosis of HCC patients[17,24,25]. The CCL5-CCR5 axis is closely correlated with liver chronic inflammation induced by different pathogens and finally participates in the development of HCC[26,27]; meanwhile, CCL3 and CCL4, the other two ligands of CCR5, show a definitive role in accelerating the course HCC[28,29]. The CCL20-CCR6 axis is a prognostic factor for HCC patients and this relates its role to recruiting regulatory T cells (Tregs)[30-32]. The other CC chemokines and receptors have also been found correlated with the clinicopathological parameters of HCC, including CCL15[33], CCL17[34], CCL22[35], CCL27[36], CCR7[37] and CCR9[38]. The CX3C subclass contains only one single member CX_3CL1 and this CX_3CL1-CX_3CR1 axis participates in HCC[39,40].

INFLAMMATION

Cancer related inflammation is the hallmark of HCC, especially for hepatitis B virus (HBV)/HCV-associated HCC, and the chemokine system has dual roles in the inflammation of HCC. On the one hand, chemokines themselves can be induced by different inflammatory cytokines such as interleukin-1 (IL-1) and IL-6, and exist as mediators for inflammation by recruiting different immune cells (details will be discussed in EFFECTS ON IMMUNE CELLS); on the other hand, chemokines can trigger the secretion of various other inflammatory cytokines from tumor cells and non-tumor cells in the microenvironment of HCC. Both of the two facets are indispensible in the inflammation of HCC.

CXCL8 is a well-defined pro-inflammatory chemokine. It is produced by HCC cells through activation of several different pathways including JNK, nuclear factor-kappa B (NF-κB), and PI3K-AKT pathways[41,42], and the elevated CXCL8 in turn induces multiple inflammatory cytokines and recruits various immune cells, all of which promotes the development of the inflammation microenvironment in HCC[43].

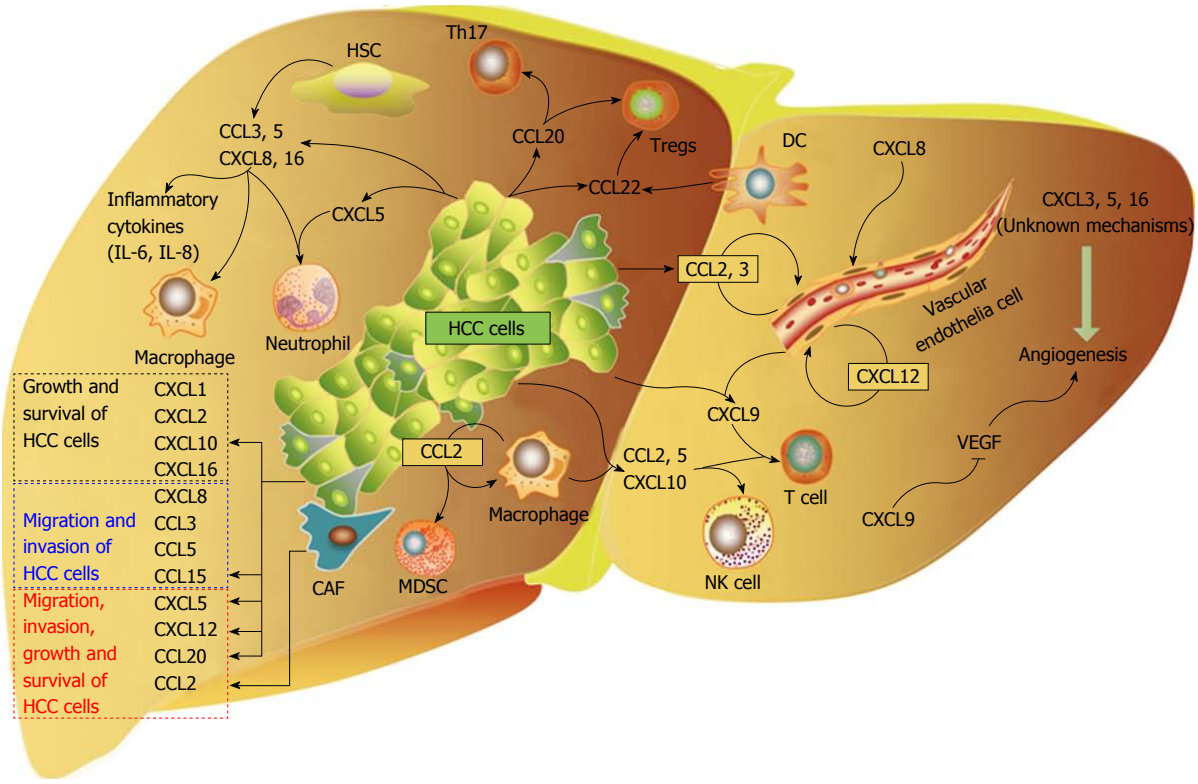

Figure 1 The complicated chemokine network in the microenvironment of hepatocellular carcinoma. The chemokine system exerts pleiotropic effects in the microenvironment of hepatocellular carcinoma (HCC). Chemokines derived from either tumor cells or non-tumor cells induce potent inflammation response, along with increased levels of cytokines and infiltration of immune cells; the potent chemotactic effects of chemokines also lead to recruitment of various immune cells into the tumor sites, exerting both anti- and pro-tumor effects. Several other chemokines such as CXCL9 and CXCL12 manifest a key role in angiogenesis of HCC *via* different mechanisms. As the HCC cells intrinsically express chemokine receptors, they are directly influenced by chemokines too, which affect the behaviors of tumor cells such as the migration, invasion, growth and survival. Both the paracrine and autocrine mechanisms constitute this mutual complex network that is indispensible in HCC. Refer to the text for abbreviations. HSC: Hepatic stellate cell; IL: Interleukin; NK: Natural killer; CAF: Cancer-associated fibroblast; MDSC: Myeloid derived suppressor cells; Tregs: Regulatory T cells; DCs: Dendritic cells; VEGF: Vascular endothelial growth factor.

CCR5 mediated inflammation is also important in the development of HCC. CCL3, one ligand for CCR5, is remarkably increased in different HCC cell lines when stimulated with IL-1α or IL-1β, which consequently attracts large amount of macrophages and neutrophils into the inflammation sites[44]. The hepatic stellate cells are capable of producing a group of inflammatory cytokines including IL-6 and transforming growth factor alpha (TGF-β); blocking the CCR5 signals with maraviroc, a CCR5 antagonist, effectively abrogates the intracellular signal transduction and inhibits the progression of HCC *in vivo*[45]. Likewise, in the CCR5-knockout mice (Mdr2:CCR5 DKO), the oval cells, which are the putative liver progenitor cells that proliferate and differentiate in response to liver damage[46,47], show decreased levels of insulin-like growth factor-binding protein 1, secreted phosphoprotein 1, CD24, keratin 19, and epithelial cell adhesion molecule, concomitant with reduced risk of HCC[48]. Besides, activation of the CXCR6 signal in HCC cells results in increased expression of IL-6 and IL-8, while disturbing the CXCL16-CXCR6 axis can potently abrogate this effect[49].

During the infection of HCV, CXCL10 and CXCL11 play a key role in the HCV-related inflammation. Either interferon (IFN)-α or IFN-γ stimulation results in a significant increase of CXCL11, and IFN-γ shows potent synergy with TNF-α in promoting the expression of CXCL11 *in vitro*[50]. Resembling this phenomenon, TLR3 and RIG-1 also potentiate the induction of CXCL10 in the course of HCV infection in hepatocytes, and IFN-α/IFN-β and IFN-γ boost this induction synergistically[51].

EFFECTS ON IMMUNE CELLS

The liver is a very special organ containing huge amount of immune cells in normal physiological state, and these immune cells consist of T cells, natural killer cells (NK cells), Kupffer cells, macrophages, neutrophils, *etc*. Therefore, it is considered to be a lymph organ[52,53]. During the development of HCC, the numbers and ratios of different immune cells have changed specifically, which exerts profound influences in the course of HCC, either promoting or inhibiting the tumor progression[54].

Regarding this issue, the first question is how these immune cells abnormally aggregate in HCC tumor tissues or peri-tumor tissues. The chemokines in the microenvironment have surely played a critical role[55]. In a CCR2-knockout mice model, intraportal injected colon cancer cells exhibit obvious delayed growth in liver; the reduced accumulation of macrophages and

Table 1 Chemokines and chemokine receptors in hepatocellular carcinoma

Chemokines	Other names	Chemokine receptors	Subclass	Ref.
CXCL1	GROα	CXCR2	CXC	[20,21,83]
CXCL2	GROβ	CXCR2	CXC	[21,83]
CXCL5	ENA78	CXCR2	CXC	[21,22]
CXCL8	IL-8	CXCR1, CXCR2	CXC	[16,17,21]
CXCL9	MIG	CXCR3	CXC	[17,18,70]
CXCL10	IP-10	CXCR3	CXC	[17,29,51,70]
CXCL11	I-TAC	CXCR3, CXCR7	CXC	[14,50]
CXCL12	SDF-1	CXCR4, CXCR7	CXC	[12,13,15,86,89,92]
CXCL14	BRAK	Unknown	CXC	[23]
CXCL16	SR-PSOX	CXCR6	CXC	[49,106]
CCL2	MCP-1	CCR2	CC	[17,56,81,116]
CCL3	MIP-1α	CCR1, CCR5	CC	[27,28,81,122]
CCL4	MIP-1β	CCR5	CC	[29,48]
CCL5	RANTES	CCR1, CCR3, CCR5	CC	[27,29,100]
CCL15	HCC-2, leukotactin-1	CCR1, CCR3	CC	[33,100]
CCL17	TARC	CCR4	CC	[34,66]
CCL19	ELC, MIP-3β	CCR7	CC	[37,117-119]
CCL20	MIP-3α	CCR6	CC	[30-32]
CCL21	SLC	CCR7	CC	[37,117-119]
CCL22	MDC	CCR4	CC	[35,66]
CCL26	Eotaxin-3	CCR3, CX3CR1	CC	[110]
CCL27	CTACK, ILC	CCR10	CC	[36]
CX3CL1	Fractalkine	CX3CR1	CX3C	[39,40]

GRO: Growth regulated oncogene; ENA78: Epithelial neutrophil-activating protein 78; IL-8: Interleukin 8; MIG: Monokine induced by IFN-γ; IP-10: IFN-γ-induced protein 10; I-TAC: IFN-inducible T cell alpha chemoattractant; SDF-1: Stromal cell-derived factor 1; BRAK: Breast and kidney expressed chemokine; SR-PSOX: Scavenger receptor that binds phosphatidylserine and oxidized lipoprotein; MCP-1: Monocyte chemotactic protein 1; MIP-1α: Macrophage inflammatory protein-1α; HCC: Hepatocellular carcinoma; TARC: Thymus activation-regulated chemokine; ELC: Epstein-Barr virus-induced molecule 1 ligand CC chemokine; SLC: Secondary lymphoid tissue chemokine; MDC: Macrophage-derived chemokine; CTACK: Cutaneous T-cell-attracting chemokine; ILC: Interleukin-11 receptor α-locus chemokine; IFN: Interferon.

hepatic stellate cells, relying on the CCL2-CCR2 signal for effective migration to the liver, accounts for this inhibitory effects[56]. Besides, the HCC cells secrete high levels of CCL2 upon up-regulation of Forkhead box Q1, and conduct a direct chemotactic effect on macrophages, which again deteriorates the progression of HCC[57]. In addition to macrophages, the CCL2-CCR2 signal also recruits myeloid derived suppressor cells (MDSCs) into tumor tissues, and maintains the immunosuppression in the microenvironment. The HCC cell line H22 produces CCL2 constitutionally and induces the migration of MDSCs significantly *in vitro*[58]. Following experiments *in vivo* confirm this observation that the increased expression of CCL2 in tumor tissues correlates with the accumulation of MDSCs in different HCC models, either DEN-induced HCC or subcutaneously implanted HCC model[59]. However, the roles of CCL2 might be both harmful and beneficial, as suggested by the finding that the reduction of intratumoral CCL2, due to nitration by reactive nitrogen species, inhibits the infiltration of tumor specific T cells and traps these T cells in the peri-tumor stroma, contributing to the immune suppression in tumor tissues[60]. Indeed in the human HCC tissues, the CCL2 produced by both tumor cells and immune cells also correlates significantly with intratumoral $CD4^+$ Th1 cells, $CD8^+$ cells and NK cells, indicating a chemotactic role for these cells that favor an anti-tumor repertoire[61]. Therefore, the thorough understanding of CCL2 in HCC needs further experiments taking into account both the models and tumor stages.

Regulatory T cells (Tregs) are key modulators in tumor-induced immune suppression and the aggregation of Tregs in HCC inevitably influences the progression of HCC[62]. Different chemokines have been found to attract Tregs into the HCC tissues. In patients infected by HCV, intrahepatic levels of CCL17 and CCL22 are significantly up-regulated, correlating with the increased number of Tregs; the *in vitro* system identifies that dendritic cells (DCs) derived CCL17 and CCL22 leads to the enhanced aggregation of Tregs[63]. Interestingly, in HBV-positive HCC, CCL22 also recruits Tregs into tumor tissues *via* the TGF-β-miR-34a-CCL22 axis[64]. The CCL20-CCR6 axis is another chemokine signal that recruits Tregs into the tumor tissues. The CCL20 is highly expressed in tumor tissues and correlates with the increased number of Tregs, and the migration experiments also confirm the direct chemotactic effects of CCL20 on Tregs[31]. In concordance with these observation, our recent results also indicate a key role of chemokine system in Tregs from peripheral blood of HCC from the perspective of microRNAs[65]. However, the highly expressed CCL20

Table 2 Pleiotropic functions of chemokines in hepatocellular carcinoma

Categories	Chemokines	Chemokine origins	Receptors participated	Functions
Inflammation	CXCL8	HCC cells	Not clarified	Increasing inflammatory cytokines
	CXCL16	HCC cells	CXCR6	(IL-6, IL-8, *etc*.) and recruiting leukocytes
	CCL3	HCC cells and HSC	CCR3	(macrophages, neutrophils, *etc*.)
	CCL5	HCC cells and HSC	CCR5	
Influences on immune cells	CXCL5	HCC cells	Not clarified	Chemotaxis of neutrophils
	CXCL9	HCC cells and endothelial cells	CXCR3	Chemotaxis of T cells
	CXCL10	HCC cells, macrophages and TILs	CXCR3	Chemotaxis of T cells and NK cells
	CXCL16	HCC cells	CXCR6	Chemotaxis of neutrophils
	CCL2	HCC cells, macrophages and TILs	CCR2	Chemotaxis of HSC, macrophages, MDSC, and T cells
	CCL5	HCC cells, macrophages and TILs	CCR5	Chemotaxis of T cells and NK cells
	CCL20	HCC cells	CCR6	Chemotaxis of Th17 cells and Tregs
	CCL22	HCC cells and DCs	CCR4	Chemotaxis of Tregs
Angiogenesis	CXCL3, CXCL5	HCC cells	Not clarified	Promoting angiogenesis *via* mechanisms not clarified
	CXCL8	CSCs	Not clarified	Promoting endothelial cell tube formation
	CXCL9	Not clarified	CXCR3	Inhibiting angiogenesis by abrogation of VEGF effects
	CXCL12	Endothelia cells	CXCR4, CXCR7	Enhancing angiogenesis through VEGF
	CXCL16	HCC cells	CXCR6	Promoting angiogenesis via mechanisms not clarified
	CCL2	HCC cells and endothelial cells	CCR2	Enhancing the proliferation of endothelial cells
	CCL3	HCC cells and endothelial cells	CCR1, CCR5	Enhancing the proliferation of endothelial cells
Direct effects on HCC cells	CXCL1, CXCL2, CXCL16	Not clarified	Not clarified	Enhancing the growth of HCC cells
	CXCL5	HCC cells	Not clarified	Enhancing the migration, invasion, and growth of HCC cells
	CXCL8	Not clarified	CXCR2	Enhancing the migration of HCC cells
	CXCL10	hepatocytes	Not clarified	Enhancing the survival of hepatocytes
	CXCL12	HCC cells, HSC	CXCR4, CXCR7	Enhancing the migration, invasion, growth and survival of HCC cells
	CCL2	WAT, CAF	CCR2	Enhancing the migration, invasion, and growth of HCC cells
	CCL3, CCL5	Not clarified	CCR1	Enhancing the migration and invasion of HCC cells
	CCL15	HCC cells	Not clarified	
	CCL20	HCC cells	CCR6	Enhancing the migration, invasion, and growth of HCC cells

HSC: Hepatic stellate cells; TILs: Tumor-infiltrating leucocytes; MDSC: Myeloid derived suppressor cells; Tregs: Regulatory T cells; DCs: Dendritic cells; VEGF: Vascular endothelial growth factor; CSCs: Cancer stem cells; WAT: White adipose tissue; CAF: Cancer-associated fibroblast; HCC: Hepatocellular carcinoma.

is also an important signal for Th17 cells infiltration into HCC[66]. Because Tregs and Th17 cells are two representative T cells with relatively opposite functions in most immune milieu, it is worth elucidating how the two subpopulations work in the same HCC micro-environment.

The CXCL16-CXCR6 and CXCL5-CXCR2 axes have a major effect on neutrophils in HCC. HCC cell lines and tumor tissues contain high levels of CXCL16 and CXCR6, and the latter correlates with increased neutrophils in tumor tissues and with a worsen prognosis of HCC patients[49]. It should be noted that the evidence for direct chemotaxis of neutrophils towards CXCL16 is still lacking, and it is not clear what and how this axis affects neutrophils. In contrast, CXCL5 shows an obvious chemotactic effect on neutrophils *in vitro*, and the level of CXCL5 in HCC tissues significantly associates with the increased neutrophils in the tumor tissues and promotes the progression of tumor[22]. CXCR3 and CCR5 are reported to facilitate different T cells traffic to the HCC, either inhibiting or promoting the progression of HCC. CXCL9 and CXCL10, the ligands for CXCR3, are produced by HCC cells and show potent attraction of $CD4^+$ and $CD8^+$ T cells[17,67]; CCL5 produced by tumor tissues has closely correlated with infiltration of CCR5 positive T cells and macrophages[48,61].

Although macrophages can be efficiently recruited into the tumor sites in HCC *via* CCL2/CCR2 and CCR5[48,56,57], their functions tightly rely on their phenotypes. Upon activation by stimuli such as antigens and cytokines,

macrophages undergo different polarization into either M1 or M2 or M2-like phenotype. M1 phenotype (classical activation, stimulated by TLR ligands and IFN-γ) shows potent anti-tumor functions *via* production of large amount of proinflammatory cytokines, while M2 and M2-like phenotype (alternative activation, stimulated IL-4/IL-13) promote tumor progression *via* tissue remodeling and immunoregulation[68,69]. Therefore, it is worth exploring exactly which phenotypes dominate in the microenvironment and its roles in the progress of HCC.

The abundant chemokines in the HCC milieu contribute greatly to the aberrant infiltration of immune cells; in the meantime, some chemokine receptors on these immune cells also alter significantly, which synergistically contributes to the abnormal migration. To gain a better understanding of the chemokine system in immune cells, these alterations of chemokine receptors should not be neglected.

The initial research finds that CCR6 is reduced significantly in $CD4^+$ and $CD8^+$ T cells from peripheral blood in HCC patients, indicating a possible role in the recruitment of lymphocytes from peripheral blood to HCC[70]; however, following studies in delineated T cell subpopulation find that CCR6 expression is not altered significantly on Th17 cells from either tumor tissues or peripheral blood[66]. In contrast, the expression of CCR6 is significantly higher on IL-17-producing $CD8^+$ T cells (Tc17 cells, derived from HCC tissues) and Tregs (derived from peripheral blood), suggesting a role of CCR6 facilitating Tc17 cells and Tregs infiltration into tumor tissues[31,71]. The origins of immune cells might affect the expression pattern of CCR6[72], but this need more evidence. Similarly, the expression of CCR5 is reduced significantly in $CD4^+$ and $CD8^+$ T cells from peripheral blood in HCC patients[70], but increased on intrahepatic $CD4^+$ and $CD8^+$ T cells, NKT cells, NK cells, and B cells in chronic HCV infection[73]. The detailed comparison of expression levels of CCR5 on $CD4^+$ and $CD8^+$ T cells from different sources yields interesting results: T cells from both tumor infiltrating leukocytes and non-tumor liver-infiltrating lymphocytes show increased levels of CCR5 compared with those from peripheral blood lymphocytes[72]. The expression of other chemokine receptors (CCR2, CCR4, CXCR3, CXCR4 and CXCR6) also exhibits certain alterations on T cells, neutrophils, NK cells, NK T cells[34,35,66,71-74].

ANGIOGENESIS

HCC is the typical tumor with hypervascular behaviors and different anti-angiogenic treatments have been utilizing in clinical practices[75,76]. In addition to the traditional angiogenic factors including vascular endothelial growth factor (VEGF) and angiopoietins, the chemokine system is also involved in this process during the development of HCC.

The CXCL12-CXCR4/CXCR7 axis exhibits a direct pro-angiogenic effect in HCC. The initial findings in the rat model demonstrate that AMD3100 (a specific CXCR4 antagonist) simultaneously decreases the size and number of blood vessels *in vivo*[77]. In support of this result, experiments *in vitro* detect large amount of VEGF produced by the HCC cell line SMCC7721 in the presence of CXCL12; the elevated CXCL12 is responsible for tube formation of endothelial cells *in vitro* and *in vivo*[14]. Importantly, cancer stem cells or tumor-initiating cells (TICs) also utilize chemokines to facilitate the angiogenesis. Previous studies have identified CD133 as a marker of TICs in HCC; the $CD133^+$ cells only account for 1.3%-13.6% of the cells in human primary HCC, whereas they have great potentials to self-renew and differentiate, and constitute the indispensible core for HCC cells[78,79]. The sorted $CD133^+$ TICs secrete high levels of CXCL8, and this chemokine consequently promotes the growth and capillary tube formation of HUVECs *in vitro*; blocking the CXCL8 signal by neutralizing antibodies or RNA interfering in HUEVCs leads to reduction of their ability to proliferate and form capillary tubes *in vitro,* and animal experiments validate the angiogenic functions of CXCL8 *in vivo*[80].

CCL2 and CCL3, which are significantly up-regulated in endothelial cells from HCC tissues, also enhance the proliferation of endothelial cells strikingly[81]; further studies facilitated by the CCR1-knockout mice demonstrate that CCR1, the putative receptor for CCL3, directly induces the growth of endothelial cells in HCC[82]. In the CCR2-knockout mice, the reduced microvessel density is correlated with decreased number of macrophages and hepatic stellate cells which are important components during the angiogenesis[56].

Utilizing a different mechanism, CXCL3, CXCL5, CXCL8, and CXCL16 are found to recruit neutrophils into the HCC tissues, and the neutrophils have a well-defined pro-angiogenic role in hepatocarcinogenesis; disrupting these signals either by antibodies or virus-mediated silencing yields potent anti-angiogenic effects[49,83].

Not all chemokines induce angiogenesis in the context of HCC. For example, in the CXCR3-knockout mice, the microvessels in the liver are much higher than the wild type counterpart; in contrast, stimulation of chemical carcinogen carbon tetrachloride leads to the increased levels of the ligand CXCL9 that efficiently ameliorates the angiogenesis in the liver[84].

DIRECT EFFECTS ON BEHAVIORS OF HCC CELLS

As the scenarios in the immune system[1,85], chemokines not only exert chemotaxis effects on HCC cells but also directly influence the properties of tumor cells. Now it is well demonstrated that chemokines directly affect the migration, invasion, growth and survival of tumor cells, which plays a critical role in the development of HCC.

Among the chemokines and receptors, the CXCL12-CXCR4 axis is of great importance. The first study of CXCR4 in HCC demonstrates that in the presence of

CXCL12 HCC cell lines show peri-nuclear translocation of CXCR4 and increase the invasion ability significantly[86], and the increased expression level of CXCR4 in HCC tissues also correlates with the tumor size, metastasis, and survival[86-88]. The binding of CXCL12 to CXCR4 on HCC cells triggers reorganization of cytoskeleton and activates matrix metalloproteinase-9 (MMP-9) and MMP-2, both of which give rise to increased migration and invasion[89-91]. In the cancerous ascitic fluid, CXCL12 is up to 8364 pg/mL, and this concentration effectively induces the migration of HCC cells[87]. During the epithelial-mesenchymal transition (EMT), the CXCL12-CXCR4 signal also plays an important role. On the one hand, in the TGF-β induced EMT system, CXCR4 is highly expressed and required for the enhanced migration and invasion of HCC cells, and further immunostaining in tumor tissues finds that CXCR4 concentrates at the tumor border and perivascular areas[92,93]; on the other hand, CXCL12 derived from hepatic stellate cells induces EMT of HCC cells *in vitro*, coinciding with the increased migration[94]. The findings that CXCR4 can be modulated by several other molecules in the microenvironment complicate its roles. TGF-β, osteopontin and astrocyte elevated gene-1 significantly up-regulate the expression of CXCR4 *via* NF-κB, PI3K-Akt and JNK pathways[91,93,95]. Glycosaminoglycans also compete with cellular heparan sulfate chains to bind CXCL12, which finally causes inhibition of CXCL12 mediated chemotaxis of HCC cells[96].

The other receptor for CXCL12, CXCR7, also has profound effects on the migration and invasion of HCC cells. Increased expression of CXCR7 is found in HCC tumor tissues and highly invasive cell lines; knockdown of its expression in different invasive cell lines results in reduced migration and invasion abilities both *in vitro* and *in vivo*, and this reduction are partially caused by decreased levels of MMP-2 and MMP-9[14,97]. However, in a large cohort of 408 HCC samples, up-regulation of CXCR7 in HCC tissues is confirmed specifically on endothelial cells, but neither human primary hepatocytes nor HCC cell lines. Furthermore, the expression level of CXCR7 on endothelial cells is regulated by hypoxia and low pH which is the typical microenvironment in HCC[15]. These controversial results need to be verified by more experiments in future.

The effects of the CXCL12-CXCR4 axis on proliferation and survival of HCC cells are examined in different cell lines. Because of the intrinsic heterogeneity of these cell lines, the data seem a little paradoxical. Therefore, when reach the conclusions, we should be more cautious. CXCL12 stimulates the proliferation of Huh7 cells[86], possibly through activation of JNK[89]; analysis of the cell cycle demonstrates that CXCL12 triggers the transition of Huh7 cells from G0 into cycle phase, and also drives those cells in G1 phase into S, G2-M phase[89]. In contrast, in other HCC cell lines such as HepG2, there exist no or subtle such effects. Although HepG2 cells express CXCR4, the binding of CXCL12 does not trigger the Ca^{2+} influx, phosphorylation or internalization of CXCR4, and finally fails to activate the following cascade signals[86,98]. Consequently, blocking this axis by other molecules, such as fucoidan, obviously prevents the growth of HCC cells induced by CXCL12[99]. In another HCC cell line FaO induced by TGF-β, CXCL12 efficiently activates extracellular signal-regulated protein kinases (ERK) pathway and enhances the survival in the absence of serum; however, the proliferation and cell cycle of FaO cells is not affected[93]. Recent data also suggest that CXCL12-CXCR7 axis, another ligation of CXCL12, has the same function on proliferation of HCC cells. Silencing CXCR7 by small interfering RNAs in HCCLM3, a highly invasive HCC cell line with abundant expression of CXCR7, decreases the growth of tumor cells both *in vitro* and *in vivo*[97].

The binding of CCL5 and CCL3 to HCC cells depends on CCR1 expression. After the ligation, CCL5 stimulates the tyrosine phosphorylation of focal adhesion kinase, activates PI3K, MAPK, and Rho kinase, leading to increased migration and invasion of HCC cells[100,101]; in contrast, knocking down the expression of CCR1 on HCC cells or disrupting the binding of CCL5 to CCR1 *via* monoclonal antibodies against SDC-1 or SDC-4 effectively abrogates this effect[101,102]. Once binding to the CCR1, CCL3 also induces the potent influx of Ca^{2+} in HCC cells and consequently stimulates the formation of various pseudopodia. These downstream effects directly enhance the migration of tumor cells[103]. Other recent reports also identify a direct effect of CXCL5, CXCL8, CCL15 and CCL20 on the migration and invasion of HCC cells[22,33,104,105], which sheds more light on this field.

CXCR2, along with its ligands CXCL1, CXCL2, and CXCL5, exhibits potent functions in promoting growth of HCC cells. CXCL5 efficiently promotes the proliferation of HCC cells by activating the PI3K-Akt and ERK1/2 pathways *via* the receptor CXCR2[22]. In another experiment *in vitro*, the addition of CXCL1, CXCL2 as well as CXCL16 significantly increases the proliferation of different HCC cell lines[106]. In addition, other chemokines belonging to the CXC family potently drive the growth and survival of hepatocytes under certain pathophysiological conditions such as toxic liver injury which increases the risk of HCC. CXCL10 is largely secreted by hepatocytes treated with CCL4 in the acute toxic liver injury model, and this increased chemokine efficiently rescues the injured hepatocytes from death in an autocrine manner[107].

CCL2 secreted by white adipose tissue induces lipid accumulation in both the primary hepatocytes and Huh7 cells, suggesting a direct role of CCL2 in the pathogenesis of liver steatosis[108]. In other studies, treating HCC cells with apigenin or co-culture them with cancer-associated fibroblasts significantly inhibits or promotes the proliferation of HCC cells, accompanied by the increase of CCR2/CCL2[109,110]. Nevertheless, direct evidence demonstrating the effects of CCL2-CCR2 axis on HCC cells is needed. In contrast, CCL20 has a definitive role and directly enhances the growth of HCC

cells through activating the p44/42 MAPK pathway[111].

THE CHEMOKINE SYSTEM AS THE COMBINATION TREATMENT TARGETS

HCC is among one of the most refractory tumors resistant to chemotherapies, and now there exist very few drugs available for systemic chemotherapies[112]. The important roles the chemokine system played pave new roads to solve this problem, albeit there are no chemokine-based therapies approved in clinical practices at present.

The roles of each single chemokine and the corresponding receptor in the development of HCC are well documented, and disruption of the signal axis indeed hinders the invasive behaviors of HCC; however, due to the complex of microenvironment in HCC, targeting the chemokines alone might not be enough for successful treatments. In contrast, many studies have already found that the chemokine-based combination therapies are promising.

The bicistronic recombinant adenovirus vector expressing HSV thymidine kinase, a suicide gene, and CCL2 on HCC cells has shown remarkable anti-tumor effects in different HCC models. The apoptosis of HCC cells and abundantly accumulated CCL2 synergistically elicits enhanced infiltration of M1 macrophages and NK cells, as well as elevated IL-12 and IL-18 in tumor tissues[113-115]. In the following work, an improved system with adenovirus vector expressing membrane-bound form of CCL2 manifests more powerful anti-tumor effects with increased intratumoral Mac-1^+ macrophages, $CD4^+$ and $CD8^+$ T cells[116].

Although the role of CCL21/CCL19-CCR7 axis in the development of HCC has not been clearly elucidated, the powerful chemotactic effects of this axis and the specific immune milieu in the liver prompt us to explore the therapeutic effects of the CCL21-CCR7 axis. Over-expression of CCL21 either in HCC cells or in DCs shows potent anti-tumor effects in HCC models. Within the tumors containing high level of CCL21, the number of $CD4^+$ and $CD8^+$ T cells and DCs significantly increases, along with elevated levels of IL-12 and IFN-γ and reduced microvessels[117,118]. To further enforce the anti-tumor effects, we devise the new treatment policy by combination of CCL21 and depleting the immunosuppressive Tregs. The combination therapy manifests better anti-tumor effects with increased intratumoral $CD4^+$ and $CD8^+$ T cells and decreased Tregs not only in the local tumor tissues but also in peripheral lymph organs; in addition, the profiles of cytokines and MMPs are also optimized in tumor tissues[119].

Combination therapies based on IL-12 treatment are very effective in different HCC models. CXCL10 is utilized and the co-transfer of IL-12 and CXCL10 yields a very powerful anti-tumor effect, in which the tumor specific cytotoxic lymphocytes and NK cells both play a key role[120]. With the same inspiration, combination of CXCL10/IL-12 expression vector with α-fetoprotein DNA vaccination also achieves better anti-tumor effects and significantly prolongs the survival of the model mice of HCC[121].

In addition, the traditional therapies of HCC can benefit from chemokine-based treatments. Radio-frequency ablation (RFA) is used to locally eradicate HCC, but the recurrence is relatively high. In the HCC mice model, RFA in combination with injection of CCL3 significantly enhances the number of $CD4^+$, $CD8^+$ T cells and $CD11c^+$ DCs in a CCR1-dependent manner, which finally leads to an obvious inhibition of tumor growth[122]. Cisplatin (cis-diamminedichloroplatinum) reduces the tumor burden by 52%, while the combination of cisplatin and G31P (the CXCL8 antagonist) remarkably enhances the suppression effects; meanwhile, the side effects of cispaltin are also released obviously[123].

CONCLUSION

During the carcinogenesis of HCC, the tumor itself needs pivotal mediators to efficiently modulate the microenvironment. These mediators should simultaneously fulfill the basics of tumor cells and steer or disable the functions of immune cells; the chemokines and their receptors are the ideal mediators. Firstly, the Morse code applied by immune cells for routine surveillance is the most effective way to patrol the body, but this code is unfortunately spied by the tumor cells, by which the tumor cells learn and gain effective invasion and dissemination. Secondly, the mechanisms and weapons gifted by the chemokine system, which are originally authorized to the normal immune cells, are excessively utilized by tumor cells in a pro-tumor way, such as modulating the cell cycle and survival, recruiting other immune cells, and secretion of MMPs, *etc*. Thirdly, the bidirectional influences between the tumor cells and the immune cells are bridged by the chemokine system, and this mutual interaction stabilizes the immunosuppression in the microenvironment of HCC. Taking advantages of the strength rooting in the chemokine system, the HCC cells achieve quick progression even when confronted with the host immune system.

Although we have not succeeded in managing HCC through targeting the chemokine system, the more we understand this system in the context of tumor, the more treatment options we will have. It is likely that the future translational research will give us more answers in verifying the therapeutic value of this complicated system in HCC.

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MINIREVIEWS

Current concepts in the immunohistochemical evaluation of liver tumors

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Abstract

Immunohistochemistry often plays an important role in the evaluation of liver tumors. Recent advances have established a classification system for hepatocellular adenomas (HCAs) based on morphology, molecular alterations, and immunohistochemistry. Specifically, loss of liver fatty acid binding protein is seen in HNF1α-inactivated HCA, staining with serum amyloid A is seen in inflammatory HCA, and diffuse staining with glutamine synthetase (GS) is seen in β-catenin activated HCA. A panel of immunohistochemical stains including glypican-3 (GPC-3), heat shock protein 70, and GS are useful in distinguishing HCC from non-malignant dysplastic nodules. Immunohistochemistry is also useful to determine whether a liver tumor is of primary hepatocellular or metastatic origin. Recently described markers useful for this purpose include arginase-1, GPC-3, and bile salt export pump. These newer markers may offer superior utility when compared to traditional markers of hepatocellular differentiation such as alpha-fetoprotein, hepatocyte paraffin-1, polyclonal carcinoembryonic antigen, and CD10. This paper will review recent advances in the immunohistochemical evaluation of liver tumors.

Key words: Immunohistochemistry; Hepatocellular adenoma; Focal nodular hyperplasia; Hepatocellular carcinoma

Core tip: Immunohistochemical stains may be an important complement to morphology in the characterization of liver tumors. Immunohistochemical stains can now be used to subtype hepatocellular adenomas. A panel of immunohistochemical stains can help distinguish hepatocellular carcinoma from dysplastic nodules and hepatocellular adenomas. Several new markers of hepatocellular differentiation have been described. These advances are reviewed.

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INTRODUCTION

Although the global incidence of hepatocellular carcinoma (HCC) varies from region to region, incidence in Europe and North America has been increasing[1]. The majority of these cancers arise in the setting of chronic liver disease, especially chronic infection by hepatitis B virus (HBV) and HCV or cirrhosis of any cause. There is a male predominance of approximately 3:1[1]. With improvements in imaging, specifically four phase multidetector commuted tomography and dynamic, contrast-enhanced magnetic resonance imaging, there has been a concomitant increase in the detection of small liver nodules. While many of these lesions can be diagnosed on imaging, histologic diagnosis remains the gold standard, especially for small nodules (< 1-2 cm), with the goal of diagnosing cancers at an early stage where treatment may be curative. Nonetheless, it can be challenging to distinguish HCC from other hepatocellular proliferations, such as focal nodular hyperplasia (FNH), hepatocellular adenoma (HCA), and dysplastic nodules, particularly when presented with small samples (*e.g.*, needle biopsy). Other primary tumors (*e.g.*, cholangiocarcinoma) and metastases may also enter the differential depending on morphology and history. In recent years, there have been a number of advances reported employing immunohistochemistry to answer such questions. These advances are reviewed herein.

FNH

FNH is a benign hepatocellular lesion thought to develop in response to localized hyperperfusion relating to the presence of an anomalous artery[2,3] with a female predominance of 8:1 and a median age of 38[4]. Histologically, the classical type is a hyperplastic nodular lesion with a central scar containing the anomalous vessel, and a ductular reaction. Most cases are asymptomatic and are often incidentally discovered[4]. Frequently, this entity can be reliably diagnosed on imaging and no treatment is required; however, some cases may be difficult to confirm with imaging, and may require biopsy to rule out HCA and HCC which could require surgical excision. In challenging cases, immunohistochemistry for glutamine synthetase (GS, an enzyme that catalyzes the synthesis of glutamine from glutamate and ammonia, important in nitrogen metabolism) is useful and shows a characteristic geographic "map-like" pattern of staining[5] (Figure 1).

HCA

In contrast to FNH, HCAs are neoplastic clonal proliferations. Resection of adenomas larger than 5 cm is recommended due to the risk of hemorrhage and potential malignant transformation in up to 7% of cases[6]. Risk factors for HCA are female gender, steroid sex hormone exposure (oral contraceptives, anabolic steroids, pregnancy), glycogen storage disease types Ⅰ and Ⅲ, maturity onset diabetes of the young type 3 (MODY3), and familial adenomatosis coli[7,8]. Adenomas can often be diagnosed on imaging, but if the differential diagnosis includes FNH or HCC, the lesion may be biopsied. Based on molecular and immunohistochemical studies, Bioulac-Sage *et al*[8] have identified 4 types of hepatocellular adenomas, now recognized by the World Health Organization. Immunohistochemical stains are therefore useful for both diagnosis and sub-classification.

HNF1α-inactivated HCA

In HNF1α-inactivated HCA (H-HCA), inactivation of both alleles of the *HNF1α* gene, which encodes hepatocyte nuclear factor 1 (a transcription factor related to hepatocyte differentiation), results in increased production of fatty acids, steatosis in hepatocytes, and loss of liver fatty acid binding protein expression, which can be appreciated by a negative immunohistochemical stain (Figure 2A and B). H-HCA accounts for 35%-40% of HCAs and is associated with MODY3 and adenomatosis[2,6,9], but is not thought to be associated with higher risk of transformation to HCC.

Inflammatory HCA

In inflammatory HCA (IHCA), activating mutations in genes (*IL6ST, STAT3, GNAS, FRK*) along the JAK-STAT pathway result in increased expression of inflammatory markers including serum amyloid A (SAA)[9,10]. Histologically, IHCA are characterized by an inflammatory infiltrate, vascular anomalies, and may exhibit a ductular reaction. In the past, this lesion was known as "telangiectatic FNH," but has now been shown by molecular and immunohistochemical analysis to be IHCA[11]. They stain with SAA and C reactive protein by immunohistochemistry (Figure 2C and D) and account for 40%-55% of HCAs[6,9,10].

β-catenin-activated HCA

β-catenin-activated HCAs (β-HCAs) are the subtype of HCA with the highest risk (4%)[12] for transformation to HCC, and account for 10%-15% of HCAs. Histologically, β-HCAs may show cholestasis and both architectural and cytologic atypia including pseudoacinar structures. In β-HCA, activating mutations (predominantly in exons 3, 7, or 8) in the *CTNNB1* gene, which encodes β-catenin, cause activation of the WNT/β-catenin pathway. This is the most commonly mutated pathway in HCC[13]. The mutations may lead to upregulation of the gene coding for GS; consequently, this subtype is expected to exhibit abnormal nuclear staining with β-catenin and diffuse GS staining by immunohistochemistry (Figure 2E and F). Staining for β-catenin is less sensitive than staining for GS, though GS is much less specific than nuclear beta-catenin. The purported sensitivity and specificity of GS in this setting is 100% and 89%[8]. However, in our experience, GS may diffusely stain many adenomas which do not exhibit atypical morphologic or clinical signs of atypia[14]. Furthermore, when our group sequenced GS overexpressing HCA, we could identify β-catenin mutations in only 1 OF 8 HCAs (unpublished

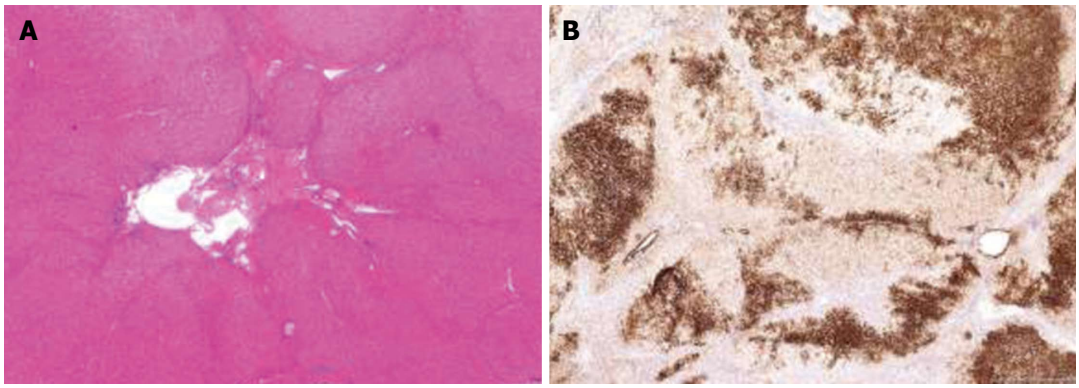

Figure 1 Focal nodular hyperplasia. A: On low-power, focal nodular hyperplasia (FNH) is characterized by nodular hepatocellular proliferation with central scar (hematoxilin and eosin stain, × 1); B: FNH showing typical map-like pattern on glutamine synthetase immunohistochemistry (anti-glutamine synthetase/diaminobenzidine chromogen, × 2).

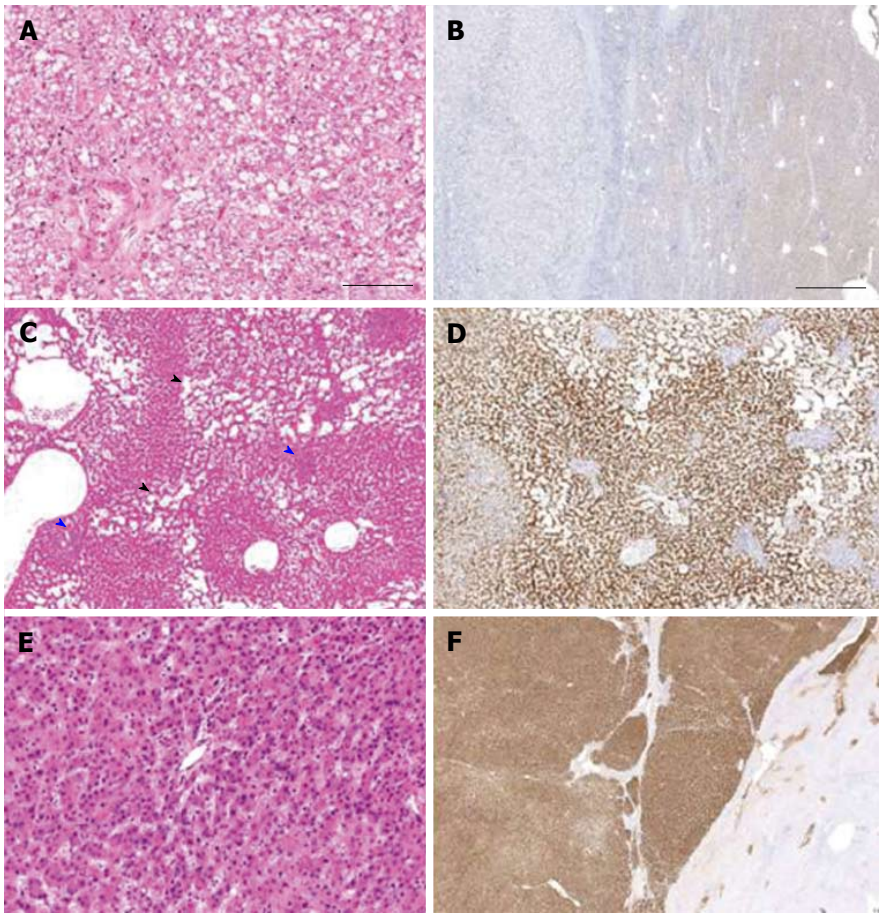

Figure 2 Hepatocellular adenoma. A and B: HNF1α-inactivated hepatocellular adenoma (HCA) with marked steatosis [A, hematoxylin and eosin (HE) stain, × 20] and loss of liver fatty acid binding protein (LFABP) expression by immunohistochemistry (left) in comparison to non-neoplastic liver (right) [B, anti-LFABP/3,3'-diaminobenzidine (DAB), × 1]; C and D: Inflammatory HCA with dilated sinusoids (telangiectasia, black arrowheads) and patchy inflammation (blue arrowheads) (C, HE stain, × 5) and diffuse serum amyloid A staining by immunohistochemistry (D, anti-serum amyloid A/DAB, × 5); E and F: β-catenin-activated HCA with strong diffuse staining for glutamine synthetase (upper left), in comparison to centrilobular staining of normal liver (lower right) (E, HE stain, × 20; F, anti-glutamine synthetase/DAB, × 1).

data). In our opinion, GS overexpression is an imperfect surrogate for β catenin mutation, and should not be a definitional characteristic.

Unclassified HCA

Unclassified HCAs represent the remaining 10% of HCAs, and lack characteristic histology, immunohistochemistry, or molecular changes.

DYSPLASTIC NODULES

The pathogenesis of HCC is thought to be a stepwise accumulation of mutations arising in a small clonal population (dysplastic nodules and a small proportion

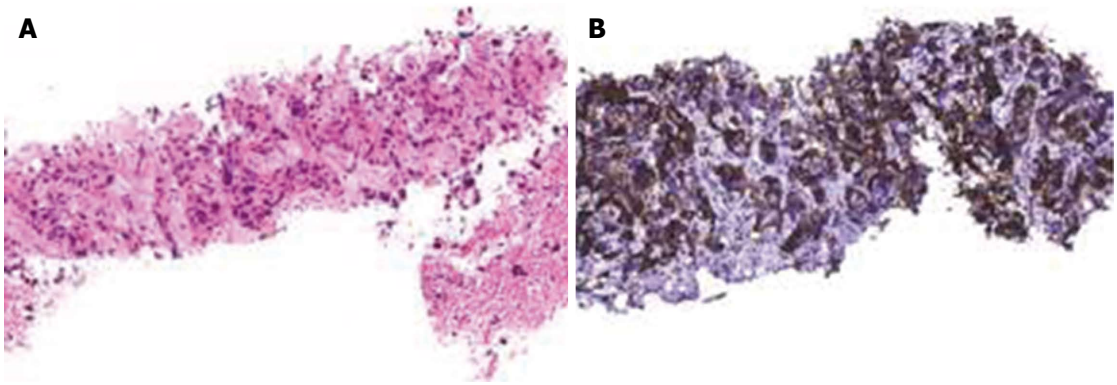

Figure 3 Scirrhous hepatocellular carcinoma. A: Cords and thin trabeculae of neoplastic hepatocytes are embedded in dense, abundant fibrous stroma (hematoxylin and eosin stain, × 20); B: By immunohistochemistry, strong glypican-3 positivity is present in tumor cells (anti-glypican-3/3,3'-diaminobenzidine, × 20).

of adenomas)[13,15]. The background liver is most often cirrhotic, with HBV as the most common underlying cause worldwide, especially in Sub-Saharan Africa and Asia where HBV is endemic, and HCV and the most common underlying cause in the United States[16]. In the cirrhotic liver, it is important to distinguish large regenerative nodules, which are benign, from low- and high-grade dysplastic nodules (H-DN), which precede HCC in a stepwise fashion, and to distinguish these from early and progressed HCC itself. Histologic criteria were established by the International Consensus Group for Hepatocellular Neoplasia in 2009, but the differences between these entities may be subtle as they lie on a continuum. The best criteria to distinguish H-DN from early HCC is the presence of invasion into portal tracts[17]. This feature may not be identifiable on biopsy material, however. Historically, thickened portal plates and subsequently a diminished reticulin framework were noted to be markers of progression from H-DN to early HCC, although an intact reticulin framework could not exclude HCC[18]. In this case, immunostains are a useful aid to histomorphology.

CD34

Normal sinusoidal endothelium does not express CD34. However, since capillarization of sinusoidal endothelium occurs during the progression of dysplastic nodules to HCC (which corresponds to the enhancement seen in HCC on the arterial phase of dynamic imaging modalities), immunostaining for the vascular marker CD34 has been found to be a useful marker of malignant transformation (since it does mark capillarized endothelial cells) . However, while increased to diffuse vascular markings with CD34 is a suspicious finding in a liver tumor, no specific cutoff has yet been established in distinguishing between H-DN and early HCC[18-21].

In studies of biopsy and resection specimens[22,23], a panel of 3 immunostains including glypican-3 (GPC-3), heat shock protein 70 (HSP70), and GS was found to be useful in distinguishing dysplastic nodules from HCC, with a combination of at least any two positive stains shown to be 72% sensitive (resection specimens; 57% on biopsy specimens) and 100% specific in distinguishing early HCC from dysplastic nodules. Overall, it was found that positive staining in at least 2/3 markers supported a diagnosis of HCC, but lack of staining was not sufficient to rule out HCC especially on biopsy specimens.

GPC-3

GPC-3 is a heparan-sulfate cell surface oncofetal proteoglycan noted to be expressed in HCC, but generally not in benign liver (normal or cirrhotic) or in metastatic carcinomas[24,25]. Immunostaining with GPC-3 in HCC may be cytoplasmic and/or membranous. Some authors have found that the sensitivity increases as the tumor becomes less differentiated[25,26]. Other studies have not found higher GPC-3 expression in poorly differentiated tumors, however, so confirmation in additional studies would be helpful[27]. GPC-3 is also useful in distinguishing HCC from HCA[14]. Anecdotally, we have seen strong positivity in a case of scirrhous variant HCC which could easily have been mistaken for metastatic adenocarcinoma (Figure 3). On the other hand, GPC-3 may be less helpful in the fibrolamellar variant of HCC[28]. GPC-3 is known to stain a few other malignancies, such as yolk sac tumor and melanoma[29]. Another caveat is the reported expression of GPC-3 in cirrhotic nodules in cases of hepatitis C infection[30].

HSP70

HSP70 is an anti-apoptotic regulator promoting cell survival and has been implicated in tumorigenesis. HSP70 expression increases stepwise as a lesion progresses from precancerous to advanced HCC[31]. Immunohistochemistry marks HCC, but not dysplastic nodules or HCA[14,23]. Staining is nucleocytoplasmic and may be focal or diffuse, and is 74% sensitive and 98% specific for HCC on resection specimens, and 48% and 94% on biopsy specimens, respectively when evaluating HCC *vs* dysplastic nodule[22,23]. However, a potential pitfall of HSP70 is that it reacts commonly with metastatic adenocarcinomas and cholangiocarcinomas[14]. Therefore,

the utility is restricted to tumors which are clearly hepatocytic in differentiation.

GS

GS catalyzes the conversion of glutamate and ammonia to glutamine in the liver[32]. As noted in the discussion of b-HCA, GS is a target of β-catenin, and is upregulated when this pathway is constitutively activated. In normal liver, GS expression is restricted to perivenular hepatocytes. In neoplasms GS expression should be strong, homogenous, and diffuse (not map-like), and should stain > 50% of the cells in question. Given these conditions, on resection specimens, the sensitivity and specificity were 70% and 94%, respectively (59% and 98% on biopsy specimens) when the consideration was HCC *vs* dysplastic nodule[22,23]. GS is frequently positive in HCA, however, and therefore not useful in the distinction of HCA from HCC[14]. An important caveat when using these 3 markers is that GS and HSP70 are frequently positive in cholangiocarcinoma and metastases, thus highlighting that the utility of this panel is restricted to the specific contexts in which evidence supports their use. GPC-3 is the only one of the three markers above which is useful in overtly malignant tumors requiring evaluation of differentiation.

ESTABLISHING HEPATOCELLULAR ORIGIN

Since poorly differentiated HCC may have histologic overlap with poorly differentiated metastatic tumors and intra-hepatic cholangiocarcinomas, there has always been interest in reliable immunohistochemical markers of hepatocytic differentiation. At a basic level, the cytokeratin profile can be helpful but in most cases of ambiguous morphology, not definitive. Hepatocytes are generally positive for CK8 and 18 and negative for both CK7 and CK20, although HCC may acquire CK7 and or CK20 positivity in some cases[33]. One caveat is fibrolamellar carcinoma, which tends to be found in younger patients without cirrhosis, and has been found to be positive for CK7[34] (as well as CD68[35]). Some HCCs may acquire biliary features (CK19 positivity) by immunohistochemistry; in one study, these patients had a higher recurrence rate of HCC after transplantation, indicating a worse prognosis in these lesions[33]. Therefore, it is often necessary to go beyond cytokeratin analysis.

MARKERS OF PRIMARY HCC

Traditionally, the most commonly used markers for this purpose include hepatocyte paraffin 1 (HepPar-1), alpha-fetoprotein (AFP), CD10, and polyclonal carcinoembryonic antigen (p-CEA)[36-39]. Each marker has drawbacks including limitations of sensitivity and specificity, as well as the requirement of a canalicular staining pattern for hepatocellular specificity in CD10 and p-CEA.

HepPar-1

HepPar-1 is an antibody to carbamoyl phosphate synthetase 1, a urea cycle enzyme in hepatocellular mitochondria, which is expressed predominantly in the liver, but also in other organs such as small intestine[40]. Developed in 1993 from a failed liver allograft[36], this antibody has been found to be relatively sensitive (70%, although some authors report higher sensitivity) and specific (84%) for hepatocellular differentiation[41], in both normal tissue and HCC, as well as hepatoblastoma. Caveats with this marker include the reported loss of sensitivity as tumors become less differentiated[36,42,43], and frequent negativity in the scirrhous variant[44]. Through many years of use, many of the pitfalls of HepPar-1 have been elucidated. For one, it marks hepatoid tumors of any organ[45-47]. Furthermore, because of the expression of carbamoyl phosphate synthetase in small bowel, it marks many small intestinal adenocarcinomas, as well as adenocarcinomas of the ampulla with intestinal morphology[48]. Additionally, a large study by Lugli *et al*[47] suggested that other tumors (notably gastric, lung, small intestinal, colonic, and pancreatic adenocarcinomas, cholangiocarcinoma, and melanoma) may have low (generally less than 15%) rates of positive staining with HepPar-1. Ovarian and neuroendocrine carcinoma have also been reported to show occasional positivity[42]. However, staining in non-hepatocellular tissues has generally been reported as weak, whereas staining in tissues of hepatocellular origin tends to be strong and cytoplasmic. Thus, despite these many well-established pitfalls, HepPar-1 remains a very useful marker.

AFP

AFP is an oncofetal glycoprotein that has been used as a tumor marker both in serum and in tissue by immunohistochemistry for some time. Although also positive in yolk sac tumors, specificity by immuno-histochemistry is high (97%) with very few metastatic adenocarcinomas or cholangiocarcinomas showing positive staining. However, sensitivity is low, around 30%[17], limiting its utility.

p-CEA

In normal liver, p-CEA stains a biliary glycoprotein similar to CEA (a fetal glycoprotein), present in the bile canaliculi and ductal epithelium. The staining pattern is characteristic: a delicate branching canalicular pattern, which has been reported as 70% sensitive and 100% specific for hepatocellular differentiation[43]. However, this pattern may be lost as HCC dedifferentiates, and in general, the staining pattern may be difficult to distinguish from non-specific membranous or cytoplasmic staining, which can be seen in some HCC, bile duct epithelium, and metastatic adenocarcinoma and cholangio-carcinoma[42,43,49].

CD10

CD10 is a membrane metallo-endopeptidase which

Table 1 Immunohistochemical stains in liver tumors

Marker	Staining pattern	Expected staining	Advantages	Disadvantages
LFABP	Cytoplasmic/nuclear	Normal: Diffuse H-HCA: Negative	Can subclassify HCA	
SAA	Cytoplasmic	Normal: Negative IHCA: Strong	Can subclassify HCA	
GS	Cytoplasmic	Normal: Perivenular FNH: Map-like β-HCA/HCC: Diffuse	Can subclassify HCA	Not specific for β-HCA (other HCA sub-types can stain) HCA and HCC have similar staining patterns Metastatic adenocarcinoma in liver usually positive
HSP70	Cytoplasmic/nuclear	Normal, HCA, H-DN: Negative HCC: Positive	Suggestive of malignancy	Staining may be focal Metastatic adenocarcinoma frequently positive
GPC-3	Cytoplasmic/membranous	Normal: Negative HCC: Positive	More sensitive in poorly differentiated tumors Marks scirrhous HCC Only single marker which supports both hepatocellular differentiation and malignancy	Low sensitivity in well-differentiated tumors May not react with fibrolamellar HCC
CD34	Cytoplasmic/membranous	Normal: Portal vessels, rare sinusoids HCC: Diffuse capillarization	Can help demarcate extent of lesion	No specific cutoff for increased staining
AFP	Cytoplasmic	Normal: Negative HCC: Positive	High specificity for HCC	Low sensitivity
p-CEA	Canalicular	Normal: Canalicular HCC: Canalicular Metastatic ca: Any pattern other than canalicular	High sensitivity and specificity for hepatocytes when definite canalicular pattern present	May be difficult to interpret, since only canalicular pattern is specific for hepatocytes
CD10	Canalicular	Normal: Canalicular HCC: Canalicular Metastatic ca: Any pattern other than canalicular	High sensitivity and specificity for hepatocytes when definite canalicular pattern present	May be difficult to interpret, since only canalicular pattern is specific for hepatocytes
HepPar-1	Cytoplasmic	Liver only	Marks cells of liver origin, both normal and neoplastic	Mediocre specificity, including any hepatoid lesion Less sensitive in poorly differentiated tumors
ARG-1	Cytoplasmic/nuclear	Liver only	More sensitive and specific than HepPar-1	Less sensitive in poorly differentiated tumors
BSEP	Usually, but not exclusively canalicular	Normal, HCC: Canalicular (usually) with occasional dot-like or incomplete membranous pattern	High sensitivity and specificity for hepatocytes Easier to interpret than other p-CEA and CD10	New marker, pitfalls and disadvantages currently unknown

HCC: Hepatocellular carcinoma; HCA: Hepatocellular adenoma; H-DN: High-grade dysplastic nodules; p-CEA: Polyclonal carcinoembryonic antigen; H-HCA: HNF1α-inactivated HCA; LFABP: Liver fatty acid binding protein; GS: Glutamine synthetase; FNH: Focal nodular hyperplasia; SAA: Serum amyloid A; IHCA: Inflammatory HCA; GPC-3: Glypican-3; HSP70: Heat shock protein 70; HepPar-1: Hepatocyte paraffin 1; AFP: Alpha-fetoprotein; BSEP: Bile salt export pump; ARG-1: Arginase-1.

cleaves the amino group of hydrophobic residues, and is expressed in multiple tissues. Although cytoplasmic, membranous, and apical staining may be seen in adenocarcinomas from multiple other primary sites, the same canalicular pattern described above for p-CEA when seen with anti-CD10 is specific for both normal and neoplastic liver, with a reported sensitivity of 68% and specificity of 100%[39]. As above, however, canalicular staining may be difficult to establish. Recently, additional markers have been described.

GPC-3

As mentioned previously, some investigators have reported a higher GPC-3 sensitivity in poorly differentiated HCC than in well differentiated tumors[26]. This could be an important finding, as HepPar-1 expression becomes less sensitive in poorly differentiated lesions. The reported overall sensitivity of GPC-3 for HCC is 75% (39% for well-differentiated, 89% for poorly differentiated)[50] and the specificity is 86%. GPC-3 staining has been reported in extragonadal germ cell tumors, melanoma, ovarian carcinoma,

and squamous cell cancer of the lung[25], and more recently, in pancreatic acinar cell carcinoma, esophageal squamous cell carcinoma and adenocarcinoma[50]. Still, most of these are rare in the liver, and so GPC-3 is now commonly used as part of the immunohistochemical panel used to establish the histogenesis of a tumor in liver.

Arginase-1

Another novel marker of hepatocellular differentiation is Arginase-1 (ARG-1). ARG-1 is a manganese urea cycle metalloenzyme isoform expressed primarily in the liver. In two series[41,51], it was been found to be more sensitive and specific (84% and 96%, respectively[41]) than HepPar-1. As with many other markers of hepatocellular differentiation, expression decreased as tumor grade increased, but ARG-1 was more sensitive grade for grade than HepPar-1 in both series. In terms of specificity, ARG-1 expression has been reported in a small subset of pancreatic, colon, gastric, and pulmonary cancers, but when HepPar-1 was also positive, specificity rose to 100%. ARG-1 does not stain non-neoplastic small intestinal and ampullary mucosa, and only rarely stains adenocarcinomas of these sites[48].

Bile salt export pump

Bile salt export pump (BSEP) is another recently described immunohistochemical marker for hepatocellular differentiation[52,53]. BSEP is a membrane-bound ATP-binding cassette transporter expressed only in hepatocytes, and functions to transport bile out of the hepatocyte. A study by Lagana *et al*[53] reported 90% sensitivity and 100% specificity for HCC. Furthermore, since it is expressed exclusively in hepatocytes, there is no requirement for a canalicular pattern of staining as there is with CD10 and p-CEA (though canalicular staining was present in 33 of 43 positive cases).

Cholangiocarcinoma

Intrahepatic cholangiocarcinoma is generally unlikely to be histologically confused with HCC. The morphology can, however, be identical to metastatic carcinomas. In the rare cases where a pathologist is entertaining the diagnosis of HCC and cholangiocarcinoma, the markers listed above, along with the addition of CK7 and CK19, should suffice.

CONCLUSION

Diagnosing liver tumors can be challenging, especially on needle biopsy specimens which may only minimally sample a lesion. Proper identification and classification is essential, as some lesions require no treatment at all, whereas in others, resection, chemotherapy, and transplantation may be offered. Recent advances in immunohistochemistry have furthered our ability to accurately characterize these lesions (summarized in Table 1).

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MINIREVIEWS

Current systemic treatment of hepatocellular carcinoma: A review of the literature

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Abstract

Hepatocellular carcinoma (HCC) is the fifth most common form of human cancer worldwide and the third most common cause of cancer-related deaths. The strategies of various treatments for HCC depend on the stage of tumor, the status of patient's performance and the reserved hepatic function. The Barcelona Clinic Liver Cancer (BCLC) staging system is currently used most for patients with HCC. For example, for patients with BCLC stage 0 (very early stage) and stage A (early stage) HCC, the curable treatment modalities, including resection, transplantation and radiofrequency ablation, are taken into consideration. If the patients are in BCLC stage B (intermediate stage) and stage C (advanced stage) HCC, they may need the palliative transarterial chemoembolization and even the target medication of sorafenib. In addition, symptomatic treatment is always recommended for patients with BCLC stage D (end stage) HCC. In this review, we will attempt to summarize the historical perspective and the current developments of systemic therapies in BCLC stage B and C in HCC.

Key words: Hepatocellular carcinoma; Transarterial chemoembolization; Sorafenib; Systemic treatment; Molecular target therapy

Core tip: Sorafenib is a multi-targeted tyrosine kinase

inhibitor that was the first systemic therapy in the world to improve the survival rate of patients with advanced hepatocellular carcinoma (HCC) in a phase Ⅲ trial. However, the overall outcomes are sometimes unsatisfactory and there is a need for second line therapies in patients with advanced HCC who still progress after the use of sorafenib. Novel systemic approaches are needed in advanced HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a primary cancer of the liver with a rate of occurrence approximately up to 90%. Clinically, HCC is the fifth most common form of cancer worldwide and the third most common cause of cancer-related deaths[1]. It is usually diagnosed as the advanced stage of the hepatic tumor and the median survival rate is poor (6-20 mo) when found[2]. The incidence and distribution of HCC varies widely among geographical locations and races in the world. For example, the incidence of HCC is highest in Asia and Africa. Now most doctors believe that the potential reason for the higher incidence rates of HCC is the prevalence of hepatitis B virus (HBV) and/or hepatitis C virus (HCV) which strongly predisposes to the development of chronic liver disease, liver cirrhosis and subsequently HCC[3]. In 1988, one large prospective HBV study in Taiwan robustly demonstrated that HBV is the primary cause of the high HCC incidence rate in regions of high HBV prevalence[4]. In Taiwan, nearly 5000 patients die from HCC every year. Although the newer treatment modalities have become more multivariate in recent years, the survival rates of patients with advanced HCC has still not significantly improved. The one year survival rate of treated patients with advanced HCC was around 25% in 1993 and 30% in 2003[5]. Until recently, there was no remarkable and effective medical therapy for patients with advanced HCC. To the best of our knowledge, HCC is a more aggressive tumor and the decision regarding therapeutic options often depends on the stage of this cancer and the patient's hepatic reserve. A number of staging systems are available[2,6-8] but there is no worldwide consensus on a single system. For instance, the Child-Pugh (C-P) classification system and the model for end-stage liver disease score can be used to assess the patient's hepatic reserve and liver function. Besides, the performance status (PS) of patients also needs to be taken into consideration. The Barcelona Clinic Liver Cancer staging and prognostic system accounts for variables related to tumor stage, physical performance, liver functional status, cancer-related symptoms and so on. It may provide the link between diseases and treatment strategies. Curative therapy, including various surgeries (*e.g.*, hepatic resection and liver transplantation), locoregional therapies (percutaneous ethanol injection and radiofrequency ablation), have been proven to have better survival benefits in the very early and early stage of HCC (such as stage 0-A). However, the intermediate stage (*i.e.*, stage B) of HCC comprises a highly heterogeneous patient population and therefore poses challenges for therapeutic management. A sub-classification B1-B4 was recently proposed, taking the C-P score, tumor burden (up to seven criteria), PS and portal vein thrombosis into account[9]. Transarterial chemoembolization (TACE) and radioembolization are the primary options for these patients with preserved liver function (C-P classification A) and PS score 0. Unfortunately, if the HCC has developed into the severely advanced stage, only systemic medical treatment is indicated and the prognosis and outcome is very poor for these patients. In this paper, we will discuss systemic treatment for patients with HCC for whom liver-directed therapy is not appropriate.

SYSTEMIC CYTOTOXIC CHEMOTHERAPY

HCC is highly refractory to conventional cytotoxic chemotherapy. In the last decade, no effective conventional systemic cytotoxic therapy has been available and no single regimen has emerged as superior to any other[10]. The substances related to sensitive of chemotherapy include P-glycoprotein[11-13], glutathione-S-transferase[14], heat shock proteins[15], topoisomerase Ⅱα[16] and p53[17]. Besides resistance, the major side effects of systemic chemotherapy are poorly tolerated by patients with severe hepatic dysfunction. One study which enrolled 147 previously untreated HCC patients demonstrated that patients with significant cirrhosis (ascites, serum total bilirubin more than 2.0 mg/dL), performance status of 2-3, a tumor occupying more than 50% of the entire liver and tumor thrombus in the main portal trunk may not be responsive to chemotherapy[18]. The regimens of systemic chemotherapy for HCC under clinical study are as follow: monotherapy regimens, including doxorubicin, mitoxantrone, fluoropyrimidines, gemcitabine, irinotecan and thalidomide; combination chemotherapy, including cisplatin-based, gemcitabine-based and oxaliplatin-based regimens; and PIAF regimen [cisplatin (P)/interferon α-2b (I)/doxorubicin (A)/fluorouracil (F)]. Most published studies of systemic chemotherapy revealed that the effective response rates were no more than 25% and there is no evidence that it may improve the overall survival rate in patients with any subset of HCC[19-21]. However, chemotherapy may still be considered for patients whose tumors progress while on sorafenib treatment. Cytotoxic

therapy should be reserved for medically appropriate patients with adequate hepatic function. The national comprehensive cancer network guidelines (version 2, 2014) recommended that systemic or intra-arterial chemotherapy can be used to treat patients with unresectable HCC by surgery and not a transplant candidate only in the context of a clinical trial[22].

MOLECULARLY TARGETED THERAPY

Hepatocarcinogenesis is a very complex system of pathways and the result of the genetic alterations that may affect multiple signaling cascades. All these pathways include various growth factors such as epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), insulin-like growth factor and regulating specific intracellular pathway (RAF/MEK/ERK pathway). For example, the activation of the RAF/MEK/ERK pathway may lead to the growth of HCC. The EGF would bind to its cognate receptor EGF receptor and trigger signal transduction through the RAF/MEK/ERK pathway. Besides, VEGF may result in HCC angiogenesis and HGF will bind to the c-MET receptor and other molecular signal pathways, including PI3K/PTEN/Akt/mammalian target of rapamycin (mTOR) and Wnt/β-catenin pathways. Recently, many medical doctors and scientists have focused on targeted molecular agents (*e.g.*, sorafenib) and tried to block one or more steps in carcinogenic pathways for retardation of tumor formation[23,24].

Until now, sorafenib has been very popular for patients with the advanced stage of HCC. Clinically, sorafenib is an oral form and belongs to the multi-targeted tyrosine kinase inhibitors (multi-kinase inhibitors) and anti-angiogenic agents. It may inhibit abnormal growth of multiple cell surfaces and intra-cellular kinases which would be involved in angiogenesis, cell proliferation and cellular differentiation. The different kinases include various VEGF receptors (VEGFR-1, 2, 3), platelet-derived growth factor receptor (PDGFR-β), c-KIT and RET. Furthermore, sorafenib was also shown to inhibit the RAF/MEK/ERK pathway[25,26].

Sorafenib is also the first medical therapy to show a statistically significant and clinically meaningful overall survival benefit in advanced HCC and is considered to be a standard therapy as it inhibits growth and angiogenesis of HCC. From the SHARP trial (phase Ⅲ) in many countries in 2008, 602 patients with advanced HCC and C-P classification A cirrhosis were randomly assigned to the sorafenib or placebo group. Improvement of median overall survival (OS) was seen in the sorafenib group (10.7 mo *vs* 7.9 mo, HR = 0.69, $P < 0.001$). Treatment was also associated with an increased time to progression (TTP) (5.5 mo *vs* 2.8 mo, HR = 0.58, 95%CI: 0.45-0.74, $P < 0.001$). Overall toxicity did not differ between the treatment and placebo arm (52% *vs* 54%)[27]. In 2009, another phase Ⅲ trial in the Asia-Pacific region (so called ORIENTAL study) reported 226 patients of advanced HCC with C-P classification A cirrhosis who received sorafenib 400 mg twice daily or placebo. Patients with sorafenib therapy had better median OS (6.5 mo *vs* 4.2 mo) and TTP (2.8 mo *vs* 1.4 mo). Only small side effects about grade 3 or 4, including hand-foot syndrome (11%), diarrhea (6%) and fatigue (3%), were found in patients[28]. These exciting results were encouraging and the better efficacy of sorafenib was validated. Thus, sorafenib was approved by the Food and Drug Administration in November 2007 in the United States and has now become the standard care for first line systemic treatment in advanced hepatocellular carcinoma.

Although sorafenib is the first and only targeted therapy approved for advanced HCC, it has also been studied in combination with other systemic chemotherapeutic agents. For example, in a phase Ⅱ trial, patients with advanced HCC were randomly assigned to receive doxorubicin in combination with sorafenib or doxorubicin alone[29]. The combination of doxorubicin and sorafenib improved median TTP (6.4 mo *vs* 2.8 mo, $P = 0.02$), median OS (13.7 mo *vs* 6.5 mo, $P = 0.006$) and progression-free survival (PFS) (6.0 mo *vs* 2.7 mo, $P = 0.006$), compared to doxorubicin alone. However, the effects and mechanisms of doxorubicin in this synergism still remained unclear. Recently, another phase Ⅲ drug trial comparing the combination of doxorubicin and sorafenib with sorafenib alone conducted by the National Cancer Institute is still ongoing. Combination of sorafenib with other systemic agents, such as octreotide[30], has been reported. All of these trials reported improved OS when compared to sorafenib alone; however, the sample sizes were small. The exact and final outcomes deserve intervention.

The worry about the drug resistance of sorafenib has attracted attention. The primary resistance mechanism is possibly due to the genetic heterogeneity and acquired resistance is possibly related to activation of the compensatory pathways, such as the PI3K/Akt and JAK-STAT pathways, tumor hypoxia, EMT, *etc.*[31]. The mechanisms for the resistance of HCC to sorafenib are complicated and remain unclear and need further study.

OTHER ANTIANGIOGENIC AGENTS IN CLINICAL DEVELOPMENT

Sunitinib

A variety of oral multiple tyrosine kinase inhibitors have been recently developed after the impact of sorafenib. Other oral, small molecule, multi-targeted receptor tyrosine kinases, so called, were developed. This agent was proved to inhibit the VEGFR (1, 2, 3), PDGFRs, KIT, RET and the fms-like tyrosine kinase-3 receptor. Some of these factors pay a role in both tumor angiogenesis and tumor cell proliferation. When we used sunitinib to treat patients with advanced HCC, the simultaneous inhibition of these targets therefore led to reduced tumor sizes, vascularization cancer cell death and even tumor shrinkage ultimately.

Indeed, most of the side effects from sunitinib are very mild, including fatigue, diarrhea, nausea and anorexia. In an initial phase Ⅱ trial, 37 patients with advanced HCC were treated with sunitinib. Only one patient had a partial response and 35% of patients were stable[32]. However, grade 3 to 4 toxicity from agents was prominent, including thrombocytopenia (37.8%), neutropenia (24.3%), asthenia (13.5%), hand-foot syndrome (10.8%) and anemia (10.8%) in some patients. Fatal treatment-related adverse events were reported in four patients (10.8%). Therefore, more attention should be paid to this event when treating patients. A phase Ⅲ trial of 1074 patients with advanced HCC disclosed that sunitinib was not superior to sorafenib, with a worse median OS (7.9 mo *vs* 10.2 mo) and more toxicity[33].

Linifanib

Linifanib is a multi-kinase inhibitor targeting VEGFR and PDGFR. In a phase Ⅱ trial involving 44 patients (of which 89% were Asian), the single agent linifanib was found to be clinically active in patients with advanced HCC, with an acceptable safety profile[34]. However, in a phase Ⅲ study, 1000 patients with advanced HCC and C-P classification A cirrhosis were randomly assigned to linifanib or sorafenib treatment. The median OS was 9.1 mo in the linifanib group, compared to 9.8 mo in the sorafenib group. TTP was 5.4 mo *vs* 4.0 mo (P = 0.001) in the linifanib group *vs* the sorafenib group[35]. Although linifanib had a longer TTP, its superiority in survival needs to be verified.

Brivanib

Brivanib is a selective dual receptor inhibitor against fibroblastic growth factor receptor and VEGFR. It was shown to have antitumor activity in patients with advanced HCC in two phase Ⅱ studies[36,37]. In a phase Ⅲ trial, brivanib was reported to have an OS of 9.4 mo *vs* 8.2 mo in the placebo group (as a second line treatment), which was not statistically significant (P = 0.33)[38]. Another phase Ⅲ trial compared brivanib with sorafenib as first line treatment[39]. Among 1150 patients with advanced HCC, the median OS was 9.5 mo in the brivanib group and 9.9 mo in the sorafenib group, with no statistically significant difference. However, brivanib was less well tolerated than sorafenib. Treatment discontinued due to side effects was 43% in the brivanib group compared to 33% of the sorafenib group[39].

OTHER INVESTIGATIONAL APPROACHES IN TARGETED KINASE INHIBITORS

Newer molecularly targeted studies are being developed in phase 1/2 studies, including everolimus, targeting inhibitors of the mTOR[40,41] and inhibitors of HGF/c-Met, such as tivantinib[42]. The single arm, phase 1/2 study of everolimus enrolled 28 patients with advanced HCC and defined 10 mg/d as the phase Ⅱ dosage[40]. The median PFS and OS were 3.8 mo and 8.4 mo, respectively. Another phase Ⅰ study enrolled 39 patients with advanced or metastatic HCC and recommended everolimus dosing of 7.5 mg daily[41]. The phase Ⅱ study reported that TTP was longer in the tivantinib group than in the placebo group (1.6 mo *vs* 1.4 mo)[42]. For patients with MET-high tumors, the TTP was longer in the tivantinib group than in the placebo group (2.7 mo *vs* 1.4 mo). The most common grade 3 adverse events in the tivantinib group were neutropenia (14%) and anemia (11%). The study recommended tivantinib as an option for second-line treatment of patients with advanced HCC. Further phase Ⅲ trials are needed.

ANTIANGIOGENIC AGENTS AS TACE ENHANCERS

TACE consumes blood and causes hypoxia in patients with HCC. However, only the deeply hypoxic area in HCC died and other limited hypoxic areas survived. This is caused by the extra-hepatic collateral arteries supply for HCC if the tumors are large or peripherally located. The development of these vessels interferes with effective control of the tumor with TACE[43]. This result of a high rate of tumor recurrence and low rate of long-term survival is still common in patients with unresectable HCC. Post-TACE recurrences may be due to angiogenesis enhancement and upregulation of VEGF induced by TACE[44,45]. Therefore, new treatment strategies for patients with unresectable HCC are needed, including the optimization of TACE with combination of other modalities. TACE has currently become the standard treatment for patients with intermediate HCC. However, for patients unsuitable for TACE or in whom TACE resulted in unacceptable toxicity, the use of oral sorafenib is another choice[46-49]. Some trials have focused on the combination of TACE and sorafenib. One meta-analysis confirmed that the combination therapy of TACE and sorafenib can improve the OS (HR = 0.65, 95%CI: 0.47-0.89, P = 0.007), TTP (HR = 0.68, 95%CI: 0.52-0.87, P = 0.003) and the objective response rate (HR = 1.06, 95%CI: 1.01-1.12, P = 0.021). Nevertheless, it did not affect the progression of free survival when compared to TACE alone[50]. Besides, the significantly increased risks of adverse reactions from combination therapy were occasionally noted. Another meta-analysis demonstrated that sorafenib combined with TACE may have superiority over TACE alone in terms of TTP. The HR for TTP was found to be 0.76 (P < 0.001) with low heterogeneity in studies (P = 0.243, I^2 = 25.5%)[51]. However, the HR for OS was found to be 0.81 (P = 0.061) with low heterogeneity in studies (P = 0.259, I^2 = 25.4%). Adverse reactions are generally manageable with dose reductions. However, one phase Ⅲ trial enrolled 458 previously TACE-treated patients and the median TTP in the sorafenib and placebo groups was 5.4 and 3.7 mo (HR = 0.87, P = 0.252). HR for OS was 1.06 (P = 0.790). Thus, sorafenib did not

significantly prolong TTP in patients who responded to TACE[52]. The efficacy of TACE plus sorafenib still needs confirmation with further studies.

NEW DIRECTION FOR MOLECULARLY TARGETED THERAPY

Both *in vitro* and *in vivo* studies have shown that sorafenib may promote anti-proliferative and pro-apoptotic effects in tumor cells as well as in endothelial cells. However, it appears that the molecular mechanisms underlying the direct effects of sorafenib in these cells are not completely understood and probably involve additional pathways. Chen *et al*[53] reported that sorafenib sensitizes HCC cells to tumor necrosis factor related apoptosis, inducing ligand (TRAIL) through the inhibition of signal transducer and activator of transcription 3 (STAT3). Tai *et al*[54] demonstrated that sorafenib would inhibit the development of HCC *via* the kinase-independent mechanism, SHP-1 dependent STAT3 inactivation. STAT3 is a transcription factor that modulates survival-directed transcription. In cancer cells, STAT3 can be activated by overactive receptors, including interleukin-6, EGF family members or HGF. In some research, it could be seen that TAT3-stimulated genes may promote angiogenesis, proliferation and survival. In addition, the STAT3 activation could also turn on the strong negative feedback loops involving tyrosine phosphatases (SHP-1 and SHP-2) and suppressors of cytokine signaling. By reducing the levels of STAT3 phosphorylation (Tyr^{705} - STAT3 phosphorylation), the phosphatases would block STAT3 dimerization and transcriptional activity[55,56]. The literature has shown that sorafenib may reduce STAT3 phosphorylation and induce cell death. A series of sorafenib derivatives were synthesized as new inhibitors for STAT3 phosphorylation. Such results provide a new direction for the designs of anti-HCC drugs[57,58]. Novel sorafenib derivatives (SC-40, SC-43 and SC-60) have been studied *in vitro* and *in vivo*. These sorafenib derivatives induced apoptotic cell death significantly by enhanced SHP-1 activity and inhibited the phosphorylation of STAT3 at the concentration of 0.5 μmol/L, which was more potent than sorafenib (5 μmol/L)[59,60]. These new compounds of sorafenib derivatives appeared to be an important different pathway to sorafenib, more potent and with not only a tumoristatic effect but also a tumorcidal effect.

OTHER AGENTS UNDER STUDY FOR ADVANCED HCC

Hormone therapies

Tamoxifen and megestrol: Many animal models of experimental liver carcinogenesis and epidemiological studies in humans have all suggested the relationship between the sexual hormones and HCC[61]. The intimate connection may be due to estrogen receptors (ERs) present in one-third of HCCs. These tumors could potentially gain benefit from ER blockade with tamoxifen. However, some large randomized trials, including CLIP-1 studies, showed no improvement in survival or functional status advantage when comparing the addition of tamoxifen to best supportive care[62,63]. The possible reasons include the presence of variant ERs in some of these tumors, lack of patient selection, the problem of dosage selection and the fact that tamoxifen in HCC could indeed act *via* an ER-independent pathway[64-66]. The efficacy of megestrol acetate has been evaluated in HCC with variant ER. Better therapeutic benefits were shown in some studies[64,67]. However, in one randomized double blind trial of megestrol acetate *vs* placebo in 204 patients with treatment-naive advanced HCC, megestrol acetate had no role in prolonging OS in advanced treatment-naive HCC[68].

Octreotide: Octreotide is an analogue of the hormone somatostatin. Over 40% of HCC patients may express specific somatostatin receptors (SSTR) and *in vitro* data showed the direct anti-tumor effect of octreotide in HCC[69,70]. The molecular mechanisms involved in the anti-neoplastic activity of somatostatin are related to the direct and indirect growth inhibition mediated by SSTR expressed in the target tissue[71]. In a phase Ⅲ study, octreotide had a favorable safety profile but did not improve OS and could have a negative impact on the quality of life for patients with advanced HCC[72]. A meta-analysis showed that the 6 and 12 mo survival rates in the octreotide group were significantly higher than those of the control group, but only in Eastern studies. They concluded that octreotide could improve the survival rates of patients with advanced HCC, but possibly not in Western countries[73]. The results are still controversial so routine administration of octreotide cannot be recommended.

Immunotherapy anti-programmed death-1

Human endogenous immunity responses can recognize many cancer cells as non-self and these kinds of responses have been observed in preclinical models and patients. However, these responses are ineffective because of the tumor's own multiple resistance mechanisms, including systemic dysfunction in T-cell signaling[74]. For instance, programmed death 1 (PD-1), the T-cell co-inhibitory receptor with its known ligand PD-L1 (known as B7-H1), plays an important role in mediating immunosuppression and has been involved in multiple immunopathological scenarios[75]. Many studies have shown that the PD-1/PD-L1 pathway was also the important factor in compromised tumor immunity. If the blockade of this pathway by anti-PD-L1 antibodies occurred, we could easily enhance the antitumor abilities and inhibit tumor growth in several cancers, such as melanoma, non-small cell lung cancer and renal cell carcinoma[76]. Thus, PD-L1 was demonstrated to deliver an inhibitory signal to PD-1 expressing T cells leading to the suppression of the immune response by inducing apoptosis, unresponsiveness and functional exhaustion

of T cells[77,78]. The dose escalation study of anti-PD-1 monoclonal antibody BMS-936558 was administered as a single dose in 39 patients with advanced solid tumors. A favorable safety profile and preliminary evidence of clinical activity were shown in this pilot study, establishing the basis for the current multiple dose trial involving patients with diverse cancers[79]. In chronic HBV patients, peripheral HBV-specific $CD8^+$ T-cells are mostly PD-1 positive and functionally impaired, with restoration of their effector function after blocking the PD-1/PD-L1 pathway. The analogous condition was also seen in chronic HCV patients[80,81]. The levels of PD-1(+)/CD8(+) T cells may apparently increase with disease progression from patients with liver cirrhosis to HCC *vs* the healthy control[82]. B7-H1, PD-1(+), CD8(+) T cells axis contributes to immune suppression in human HCC, with blockade of this pathway carrying therapeutic implications. Various studies have demonstrated the relationships between the expression of intrahepatic PD-1 on T-cells and postoperative recurrence, the stage of tumor and the prognosis of diseases[80,82]. Thus, monoclonal antibodies against both PD-L1 and PD-1 have been developed.

Oncolytic virotherapy

Replication-selective tumor-specific viruses present a new treatment direction for neoplastic disease which is facilitated by virus-mediated lysis of tumor cells after selective viral propagation within the tumor. The selectivity for cancer cells is derived from a human telomerase reverse transcriptase (hTERT) promoter-driven active viral replication, which only occurs in cancer cells with high telomerase activity. For example, telomelysin is a telomerase-specific replication-competent oncolytic adenovirus that may replicate efficiently and induce marked cell killing in human cancer cells[83]. However, the TERT activity is elevated in most cases of HCC and thus all current studies aim to investigate whether telomelysin can be used for the treatment of HCC or not[84]. Telomerase-specific oncolytic virotherapy has been studied *in vivo* to show that it is cancer-selective, replication-competent and causes the oncolysis of liver cancer cells[85]. In a preclinical *in vivo* study of the orthotopic HCC model, the telomelysin agent showed the potent oncolytic effect on HCC but spared normal liver tissue. The effects of multiple injections of telomelysin were also evaluated recently. Lin *et al*[86] concluded that telomelysin can be used for treatment of human HCC at an appropriate dosage and that its tumor-killing activity persists after multiple injections.

CONCLUSION

Treatment of human HCC is a multidisciplinary, patient-oriented strategy and must take the patient's clinical stage, liver function reserve and performance status into consideration in detail. Multiple chemotherapeutic agents have been used both as single agents and in combination to treat advanced HCC but, until recently, none of them had been shown to improve overall survival effectively. Now, the multi-kinase inhibitor "sorafenib" is still the only approved drug for patients with advanced HCC. However, there are also many mechanisms involving molecular signaling pathways which may identify different targets for novel molecular therapies that remain unknown. Thus, the efficacy of combination of anti-angiogenic agents plus TACE still needs more confirmation. Besides, immunotherapy with anti-PD-1 and oncolytic virotherapy also have therapeutic potential but need approval by further clinical studies and clues. To our knowledge, the importance of effective systemic therapies for patients with advanced HCC is clear now and more effort is required to advance talent in the future.

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MINIREVIEWS

Management of chronic hepatitis B before and after liver transplantation

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Abstract

Liver transplantation remains the only curative option for eligible patients with complications of chronic hepatitis B (CHB) infection, including severe acute hepatitis flares, decompensated cirrhosis, and hepatocellular carcinoma. In general, all patients with CHB awaiting liver transplantation should be treated with oral nucleos(t)ide analogs (NAs) with high barriers to resistance to prevent potential flares of hepatitis and reduce disease progression. After liver transplantation, lifelong antiviral therapy is also required to prevent graft hepatitis, which may lead to subsequent graft loss. Although combination therapy using NA and hepatitis B immune globulin (HBIG) has been the regimen most widely adopted for over a decade, recent studies have demonstrated that newer NAs with low rates of resistance are effective in preventing graft hepatitis even without the use of HBIG, achieving excellent long term outcome. For patients without pre-existing resistant mutations, monotherapy with a single NA has been shown to be effective. For those with resistant strains, a combination of nucleoside analog and nucleotide analog should be used. To date, clinical trials using therapeutic vaccination have shown suboptimal response, as CHB patients likely have an immune deficit against HBV epitopes. Future strategies include targeting different sites of the hepatitis B replication cycle and restoring the host immunity response to facilitate complete viral eradication.

Key words: Hepatitis B; Liver transplantation; Antiviral therapy; Prevention; Prophylaxis; Hepatitis B immune globulin

Core tip: In the era of highly potent nucleoside and nucleotide analogues for the treatment of hepatitis B, long term viral suppression can be achieved with minimal risk of drug resistance. The use of these agents without hepatitis B immune globulin has been shown to be highly effective in preventing hepatitis B-related graft hepatitis, with excellent long-term outcome. Complete eradication of hepatitis B from the host however is unlikely, and long-term therapy is therefore required. Future developments aiming at different target sites together with immune restoration of the host against hepatitis B may make this elusive goal possible.

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INTRODUCTION

Chronic hepatitis B (CHB) remains a major health burden worldwide, with an estimated 400 million people affected. Of these, an estimated 15%-40% may develop complications of hepatitis B virus (HBV) infection, including cirrhosis, decompensation, and hepatocellular carcinoma (HCC)[1]. For those with life-threatening complications, liver transplantation (LT) remains the only curative option for those who are eligible and in regions where LT is available. The indications for LT for CHB-related complications fall into three major categories. Firstly, LT is indicated for those with cirrhosis with evidence of decompensation. The second indication for LT includes those patients with severe hepatitis flares with evidence of liver failure. Finally, for those patients with HBV-related HCC within transplantable criteria, and where resection is not feasible, LT is indicated.

The use of nucleos(t)ide analogs (NA) is currently the cornerstone of CHB management. The use of interferon-based regimen is largely contra-indicated in the setting of decompensated cirrhosis, and also not proven in the post transplant setting for CHB. There are currently 5 NAs approved for treating CHB, of which three are nucleoside analogs (lamivudine, telbivudine, and entecavir) and two are nucleotide analogs (adefovir and tenofovir). The major difference between the various NAs is the susceptibility to the development of drug resistant mutations. Lamivudine, the first NA to be approved, is associated with the highest resistance rate, with approximately 70% developing drug resistance after 5 years of therapy. In contrast, newer NAs including entecavir and tenofovir are associated with extremely low risk of resistance, and should be considered for first line therapy if available[2].

TREATMENT OF CHB BEFORE LT

There are major regional guidelines with treatment recommendations regarding criteria for therapy, duration of therapy, and treatment endpoints[3-5]. Although the indications for LT may be different, the principles of CHB treatment for those waitlisted for transplantation remain identical. As all patients will have advance liver disease with liver failure, established cirrhosis, or both, the goal of antiviral therapy is to prevent further damage from ongoing hepatitis or from acute flares. Therefore, all CHB patients who are waitlisted or being worked up for LT should be treated with antiviral therapy. The major limitation of NAs is the development of drug resistant mutation, with subsequent virological rebound leading to hepatitis flares. Patient who require LT are unlikely to tolerate further insult to their compromised liver. Therefore, these patients should be treated with NAs such as entecavir or tenofovir with high barriers to resistance. For those with pre-existing drug-resistant mutations, a combination of a nucleoside and nucleotide analog is preferred. NAs are generally well tolerated with minimal adverse effects. As they undergo renal excretion predominantly, it is important to adjust the doses accordingly for those patients with impaired renal function. For patients with decompensated liver disease, an association with the development of severe lactic acidosis has been reported with the use of entecavir, especially for those with underlying renal impairment[6]. Nucleotide analogues can also directly be nephrotoxic causing proximal tubule dysfunction[7].

TREATMENT OF CHB AFTER LT

In CHB infection, complete eradication of HBV from the host is exceedingly rare. Even after hepatitis B surface antigen (HBsAg) seroclearance, it is likely that HBV still persists within the host[8,9]. This is the reason why lifelong antiviral therapy is required. Despite the removal of the liver, HBV may still persist in extrahepatic sites such as the lymph nodes, spleen, peripheral blood mononuclear cells, and other organs[10-13]. HBV may also persist in the circulation at the time of transplantation. These sites serve as reservoirs for re-infection of the new graft. Furthermore, reactivation of latent HBV occurs with the use of immunosuppressive therapy after transplantation[14]. Therefore, in the absence of effective antiviral therapy, severe hepatitis leading to graft failure from recurrent HBV infection is almost universal[15-17]. In fact, prior to the advent of effective HBV therapy, liver transplantation was considered a contraindication because of untreatable HBV recurrence.

Hepatitis B immune globulin

Hepatitis B immune globulin (HBIG) is pooled human immune globulins from donors with high antibody titers, and is administered as a form of passive immuno-prophylaxis. The exact mechanism as to how HBIG reduces recurrence rate after liver transplantation is unclear. Several proposed mechanisms include the blockage of putative receptors involved in the exportation of virions from extrahepatic sites, and in the formation of immune complexes with subsequent neutralization of viral particles[18]. In a landmark study published in 1993 of 372 consecutive HBsAg-positive patients undergoing liver transplantation across 17 European centers, the recurrence rate was significantly less for patients treated with HBIG for 6 mo or longer compared with those receiving no treatment (36% *vs* 75% respectively, $P < 0.001$)[16].

Several strategies have been adopted to maintain a protective level of antibody titer in the circulation. These include injecting at a regular interval (usually monthly) or administering when the antibody level falls below an arbitrary cut-off level that is considered protective. Higher doses have been shown to be more protective, but at a much higher cost[19-21]. The major disadvantage of using HBIG is the need for regular parental injections, its high costs, and the risk for developing escape mutations. The G145A mutation at the antigenic loop of the "a" determinant is the most common mutation

associated with immune escape, and may diminish the efficacy of HBIG[22-24].

NA

Although the rate of HBV recurrence was reduced with the use of HBIG, a significant proportion still developed recurrence of hepatitis. The most widely adopted use of HBIG as post transplant prophylaxis has been as part of a combination regimen with oral NA. Lamivudine was the first oral antiviral agent approved for CHB treatment. By itself, it reduced the recurrence of HBV to 3.8%-40.4%[25-28]. The combination of HBIG and lamivudine was synergistic as either agent was suboptimal when administered alone in preventing HBV recurrence. The recurrence rate was further reduced to less than 5% with combination therapy. Subsequently, a lower dose of HBIG given intramuscularly in combination with lamivudine was shown to be effective[29,30]. Although the addition of NAs to HBIG significantly reduces the rate of recurrence after transplantation, the reverse may no longer be true with the newer and more potent NAs. An earlier study of 33 CHB patients receiving combination therapy with lamivudine + HBIG for at least 12 mo after liver transplantation without evidence of HBV recurrence, patients were randomized to either continue HBIG or replace HBIG with adefovir[31]. At the end of follow up, only 1 patient developed HBsAg positivity without evidence of detectable HBV DNA in the adefovir arm. In another study of 37 CHB patients treated with tenofovir + emtricitabine + HBIG for at least 12 wk after liver transplantation, participants were randomized to either continue or stop HBIG[32]. At 96 wk, there was no HBV recurrence reported in both arms. These studies demonstrated that HBIG withdrawal was safe and effective for those receiving combination NA therapy.

The effectiveness of NAs alone without HBIG can be primarily attributed to newer NAs having significantly lower rates of resistance. The major disadvantage in using lamivudine is due to its high resistance rate, with up to 70% after 5 years of therapy in non-transplant patients[33]. In contrast, both entecavir and tenofovir have been associated with resistance rates of < 2% after 5 years in patients without pre-existing resistant mutations[34,35]. Therefore these newer agents would appear to be ideal agents to prevent HBV recurrence after transplantation. In this instance, the role of HBIG in the era of these new NAs become less obvious, as resistance and virological breakthrough rates are extremely low. In a study of 80 CHB patients undergoing liver transplantation, entecavir was used as monotherapy in a completely HBIG-free regimen with a median follow up of 26 mo[36]. There was a high HBsAg seroclearance rate of 86% and 91% after 1 and 2 years respectively despite the absence of HBIG administration. Although a small proportion (13.7%) had HBsAg positivity either from re-appearance of HBsAg after initial seroclearance, or from persistence of HBsAg status after transplantation, none of the patients had evidence of virological rebound or resistance. Importantly, there was no incidence of HBV related hepatitis, graft loss, or mortality. Furthermore, quantitative HBsAg measurements in those with positive HBsAg revealed a persistently low level in the absence of HBV DNA. A larger study of 362 patients using a completely HBIG free regimen also demonstrated excellent long-term survival of 83% at 8 years[37]. In this particular study, there was no difference in the rate of HBsAg seroclearance and HBV DNA suppression between those on lamivudie, combination NA, or entecavir. The key difference was the significantly lower rate of virological rebound observed at 3 years after liver transplantation with entecavir when compared to those treated with combination NA therapy and lamivudine (0%, 7% and 17% respectively, $P < 0.001$).

Vaccination

The use of vaccination after liver transplantation is currently not standard practice, and can only be recommended within the clinical trials setting. Results so far have been unconvincing. Although initial studies have been encouraging, subsequent studies have demonstrated low response rates of 7.7%-17.6% only[38-44]. Perhaps this is not surprising given that CHB patients have been exposed to high levels of HBsAg epitopes for most of their lives with the inability to mount an effective immune response. Therefore, further exposure to HBV antigens *via* vaccination is unlikely to trigger a novel and effective response, especially in an immunosuppressed host. However, studies using higher doses of pre-S vaccines have shown that a proportion of patients may develop an immune response[44]. Whether the anti-HBs titers induced by vaccination strategies can be maintained and confer protection also remains to be determined. The use of vaccination may also be associated with the potential risk of developing escape mutations, rendering the antibody production ineffective.

Adoptive transfer of immunity

In addition to vaccination, adoptive transfer of immunity against HBV represents another strategy to restore the immune deficit against HBV. This phenomenon was initially described in recipients of bone marrow transplants from donors immune to HBV[45]. In liver transplant recipients, the anti-HBs have been frequently detectable after transplantation even without the administration of HBIG[36,37]. Furthermore, donor-derived HBV-specific lymphocytes were found to be present in the liver graft[46]. However, the antibody titers that is observed appears to be transient in the majority of liver recipients, suggesting that in a proportion of these patients, passive transfer of antibody and donor lymphocytes at the time of transplant may occur.

FUTURE STRATEGIES

New approaches for CHB therapy continue to build on

the current strategies of either targeting factors involved in viral replication or at the host immune response[47]. Potential target sites for the former includes methods to prevent viral entry, lower covalently closed circular DNA levels, disrupt viral transcription, assembly, and exportation of virions. To restore or enhance the immune response against HBV, other strategies apart from therapeutic vaccination have been explored. These include targeting T-cell inhibitor receptors and blocking suppressive cytokines and regulatory T-cells in the liver.

DISCUSSION

In the last 15 years, NAs have revolutionized the treatment paradigm of CHB infection. The major limitation with early NAs was the associated high resistance rate associated with increase risk of virological breakthrough and hepatitic flare. This risk has largely been diminished by the approval of highly potent NAs with high barriers to resistance. For patients awaiting liver transplantation with CHB infection, it is recommended that all should be on NAs irrespective of the viral load. As most of the patients will already have established cirrhosis, hepatitic flares may result in further decompensation. This is also the main reason why NAs with high barriers should be used to lower the risk of virological breakthrough and subsequent flare.

After liver transplantation, the choice of NAs will be dependent on the NA used prior to surgery, and also whether drug-resistant mutations are present. The role of HBIG has become diminished with newer NAs, and studies have already demonstrated the efficacy of an HBIG-free regimen as antiviral therapy after transplantation. Although several meta-analyses have demonstrated that combination therapy with HBIG appears to be more efficacious than NA alone, it has to be said that the overwhelming majority of the studies included in these meta-analyses were patients using the early NAs such as lamivudine[48-51]. Therefore it is not surprising that the additional of HBIG would be beneficial.

As complete eradication of HBV from the host is still not attainable with current antiviral regimens, life long treatment is required. Intrahepatic HBV remains detectable even if there is no serological evidence of HBV infection[52,53]. In this respect, antiviral therapy after liver transplantation does not prevent recurrent infection. The recipient is unlikely to have complete clearance of HBV even with removal of the infected liver, as HBV may be present in the circulation and extra-hepatic sites. Therefore, antiviral therapy serves to prevent recurrent hepatitic flares, rather than recurrent infection, given the patient is already chronically infected. The significance of HBsAg positivity remains unclear, especially when it remains at very low levels in the absence of virological rebound or detectable HBV DNA. The administration of HBIG will bind on to HBsAg, leading to its undetectability, and a higher rate of HBsAg negativity compared to HBIG-free regimens. The level of hepatitis B core related antigen however remains unchanged by the administration of HBIG, and has been shown to persist despite maintaining an anti-HBs titer of > 100 U/L[54].

In summary, all patients wait-listed for liver transplantation with CHB infection should receive antiviral therapy with high barriers to resistance. After transplantation, life-long prophylaxis to prevent recurrent hepatitis flares is required. A shifting paradigm of using high dose HBIG, low dose HBIG, limited-duration HBIG, to no HBIG using combination of NAs or monotherapy with NA with high barrier to resistance has been observed, with the latter showing excellent clinical outcomes. In the future, other novel methods targeting different sites of viral replication cycle together with restoration of the host immune response may allow complete eradication or long-term immune control of HBV, thereby obviating the need for long-term therapy.

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ORIGINAL ARTICLE

Retrospective Study

Alpha-1 antitrypsin deficiency and the risk of hepatocellular carcinoma in end-stage liver disease

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Ethics approval: The study was reviewed and approved by the Cleveland Clinic Institutional review board.
Informed consent: This is a minimal risk study using data collected for routine clinical practice. A waiver of Informed Consent and waiver of HIPAA authorization were approved by IRB. The presented data are anonymized and risk of identification is low.
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Abstract

AIM: To evaluate the association between alpha-1 antitrypsin deficiency (A1ATD) and hepatocellular carcinoma (HCC) in patients with end-stage liver disease (ESLD).

METHODS: Patients with cirrhosis and ESLD referred to the Cleveland Clinic Foundation for liver transplantation between 2003 and 2014 were included in the study (*N* = 675). ESLD was defined as having histological features of cirrhosis and/or radiological evidence of cirrhosis in the context of portal hypertension (ascites, variceal bleeding, thrombocytopenia, or hepatic encephalopathy). A1ATD was diagnosed using phenotype characterization (MZ or ZZ), liver biopsy detection of PAS-positive diastase-resistant (PAS+) globules, or both. Patients with other causes of liver diseases such as hepatitis C virus (HCV), alcoholic liver disease and non-alcoholic steatohepatitis (NASH) or NASH were also included in the study. HCC was diagnosed by using imaging modalities, biopsy findings, or explanted liver inspection. Follow-up time was defined as the number of years from the diagnosis of cirrhosis to the diagnosis of hepatocellular carcinoma, or from the diagnosis of cirrhosis to the last follow up visit. The rate of HCC was assessed using time-to-interval analysis for interval censored data.

RESULTS: This study included 675 patients. 7% of subjects had A1ATD (n = 47). Out of all subjects who did not have A1ATD, 46% had HCV, 17% had alcoholic liver disease, 19% had NASH and 18% had another primary diagnosis. Of the 47 subjects with A1ATD, 15 had a primary diagnosis of A1ATD (PI*ZZ phenotype and PAS+ globules), 8 had a PI*MZ phenotype alone, 14 had PAS+ alone, and 10 had both the PI*MZ phenotype and PAS+. Median follow-up time was 3.4 (25^{th}, 75^{th} percentiles: 1, 5.2) years. The overall rate of hepatocellular carcinoma in all subjects was 29% (n = 199). In the A1ATD group, the incidence rate of HCC was 8.5% compared to 31% in the group of patients with other causes of cirrhosis (P = 0.001). Patients with ESLD due to A1ATD had the lowest yearly cumulative rate of hepatocellular carcinoma at 0.88% per year compared to 2.7% for those with HCV cirrhosis, 1.5% in patients with NASH and 0.9% in alcohol-induced liver disease (P < 0.001).

CONCLUSION: Within this group of patients with ESLD, there was no significant association between A1ATD and increased risk of HCC.

Key words: Hepatocellular carcinoma; Liver cirrhosis; End-stage liver disease; Hepatitis C virus; Alpha-1 antitrypsin deficiency

Core tip: It's been postulated that alpha-1 antitrypsin deficiency (A1ATD) and the presence of the Z allele increase the risk of hepatocellular carcinoma (HCC) above that attributable to cirrhosis alone. Our study showed that the occurrence of HCC in subjects with cirrhosis due to A1ATD was 0.88%/year. This incidence rate was considerably lower than that among patients with other causes of liver disease including hepatitis C, alcoholic liver disease and non-alcoholic liver steatohepatitis. This challenges the view that A1ATD confers a disproportionate risk of HCC.

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INTRODUCTION

Alpha-1 antitrypsin deficiency (A1ATD) is an autosomal codominant genetic disease affecting 1 per 3000-5000 individuals[1-3]. Twenty-five million people have been estimated to carry at least one defective gene[4]. A1ATD has been linked to severe pulmonary and liver disease.

The most common, normal allele of the *A1AT* gene is called M and produces normal levels of alpha-1 antitrypsin protein. The Z mutation is a single base substitution that alters the A1AT molecular structure and promotes intra-hepatocyte polymerization, thereby trapping A1AT in the endoplasmic reticulum of hepatocytes and causing serum A1AT levels to be low[5]. This mechanism of liver disease has been called a "toxic gain of function" and the resulting deficient proteolytic screen in the lung has been called a "toxic loss of function." The intracellular accumulation of the A1AT molecule in hepatocytes triggers apoptosis of liver cells, instigating chronic hepatocellular death and regeneration[6]. This can manifest in a wide range of symptoms and signs from abnormal liver enzyme tests to liver fibrosis, cirrhosis and the dreaded hepatocellular carcinoma (HCC).

There is marked variability in the degree of liver disease among homozygous individuals for the Z allele (PI*ZZ). Several studies have examined the prevalence of abnormal liver function tests in these individuals and found only 7.7%-10% with mild increases in alanine aminotransferases levels[7,8]. The prevalence of cirrhosis in PI*ZZ individuals varies between 2%-43% and seems to be mostly driven by age[9-11]. Some studies have also reported a similar association between heterozygous PI*MZ individuals and cirrhosis[12-14], though this has been challenged more recently[15]. The most feared complication of A1ATD cirrhosis is hepatocellular carcinoma or HCC. HCC is the sixth most common neoplasm worldwide[16]. It is the third most frequent cause of cancer deaths and is a leading cause of death among patients with cirrhosis[17]. An autopsy study from Sweden demonstrated that homozygotes for the Z allele had increased risk for primary liver cancer, especially in male patients[11]. In addition, it has been suggested in recent studies that heterozygotes for the Z allele or PI*Z (such as PI*MZ) had an increased risk of primary liver carcinoma that developed even in the absence of cirrhosis and was frequently characterized by cholangiocellular differentiation[18,19]. However, the view that A1ATD confers a substantially higher risk for liver cancer remains controversial and studies addressing the occurrence rate of HCC in patients with end-stage liver disease (ESLD) caused by A1ATD are sparse.

In this context, the specific aims of the current study were to: (1) estimate the incidence and cumulative annual risk of HCC in subjects with A1ATD-associated cirrhosis; and (2) compare this incidence rate to that of patients with other causes of liver disease, including hepatitis C virus (HCV), alcoholic liver disease (ALD) and non-alcoholic steatohepatitis (NASH).

MATERIALS AND METHODS

Patients

The study was approved by the Cleveland Clinic Institutional Review Board. Patients with cirrhosis and ESLD referred to the Cleveland Clinic Foundation for liver transplantation between January 2003 and January 2014 were identified (N = 675) and their medical records were retrospectively reviewed using our electronic medical record (EPIC, Verona, WI).

Table 1 Patient characteristics

Factor	No A1ATD (n = 628)		A1ATD (n = 47)		P-value
	n	Summary	n	Summary	
Female	628	194 (30.9)	47	13 (27.7)	0.64[c]
Age at cirrhosis diagnosis	628	54.6 ± 9.5	47	55.2 ± 10.3	0.68[a]
Race	615		47		0.14[c]
Caucasian		513 (83.4)		45 (95.7)	
African-American		65 (10.6)		1 (2.1)	
Hispanic		19 (3.1)		1 (2.1)	
Other		18 (2.9)		0 (0.0)	
HCC	628	195 (31.1)	47	4 (8.5)	0.001[c]
Follow-up (yr, median)	628	3.2 (1.00, 5.1)	47	4.2 (1.9, 5.2)	0.090[b]

Values presented as mean ± SD, median (P25, P75), or n (column%). P-values: [a]ANOVA, [b]Kruskal-Wallis test, [c]Pearson's χ^2 test. A1ATD: Alpha-1 antitrypsin deficiency; HCC: Hepatocellular carcinoma.

Demographic features such as age, sex and race were extracted.

ESLD was defined as having histologic features of cirrhosis and/or radiologic evidence of cirrhosis in the context of portal hypertension (ascites, variceal bleeding, thrombocytopenia, or hepatic encephalopathy).

The diagnosis of A1ATD was based on phenotype characterization using isoelectric focusing (MZ or ZZ), liver biopsy detection of PAS-positive diastase-resistant (PAS+) globules, or both.

Patients with other causes of liver diseases were also included in the study. The diagnosis of alcohol-induced cirrhosis was based on history and histological findings on biopsy. The diagnosis of hepatitis C was based on detecting hepatitis C antibody and RNA, that of NASH was based on histological features. The diagnosis of hepatitis B was established with seropositivity for hepatitis B surface antigen, hepatitis B core antibody, and hepatitis B DNA. Autoimmune hepatitis was established based on finding serum autoantibodies and consistent histology. Primary biliary cirrhosis was diagnosed based on finding a serum mitochondrial antibody and compatible histology. Hereditary hemochromatosis was established with serum iron studies and genetic testing and if suspected, primary sclerosing cholangitis was diagnosed by cholangiogram.

HCC was diagnosed by using imaging modalities, biopsy findings, or explanted liver inspection.

Follow-up time was defined as the number of years from the diagnosis of cirrhosis to the diagnosis of hepatocellular carcinoma, or from the diagnosis of cirrhosis to the last follow-up visit.

Subjects with A1ATD were recognized and the incidence of HCC was calculated in this group. The incidence of hepatocellular carcinoma in patients with ALD, HCV, NASH and a group of other causes of cirrhosis (which included hepatitis B, hereditary hemochromatosis, primary biliary sclerosis, primary sclerosing cholangitis and autoimmune hepatitis) was assessed using time-to-event analysis for interval-censored data.

Statistical analysis

Data are presented as mean ± standard deviation or N (%). Differences between subjects with and without A1ATD were determined using univariate analysis. Analysis of variance was used for continuous variables and Pearson's chi-square tests were used for categorical factors. Time-to-event analysis for interval-censored data was carried out to determine the incidence of hepatocellular carcinoma in each group because only the year of diagnosis was known for many subjects. A cumulative incidence plot was constructed and the generalized log-rank test for interval-censored data was used. Values of $P < 0.05$ were considered statistically significant. All analyses were performed using R (version 3.0.1, The R Institute for Statistical Computing, Vienna, Austria).

The statistical review of the study was performed by a biomedical statistician.

RESULTS

Demographic, clinical, and histologic characteristics of the patients

Demographic features of our patient population are summarized in Table 1. Most (91%) patients were Caucasian. At time of cirrhosis diagnosis, average age was 55 ± 10 years; 31% of cirrhotics were female. The ages and gender distribution were similar between the A1ATD group and the non-A1ATD group (P = 0.64 and P = 0.68 for age and gender, respectively). Furthermore, there was no significant age difference between A1ATD group with or without HCC (53 ± 11 years and 55 ± 10 years respectively) or between HCC patients with A1ATD or without A1ATD (53 ± 11 years and 58 ± 8 years respectively). The overall number of deaths in the cohort was 24.6% (n = 166). Of subjects with HCC, 30.15% were diseased (60/199).

Seven percent of subjects had A1ATD (n = 47). Out of all subjects who did not have A1ATD, 46% had HCV, 17% had ALD, 19% had NASH and 18% had other primary diagnoses.

Of the 47 subjects with A1ATD, 15 had severe deficiency of A1ATD (based on a confirmed PI*ZZ phenotype and PAS+ globules), 8 had a PI*MZ phenotype only, 14 had PAS+ globules only, and 10

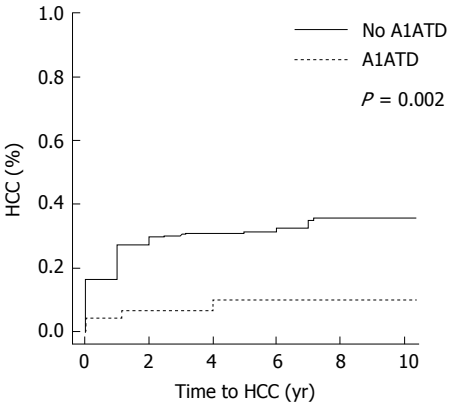

Figure 1 Cumulative incidence of hepatocellular carcinoma. HCC: Hepatocellular carcinoma; A1ATD: Alpha-1 antitrypsin deficiency.

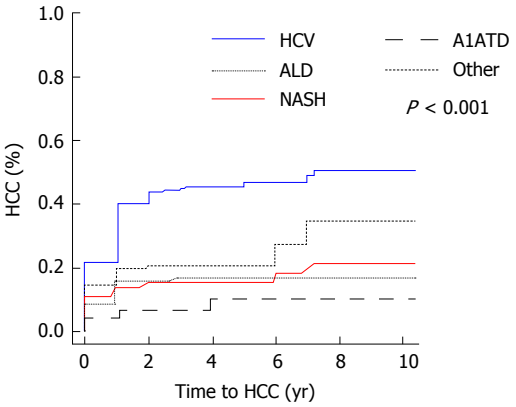

Figure 2 Cumulative incidence plot for hepatocellular carcinoma by liver disease etiology. HCC: Hepatocellular carcinoma; A1ATD: Alpha-1 antitrypsin deficiency; HCV: Hepatitis C virus; ALD: Alcoholic liver disease; NASH: Non-alcoholic steatohepatitis.

had a PI*MZ phenotype and PAS+. Overall, 32% had the PI*ZZ phenotype, 38% were PI*MZ, and 83% had PAS+ globules on liver biopsy.

HCC incidence

Median follow-up time was 3.4 years (25th, 75th percentiles: 1, 5.2). The overall rate of hepatocellular carcinoma in all subjects was 29% (n = 199). Of the 47 patients with A1ATD, 8.5% (4/47) developed HCC compared to 31.1% (195/628) of patients with other causes of liver disease (P = 0.001, Figure 1). When comparing the incidence rates of HCC in each subgroup of subjects, the highest yearly cumulative incidence of hepatocellular carcinoma was found in HCV subjects (2.7%/year, Figure 2). This was followed by a yearly cumulative incidence rate of 2.3% in patients with other etiologies of cirrhosis, 1.5% in NASH, 0.9% in alcohol-induced liver disease. The rate was lowest (0.88%/year) in patients with A1ATD cirrhosis (P < 0.001). Among those with confirmed PI*ZZ A1ATD, the rate of developing HCC was similar to patients with PI*MZ and those with PAS+ globules only.

The average AFP value at time of diagnosis of HCC in the A1ATD group was 27.6. Of the four patients with A1ATD/HCC, two had a single HCC focal lesion with BCLC stage A3, one subject had bifocal disease and one subject had multi-nodular HCC (BCLC stage B).

DISCUSSION

The principle finding of this study is that the incidence of HCC among patients with A1ATD was lower than that among patients with other causes of liver disease, thereby challenging the view that A1ATD confers a disproportionate risk of HCC. Our study also confirms that patients with cirrhosis due to hepatitis C have the highest risk of hepatocellular carcinoma compared to patients with NASH or alcohol-induced liver cirrhosis.

In contrast to our findings regarding a low risk of HCC with A1ATD, previous studies have reported a higher risk of HCC in A1ATD cirrhotic patients, with prevalence estimates of 10% to almost 50%. Two autopsy studies from Sweden assessed the prevalence of cirrhosis and hepatocellular carcinoma in PI*ZZ elderly individuals. The first study found that 43% of PI*ZZ patients had liver cirrhosis and 28% had HCC[20]. The second study only found that 16.1% of PI*ZZ patients had HCC at the time of autopsy[21]. A case series of 19 patients with A1ATD-associated liver disease found that 10% of patients had HCC (n = 2, both of whom were PI*ZZ)[22]. Several studies have also reported a similar association between heterozygous PI*MZ individuals and HCC. In a series of 61 patients with A1ATD-associated cirrhosis, most (89%) of whom were PI*MZ, Propst *et al*[23] reported that 10% had HCC. Several methodological differences could account for the discordance between our findings and prior reports and must be considered. First, our study was aimed at investigating the incidence and annual cumulative risk of developing HCC in A1ATD; whereas the other studies assessed the prevalence of HCC at one time point. Second, HCC was ascertained through imaging studies in our series *vs* by post-mortem in earlier studies. To the extent that imaging may fail to detect HCC, the frequency would be under-estimated here. Given the sensitivity of computed tomography for HCC, this seems an unlikely source of bias, however. Another possible explanation is that the universe of A1ATD patients in this series includes PI*MZ heterozygotes *vs* prior observations in all PI*ZZ patients. Earlier series have discounted the risk of cirrhosis in PI*MZ heterozygotes[15]. At the same time, the incidence of HCC among the 15 confirmed PI*ZZ individuals in this series is also low, making this less likely as a source of the discordance with prior reports. While a third potential source of under-recognizing HCC in the patients with A1ATD *vs* among other patients with other causes of liver disease might be that the duration of follow up was shorter in the A1ATD group or that they were under less close surveillance for the occurrence of HCC, the follow up duration in A1ATD patients was actually longer (median 4.2 years *vs* 3.2 years) than in others. Other

potential limitations of this study include the difficulty of generalizing the findings from this large tertiary care setting to a community context and the relatively small sample size upon which to make the frequency estimates. Knowing the exact risk of occurrence of HCC in this population is important as it can help guide management of these patients.

In summary, our findings suggest that out of all patients with chronic liver disease, HCC incidence was the lowest among patients with A1ATD. Further study is needed to confirm these findings and to enhance generalizability.

COMMENTS

Background

Alpha-1 antitrypsin is a molecule that is made in the liver, is then released into the systemic circulation where it travels to the lungs where it has its main function. In alpha-1 antitrypsin deficiency (A1ATD), the molecule is unable to leave the liver, ends up accumulating in the liver cells causing damage, liver fibrosis, cirrhosis and potentially hepatocellular carcinoma (HCC). The view that A1ATD confers a significantly higher risk of liver cancer remains controversial and studies regarding the incidence of HCC in patients with cirrhosis caused by A1ATD are sparse. In this study, the authors compared the rate of developing HCC in patients with liver cirrhosis due to A1ATD to patients with other causes of cirrhosis.

Research frontiers

Knowing the exact risk of occurrence of HCC in patients with A1ATD can help guide management of these patients; such as amount of surveillance and screening they would need over their lifetime.

Innovations and breakthroughs

A1ATD was associated with a lower risk of HCC compared to other causes of liver disease. This challenges the view that A1ATD confers a disproportionate risk of HCC.

Applications

A1ATD does not confer a higher risk of HCC compared to other causes of liver disease. Therefore, management of these patients should be tailored accordingly. Further research should be carried out to confirm their findings and generalize their data.

Terminology

Liver disease is characterized by different stages. Injury to liver cells can lead to inflammation followed by liver fibrosis or the development of scar tissue. If the injury continues, the liver can lose its normal function and become permanently cirrhotic.

Peer-review

This retrospective study, examining HCC in cirrhosis, is well written, clear, and appropriately analysed. It also answers an important clinical question.

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ORIGINAL ARTICLE

Observational Study

Genetic ancestry analysis in non-alcoholic fatty liver disease patients from Brazil and Portugal

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Data sharing: Technical appendix, statistical code, and dataset available from the corresponding author at email lourianne@gmail.com. All participants gave written informed consent for data sharing. Presented data were anonymized.

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Abstract

AIM: To study the association between genetic ancestry, non-alcoholic fatty liver disease (NAFLD) metabolic characteristics in two cohorts of patients, from Brazil and Portugal.

METHODS: We included 131 subjects from Brazil [(n = 45 with simple steatosis (S. Steatosis) and n = 86 with nonalcoholic steatohepatitis (NASH)] and 90 patients from Portugal (n = 66, S. Steatosis; n = 24, NASH). All patients had biopsy-proven NAFLD. In histologic evaluation NAFLD activity score was used to assess histology and more than 5 points defined NASH in this study. Patients were divided into two groups according to histology diagnosis: simple steatosis or non-alcoholic statohepatitis. Genetic ancestry was assessed using real-time polymerase chain reaction. Seven ancestry informative markers (AT3-I/D, LPL, Sb19.3, APO, FY-Null, PV92, and CKMM) with the greatest ethnic-geographical differential frequencies (≥ 48%) were used to define genetic ancestry. Data were analyzed using R PROJECTS software. Ancestry allele frequencies between groups were analyzed by GENEPOP online

and the estimation of genetic ancestry contribution was evaluated by ADMIX-95 software. The 5% alpha-error was considered as significant ($P < 0.05$).

RESULTS: In the Brazilian sample, NASH was significantly more frequent among the elderly patients with diabetes (NASH 56 ± 1.1 years old *vs* S. Steatosis 51 ± 1.5 years old, $P = 3.7 \times 10^{-9}$), dyslipidemia (NASH 63% *vs* S. Steatosis 37%, $P = 0.009$), higher fasting glucose levels (NASH 124 ± 5.2 *vs* S. Steatosis 106 ± 5.3, $P = 0.001$) and Homeostatic Model of Assessment index > 2.5 [NASH 5.3 (70.8%) *vs* S. Steatosis 4.6 (29.2%) $P = 0.04$]. In the Portuguese study population, dyslipidemia was present in all patients with NASH ($P = 0.03$) and hypertension was present in a larger percentage of subjects in the S. Steatosis group ($P = 0.003$, respectively). The genetic ancestry contribution among Brazilian and Portuguese individuals with NASH was similar to those with S. Steatosis from each cohort (Brazilian cohort: $P = 0.75$; Portuguese cohort: $P = 0.97$). Nonetheless, the genetic ancestry contribution of the Brazilian and Portuguese population were different, and a greater European and Amerindian ancestry contribution was detected in the Portuguese population while a higher African genetic ancestry contribution was observed in Brazilian population of both NASH and S. Steatosis groups.

CONCLUSION: There was no difference between the genetic ancestry contribution among Brazilian and Portuguese individuals with NASH and S. Steatosis from each cohort.

Key words: Ancestry; Nonalcoholic fatty liver disease; Simple steatosis; Nonalcoholic steatohepatitis; Admixed population

Core tip: Nonalcoholic fatty liver disease (NAFLD) is frequent and may lead to cirrhosis and hepatocellular carcinoma. Awareness about its risk factors and predictive markers of severity is important in the management of this infirmity. Self-reported ancestry may also influence NAFLD outcomes in homogeneous populations and African descendants appear to have milder disease than Caucasians. However, there are no available data that demonstrate the relationship between ancestry and NAFLD in admixed populations. This is the first study to evaluate the possible association between ancestry analyzed for genetic markers and biopsy-proven NAFLD in a homogeneous and a highly admixed population.

Cavalcante LN, Stefano JT, Machado MV, Mazo DF, Rabelo F, Sandes KA, Carrilho FJ, Cortez-Pinto H, Lyra AC, de Oliveira CP. Genetic ancestry analysis in non-alcoholic fatty liver disease patients from Brazil and Portugal. *World J Hepatol* 2015; 7(10): 1433-1438 Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i10/1433.htm DOI: http://dx.doi.org/10.4254/wjh.v7.i10.1433

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) may vary from simple steatosis across steatohepatitis (NASH) to hepatic fibrosis and, finally, cirrhosis and hepatocellular carcinoma. NAFLD is characterized by a potential and substantial seriousness and by variability inter-patient disease progression degree. However, while a meaningful percentage of the population is in risky of worsening condition, just a subgroup of patients will advance to NASH, liver fibrosis and cirrhosis[1].

Several studies suggest the presence of diverse risks factors for NAFLD and differences in clinical features based upon ancestry, as well as the potential role of ancestry as an independent risk factor associated to disease gravity[2]. In particular, Hispanic Americans and Caucasians have the larger frequency of NAFLD while African Americans have the lowest[2]. However, few data are available about ancestry contribution in NAFLD using ancestry informative markers (AIMs). These AIMs are powerful tools for inferring the genetic composition of admixed populations. The assessment of accurate admixture estimates is important in population genetic studies, particularly in the context of highly admixed populations as those of Brazil and most American countries[3-5]. In Brazil, a country that was colonized by Portugal, the admixtures of three main parental groups (Amerindians, Europeans and Sub-Saharan Africans) have originated the current Brazilian population. Thus, Brazilian population is admixed, tri-hybrid, with a great heterogeneity, resulting of inter-ethnic mating between individuals from three major ancestries contributors: the Amerindians, the Europeans who colonized South America in the 1500s and the Africans who arrived through the slave trade over a span of more than 300 years[3,6]. Furthermore, the diverse regions of the country underwent different colonization processes, which to some extent shaped their genetic backgrounds, nowadays characterized by different proportions of Amerindian, European and African contribution[7].

To investigate the possible association between genetic ancestry, NAFLD severity (simple steatosis or NASH) and metabolic characteristics in two cohorts with biopsy-proven NAFLD from Brazil and Portugal we then aimed to identify, for the first time, the ancestry influence in NAFLD and the possible role of genetic ancestry as an independent risk factor associated with non-alcoholic fatty liver disease.

MATERIALS AND METHODS

Subjects

We investigated 131 NAFLD patients whose diagnosis was confirmed by liver biopsy, ($n = 45$ had simple steatosis and $n = 86$ had NASH) from Brazil and 90 NAFLD patients, ($n = 66$, simple steatosis and $n = 24$, NASH) who had undergone bariatric surgery from Portugal. NASH was diagnosed based upon the pathologic criteria, and NAFLD activity score (NAS) was used to evaluate NASH diagnosis. All included patients

were at least 18 years old. The inclusion criterion was to have a liver biopsy-proven NAFLD. Exclusion criteria were the presence of concomitant known liver disease comprising viral hepatitis, Wilson's disease, hemochromatosis, or/and autoimmune liver diseases; reporting of methotrexate, tamoxifen or corticosteroids intake; or alcohol drinking ⩾ 140 g ethanol weekly.

Ethics Committees from University of São Paulo School of Medicine and from the Hospital of Santa Maria University Lisbon have approved this study. The written informed consent was obtained from subjects, or their legal guardian, prior to study inclusion.

Laboratory evaluation

Laboratory tests were performed from peripheral blood sample, including: fasting glucose, plasma insulin, total cholesterol, high density lipoprotein-, low density lipoprotein (LDL)-, very LDL-cholesterol, triglycerides, aspartate aminotransferase and alanine aminotransferase (ALT). Insulin resistance calculi was performed using Homeostatic Model of Assessment (HOMA) [(fasting insulin mU/L) × (fasting glucose mmol/L)/22.5]. The cut-off of HOMA ⩾ 2.5 was used to define insulin resistance, a value that has been assessed at prior studies realized in Brazilian population[8].

Histological evaluation

Hepatic biopsy fragments were fixed in formaldehyde saline (4%) and processed using hematoxylin-eosin and picrosirius stains. A blinded single pathologist with liver expertise in each center performed histological analyzes.

NASH was described as increased hepatic steatosis with centrilobular ballooning and/or Mallory-Denk bodies or any level of steatosis beside pericellular or centrilobular fibrosis perisinusoidal or bridging fibrosis[9,10]. We used the NAS to assess histology and more than 5 points defined NASH in this study.

DNA extraction and AIMs

Peripheral blood samples were collected and DNA extraction was performed from mononuclear cell by Pure Link Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, United Sates) following the manufacturer's guidelines.

Ancestry for genetic markers was evaluated by analysis of seven specific alleles of population. These alleles, AIM, were chosen based on the earlier studied panel of 48 AIMs determined in diverse populations. The AIM allele* 1 was defined as the existence of insertion or the absence of restriction enzyme site[4,5].

Seven AIM with the greatest ethnic-geographical differential frequencies (⩾ 48%) were chosen in this study. Polymorphisms as *Alu* insertion and *insertion-deletion* (*indel*, I/D) were identified by conventional polymerase chain reaction (PCR); single nucleotide polymorphisms were performed by real-time PCR (TaqMan™ System, Applied Biosystems).

The examined AIM were: African ancestry *AT3-I/D* (rs3138521) and *LPL* [rs285][11]; European ancestry *Sb19.3* (rs3138524), *APO* (rs3138522) and *FY-Null* (rs2814778); and Amerindian ancestry markers *PV92* (rs3138523) and *CKMM* (rs4884)[4,11]. The frequency of allele* 1 in AIM was considered in the analysis.

Ancestral population characteristics

Four hundred eighty-two (n = 482) subjects from homogeneous populations comprised ancestral populations. These groups were used as a referral to genetic ancestry assessment. Ancestral African population included 134 Nigerians subjects; European ancestral population included 23 Germans and 83 Spanish; and Amerindian ancestral population had 242 Native Americans. The ancestral samples were friendly provided by Mark Shriver, MD. The analyzed AIM from NAFLD studied samples were verified in the ancestral population.

Statistics analysis

Data were analyzed using R PROJECTS software version 2.11.1 for Windows (May 31 2010, Statistics Department of the University of Auckland, Auckland, New Zealand, http://www.r-project). The continuous variables were expressed as mean ± SD; data comparison was executed by the Mann-Whitney *U*-test. Categorical variables were presented as number of cases and percentage and analyzed by Pearson's χ^2 test. Since the sample size assessment could not be performed previously, power was calculated posteriorly. The 5% alpha-error was considered as significant ($P < 0.05$) (two-sided).

The Hardy-Weinberg equilibrium assessment was calculated by GENEPOP online version 4.0.10 (Laboratiore de Genetique et Environment, Montpellier, France)[12]. Ancestry allele frequencies evaluation and variation between groups were analyzed utilizing GENEPOP online version 4.0.10[12]. Estimation of genetic ancestry contribution was computed by ADMIX-95 software (Departamento de Genética de la Facultad de Medicina, Universidad de la Republica, Montevideo, Uruguay, http://www.genetica.fmed.edu.uy)[13]. Stimatives of genetic ancestry contribution from each individual were determined through STRUCTURE software version 2.2 (Human Genetics Department, University of Chicago, Chicago, IL, United States).

A biomedical statistician performed the statistical review of the study.

RESULTS

We analyzed 131 Brazilian patients with NAFLD. Forty-five patients were identified with simple steatosis and 86 had NASH by histologic liver analysis (Table 1). These results were compared with patients from Portugal who had undergone bariatric surgery; 90 had histologic liver information available to analysis. Among Portuguese subjects 66 had simple steatosis, and 24 had NASH (Table 1). In this sample, NAS criteria were used as parameters to Simple Steatosis and NASH

Table 1 Baseline characteristics of Brazilian and Portuguese study population according to non-alcoholic fatty liver disease status *n* (%)

	Brazil (*n* = 131)			Portugal (*n* = 90)		
	NASH	S. Steatosis	*P*-value	NASH	S. Steatosis	*P*-value
	86 (65.6)	45 (34.4)		24 (26.7)	66 (73.3)	
Gender						
Female	61 (70.1)	26 (29.9)	0.2	20 (28.6)	50 (71.4)	0.6
Male	25 (56.8)	19 (43.2)		4 (20.0)	16 (80.0)	
Age (yr)	56 ± 1.1	51 ± 1.5	[2]0.006	47 ± 12.4	47 ± 10.2	0.3
Diabetes-2	71 (82.4)	8 (17.6)	[2]3.7×10^{-9}	14 (33.3)	28 (66.7)	0.2
Fasting glucose (mg/dL)	124.9 ± 5.2	106.0 ± 5.3	[2]0.001	97.3 ± 28.2	92.6 ± 18.4	0.3
HOMA ⩾ 2.5 - mean	5.3	4.6	[2]0.04	4.9	4.8	0.5
HOMA ⩾ 2.5 - *n*	75 (70.8)	31 (29.2)	[2]0.02	15 (45.5)	18 (54.5)	0.5
Hypertension	59 (68.2)	14 (31.8)	[2]8.0×10^{-5}	12 (20.1)	34 (73.9)	[2]0.003
ALT (IU/L)	38.3 ± 0.6	33 ± 5.2	0.2	31.7 ± 30.6	30.56 ± 15.9	0.7
AST (IU/L)	22 ± 22.5	32.8 ± 3.2	[2]0.009	25.9 ± 24.8	25.06 ± 11.2	0.7
[1]Dyslipidemia	54 (63.0)	17 (37.0)	[2]0.009	24 (100)	55 (83)	[2]0.03

[1]High levels of cholesterol and/or triglyceride; [2]Statistically significant results, $P < 0.05$. NASH: Nonalcoholic steatohepatitis; S. Steatosis: Simple steatosis; HOMA: Homeostatic Model of Assessment; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

Table 2 Genetic ancestry contribution in Brazilian and Portugal populations with non-alcoholic fatty liver disease

	Brazil			Portugal		
	NASH	S. Steatosis	*P*-value	NASH	S. Steatosis	*P*-value
African	41.7%	37.7%	0.75	6.4%	6.1%	0.97
Amerindian	9.5%	12.7%		37.8%	39.4%	
European	48.8%	49.6%		55.9%	54.5%	

NASH: Nonalcoholic steatohepatitis; S. Steatosis: Simple steatosis.

diagnosis: 65.6% of Brazilians (*n* = 86) had NAS score ⩾ 5 defining NASH and 26.7% Portuguese people had NAS score ⩾ 5, classified as NASH.

In the Brazilian sample, NASH was more frequent among the elderly with diabetes, dyslipidemia, high fasting glucose levels and HOMA index ⩾ 2.5. In the Portuguese study population, dyslipidemia was present in all patients (*n* = 24) with NASH and hypertension was present in a superior number of patients in the simple steatosis group compared to NASH (Table 1). ALT levels were similar between NASH and simple steatosis patients.

The genetic ancestry contribution among Brazilian and Portuguese individuals with NASH was similar to those with simple steatosis from each cohort (Table 2). Furthermore, the genetic ancestry contribution of the Brazilian and Portuguese population were different and a greater European and Amerindian ancestry contribution was detected in the Portuguese population while a higher African genetic ancestry contribution was observed in Brazilian population of both NASH and simple steatosis subgroups (Table 2).

In Brazilian population, genetic ancestry did not influence known risk factors for NAFLD as gender, hypertension, diabetes, HOMA-index or dyslipidemia. However, in Portugal sample there was a trend for European genetic ancestry being greater among subjects with HOMA ⩾ 2.5 and Amerindian ancestry contribution among those with HOMA-index < 2.5 (Table 3).

DISCUSSION

Of our knowledge, this is the first study that investigated the possible association between genetic ancestry, NAFLD and metabolic characteristics using AIM in two cohorts with biopsy-proven NAFLD from Brazil and Portugal. However, our results showed that these genetic ancestry differences do not appear to be associated with the development of NASH. Genetic ancestry distribution was quite different between Brazilian and Portuguese patients. Unexpectedly, there was a higher prevalence of an Amerindian ancestry in the Portuguese group than in the Brazilian. On the other hand, as expected there was a high prevalence of African ancestry in the Brazilian patients. However, interestingly, no differences in this distribution were found when NASH or simple steatosis was compared, either in the Brazilian or the Portuguese group.

In the Brazilian sample, known risk factors as diabetes, dyslipidemia, higher fasting glucose levels and HOMA index ⩾ 2.5 were more frequent among patients with NASH than simple steatosis. In the Portuguese study population, dyslipidemia was frequent among patients with NASH; and hypertension was present in a higher proportion among subjects with simple steatosis in comparison to those with NASH, possibly due to larger sample size in simple steatosis group. However, there were no differences regarding other metabolic risk factors, what may be due to the fact that the entire Portuguese cohort had morbid obesity. In the Portugal sample, the genetic ancestry did not have a significant influence in the prevalence of insulin resistance as evaluated by HOMA. Several studies have showed that individuals with European ancestry have a higher risk of progression to NASH; however, these studies used self-reported ancestry classification. To our knowledge, no previous study has evaluated the influence of genetic ancestry in NASH progression in admixed populations.

The heterogeneity of the Brazilian population presents an additional difficulty in pharmacogenetic and clinical

Table 3 Genetic ancestry influence in risk factors for non-alcoholic fatty liver diseases in Brazilian and Portuguese study populations

	Genetic ancestry contribution							
	Brazil				Portugal			
	African	Amerindian	European	*P*-value	African	Amerindian	European	*P*-value
Diabetes								
No	11.7%	39.8%	48.5%	1.00	6.1%	37.5%	56.4%	0.69
Yes	12.1%	39.6%	48.3%		7.2%	31.6%	61.2%	
Dyslipidemia								
No	11.8%	39.5%	48.6%	0.99	6.0%	37.4%	56.5%	0.90
Yes	11.7%	39.6%	48.7%		6.6%	33.6%	59.8%	
Gender								
No	11.8%	39.3%	48.9%	0.99	6.2%	37.9%	55.9%	0.97
Yes	11.7%	39.4%	48.9%		6.2%	39.5%	54.3%	
HOMA-index								
> 2.5	11.7%	39.7%	48.6%	0.94	3.4%	33.9%	62.7%	0.07
< 2.5	10.6%	38.3%	51.1%		7.8%	43.5%	48.7%	
Hypertension								
No	45.9%	20.4%	21.0%	0.16	4.7%	31.4%	63.9%	0.18
Yes	44.0%	18.7%	37.3%		7.6%	41.2%	51.1%	

genetic research. Studies have demonstrated only a weak association between skin pigmentation, self-reported ancestry and the ancestrality determined by DNA markers in admixed populations, as is the Brazilian case[3,6,14]. Therefore, assessment of an individual's genetic ancestry might be the best way to assess the possible relation among disease factors and ethnic influence in association studies from admixed populations as the Brazilian.

Brazilian and Portuguese populations have different ancestry's contributions. More even proportions of ethnic contribution were observed in the Brazilian population, reflecting the greater racial admixing, while a higher genetic European contribution and a small African contribution was observed among the Portuguese, probably reflecting the historic context of both countries.

European and Amerindian come from the same chain migration and then some specific allele populations could be difficult to differentiate similar ancestries. So we believe that a larger number of AIMs with a great differential of frequency (> 40%) among Amerindians and Europeans or mitochondrial DNA analysis could be able to better differentiate the contribution of each of these ancestries in the Portuguese sample[15,16].

Limitations of the present study could be the differences in the studied cohorts. While the Brazilian cohort consisted of a group of patients being evaluated for suspected NAFLD, the Portuguese cohort was a cohort of morbid obese patients, with the usual distribution of about a third having NASH and two thirds having simple steatosis. Also, we are not aware what the ancestry distribution of the normal population of either country is. Regarding the Portuguese cohort it would be tempting to speculate that this high prevalence of Amerindian ancestry could be involved in the fact that they have morbid obesity, since it has been recognized that Amerindian subjects have an increased susceptibility to develop obesity[17,18].

In summary, the genetic ancestry contribution among Brazilian and Portuguese individuals with NASH was similar to those with simple steatosis from each cohort. On the other hand, a greater European and Amerindian ancestry contribution was detected in the Portuguese population while a higher African genetic ancestry contribution was observed in Brazilian population of both NASH and simple steatosis groups.

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COMMENTS

Background

Nonalcoholic fatty liver disease (NAFLD) is a frequent liver disease and may vary from simple steatosis through steatohepatitis, fibrosis and, finally, cirrhosis and hepatocellular carcinoma. Several studies suggest the presence of diverse risks factors for NAFLD and differences in clinical features based upon ancestry, as well as the potential role of ancestry as an independent risk factor for disease severity. Hispanic Americans and Caucasians have the highest prevalence of NAFLD whereas African Americans have the lowest; however, few data about ancestry contribution in NAFLD patients are available. Brazil is a country that was colonized by Portugal and its population genetic background ancestry is an admixture of three main parental groups (Amerindians, Europeans and Sub-Saharan Africans). Portugal has a more homogeneous population of European ancestry. In order to analyze the influence that ancestry of admixed populations has over a specific disease it is necessary to assess genetic ancestry markers. The ancestry informative markers (AIMs) is powerful tools for inferring the genetic composition of admixed populations. In this study the authors investigated the possible association between genetic ancestry, NAFLD severity [simple steatosis and nonalcoholic steatohepatitis (NASH)] and metabolic characteristics in two cohorts with biopsy-proven NAFLD from Brazil and Portugal.

Research frontiers

To their knowledge this is the first study that have utilized genetic ancestry markers to compare the ancestry influence of an admixed population with a more homogeneous one such as the Portuguese population.

Innovations and breakthroughs

The authors results showed that these genetic ancestry differences do not appear to be related with NASH; no differences in genetic ancestry distribution were found when NASH or simple steatosis were compared, either in the

Brazilian or the Portuguese study samples. The genetic ancestry distribution among Brazilian and Portuguese patients were different and they observed a greater prevalence of Amerindian ancestry among Portugueses compared to the Brazilians. On the other hand there was a marked prevalence of African ancestry in the Brazilian patients. However, interestingly, no differences in this distribution were found when NASH or simple steatosis was compared, either in the Brazilian or the Portuguese samples. Previous studies have showed that individuals with European ancestry have a higher risk of progression to NASH; however, these studies used self-reported ancestry classification, which may not be accurate when analyzing admixed populations. Known risk factors as diabetes, dyslipidemia, higher fasting glucose levels and Homeostatic Model of Assessment index $\geqslant$ 2.5 were more frequent among patients with NASH than simple steatosis in the Brazilian sample. In the Portuguese analyzed sample, dyslipidemia was present in a higher proportion in the NASH group compared to simple steatosis. They did not find any association among genetic ancestry and those risk factors.

Applications

This study suggests that ancestry analyzed by genetic markers possibly do not add as a risk factor in the evaluation of NAFLD disease. However, other known risk factors were present in this study. The high Amerindian ancestry among Portugueses is an issue to be studied in future analyses.

Terminology

NAFLD comprises two major presentations, simple steatosis and NASH. The former is a mild clinical condition characterized by fatty deposits in the hepatocyte cytoplasm while the latter is defined by the presence of any degree of steatosis along with centrilobular ballooning and/or Mallory-Denk bodies or any degree of steatosis along with centrilobular pericellular/perisinusoidal fibrosis or bridging fibrosis. The NAFLD activity score is a tool to assess NASH diagnosis and NAFLD severity. AIMs are genetic polymorphisms, which have a frequency differential of at least 30% in order to differentiate two distinct populations. They have chosen AIMs with high frequency differential in this analysis ($\geqslant$ 48%).

Peer-review

In the original article of Cavalcante *et al*, the authors investigated the possible association between genetic ancestry, NAFLD severity and metabolic characteristics in two cohorts with biopsy-proven NAFLD from Brazil and Portugal. They found that ancestry markers are not different between subjects with steatohepatitis and ones suffering from hepatic steatosis in the investigated populations. They concluded that genetic ancestry is not associated with a higher risk of NASH in their study.

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