REVIEW

Hepatocellular carcinoma: Review of disease and tumor biomarkers

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Abstract

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

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

Core tip: Many independent authors have utilized

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

INTRODUCTION

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

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

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

PATHOGENIC MECHANISMS WITH METABOLIC IMPLICATIONS

Altered tumor metabolism

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

"Warburg effect" and glycolysis

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

There is now some consensus that the major role of heightened glycolysis in tumor cells is to provide substrates to the pentose phosphate pathway for nucleotide synthesis, rather than energy provision in the form of ATP[12,13]. In essence, the tumor is maximizing production of cellular constituents for proliferation at the expense of energy production.
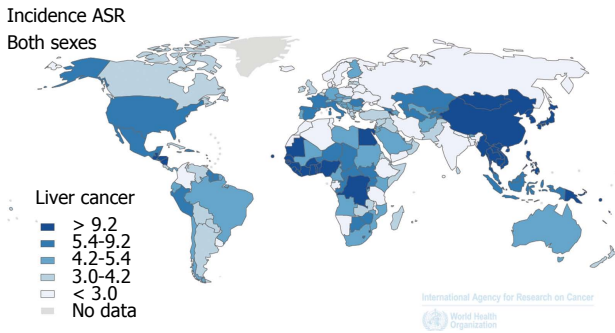
Figure 1 Global incidence of hepatocellular carcinoma. Sourced from GLOBOCAN 2012.
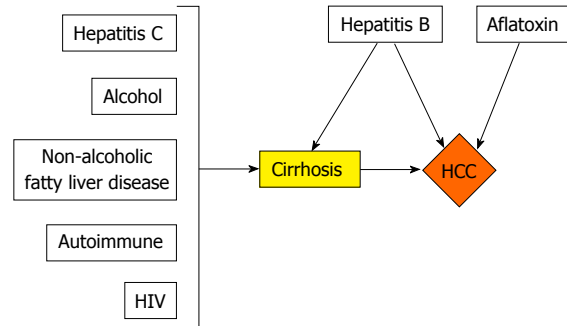

Figure 2 Independent risk factors of cirrhosis and hepatocellular carcinoma. HIV: Human immunodeficiency virus; HCC: Hepatocellular carcinoma.
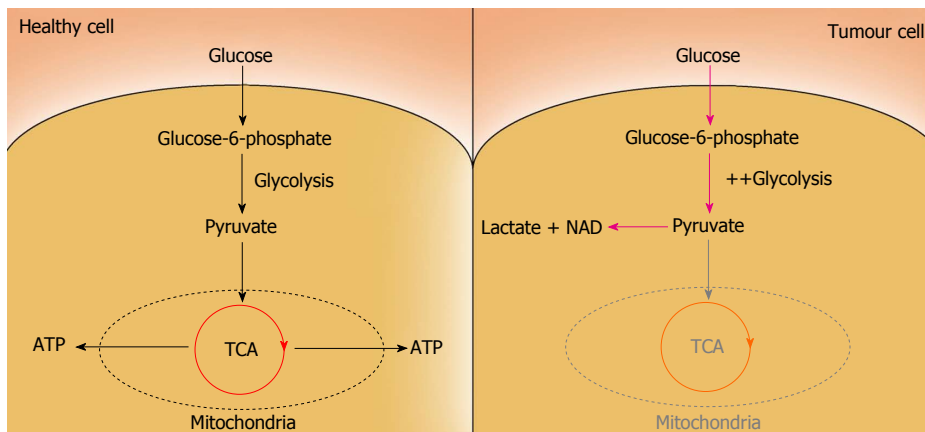

Figure 3 Warburg theory of heightened glycolysis in tumor cells. TCA: Tri-carboxylic acid cycle; ATP: Adenosine triphosphate; NAD: Nicotinamide adenine dinucleotide.

MOLECULAR EFFECTORS AND TUMOUR METABOLISM

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

HIF

HIF 1 is a heterodimeric protein complex transcription factor that is activated by hypoxic, inflammatory, metabolic and oxidative stress[10,12,14]. The HIF 1 heterodimeric complex (HIF 1α + HIF 1β) is stabilized at low oxygen levels, but degraded by the proteasome in normoxic conditions. The HIF 1 heterodimer stimulates glycolysis by increasing the expression of pro-glycolytic uptake enzymes and transport molecules, such as glucose transporter 1 (GLUT 1) and hexokinase[12]. HIF 1β deficient hepatoma cells grown as solid tumors in mice were found to have reduced rates of growth and glycolytic intermediates compared to wild type hepatoma cells[15]. It would therefore appear that HIF 1 may play a central role in the Warburg model. However, HIF 1 is only stable in hypoxic conditions and Warburg's model describes heightened glycolysis in normoxic conditions.

Only a minority of cancers display aberrant HIF 1 function in normoxia, such as renal cell carcinoma[16]. The role of HIF 1 in HCC is still under investigation but a number of recent studies, mostly in animal models, have observed high HIF 1 activity and its downstream counterparts, such as GLUT1, in hepatoma cells[17-19]. Recent studies have also identified association between HIF 1 and the prognosis of HCC, where HIF 1α levels have been found to be significantly raised in HCC, compared to benign liver disease[19]. Furthermore, it appears that HIF 1 inhibition may be a potential target of therapeutic benefit in HCC by down-regulating its role in tumorigenesis. There have been several proposals to incorporate HIF 1 inhibition as adjunct to the current treatment pathways, but further investigations are required before its clinical application[20].

p53

Tumor suppressor genes, such as *p53*, have also been implicated in alterations in metabolism. Inactivation of *p53* can cause the Warburg phenomenon. *p53* positively regulates the expression of the protein synthesis of cytochrome C oxidase 2, which is required for the assembly of the oxidative phosphorylation enzyme, cytochrome C oxidase[21] and also negatively regulates phosphoglycerate mutase, a key glycolytic enzyme[22]. In addition, *p53* transcriptionally activates TP53-induced
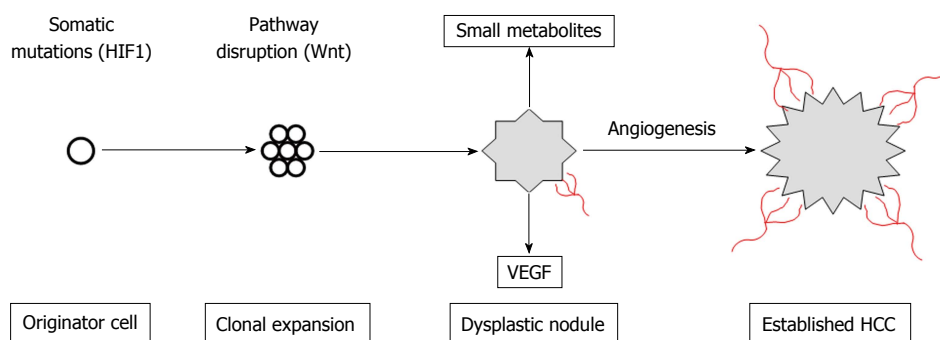
Figure 4 **"Angiogenic switch" in hepatocellular carcinoma.** VEGF: Vascular endothelial growth factor; HCC: Hepatocellular carcinoma; HIF 1: Hypoxia inducible factor 1.

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

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

METABOLITE EFFECTS ON CARCINOGENESIS

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

OTHER PATHOGENIC MECHANISMS

Genetic profiling studies of HCC tissue have shown several genes to be disrupted through somatic mutations, chromosomal disruption and epigenetic aberration through methylation abnormalities including *p53*, *Rb1*, *β-catenin, CMYC* and *survivin*. The Wnt-β catenin pathway is the most commonly disrupted pathway,

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

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

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

Table 1 Comparison of nuclear magnetic resonance and mass spectrometry

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

NMR: Nuclear magnetic resonance; MS: Mass spectrometry.

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

CURRENT SURVEILLANCE AND DIAGNOSIS

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

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

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

METABOLIC PROFILING TO FIND BIOMARKERS

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

PROTON NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

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

MASS SPECTROMETRY

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

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

CLINICAL APPLICATION OF BIOMARKERS

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

SERUM MARKERS OF HCC

Serum AFP
Serum AFP is the most widely used marker of HCC. It is a fetal glycoprotein, which is synthesized *in utero* by the embryonic liver, cells of the vitelline sac and the fetal intestinal tract. Serum AFP is usually undetectable in healthy adults[54]. The production of AFP by HCC cells has been seen as confirmation that the tumor arises from hepatic stem cells as a form of maturation arrest, akin to an embryonic state[55]. Not all HCCs secrete AFP and its diagnostic accuracy is variable. A meta-analysis of AFP for HCC surveillance found that it displayed a sensitivity of 39% to 65% and a specificity of 76% to 94% for tumor diagnosis[56]. The cut-off level of AFP was important in determining the diagnostic power.

A cut-off of 20 ng/mL resulted in a sensitivity of 64% and specificity of 91%[57], while a cut-off of 400 ng/mL resulted in a sensitivity of 17% and specificity of 99%[58]. Values of over 400 ng/mL are generally considered diagnostic of HCC, although only about 20% of patients with HCC display values this high. Furthermore, patients with chronic viral hepatitis may display a raised AFP during viral flares without the presence of HCC. In a study of 290 Chinese patients with chronic HBV, 44 were found to have elevated serum AFP levels (> 20 ng/mL) and only six (13%) had HCC. The remaining 38 had elevated serum AFP, either due to viral flares or due to unknown causes[59]. Trevisani *et al*[58] also observed that an AFP elevation in non-infected patients could be more indicative of HCC when compared to infected patients.

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

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

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

Table 2 Diagnostic performance of serum markers of hepatocellular carcinoma

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

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

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

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

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

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

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

Tumor-specific growth factor
Tumor-specific growth factor (TSGF) is released by malignant tumors, and has been shown to correlate with tumor growth and surrounding vascularization.

Therefore, it is reasonable to suggest that TSGF could be a potential biomarker that may be used for HCC grading in populations around the world. TSGF has been approved for use by the Chinese government following study results that showed a sensitivity of 82% in HCC diagnosis at the cut-off of 62 U/mL[77].

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

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

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

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

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

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

URINARY MARKERS OF HCC

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

Nucleosides

Studies in the 1970s observed elevated levels of the methylated purines 7-methylguanine, 1-methylguanine, *N*-dimethylguanine, 1-methylhypoxanthine and adenine in the urine of patients with HCC. In 1976, it was found

that urine levels of cyclic guanosine 3':5' monophosphate (cGMP) were elevated in rats with transplanted liver and renal tumors[109]. In 1982, Dusheiko *et al*[110], found parallels in human studies, observing elevated urinary cGMP levels in patients with HCC. In the same study, cGMP was also elevated in the urine of patients with liver disease and other non-HCC tumors, reducing the specificity of the marker considerably.

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

TGFα and β

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

Neopterin

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

Polyamines

The polyamines, organic compounds containing two or more amine groups, include putrescine, spermine, and spermidine. Their exact cellular role is unclear but they are required for cellular proliferation. Putrescine acts on *S*-adenosylmethionine (SAMe), a methylating molecule, to produce spermine which in turn acts on further SAMe molecules to produce spermidine[119]. Antoniello *et al*[120] reported increased urinary levels of free and acetylated polyamines using HCC patients compared to healthy controls and patients with cirrhosis, although the sensitivity of these markers was found not to be high enough for early tumor detection.
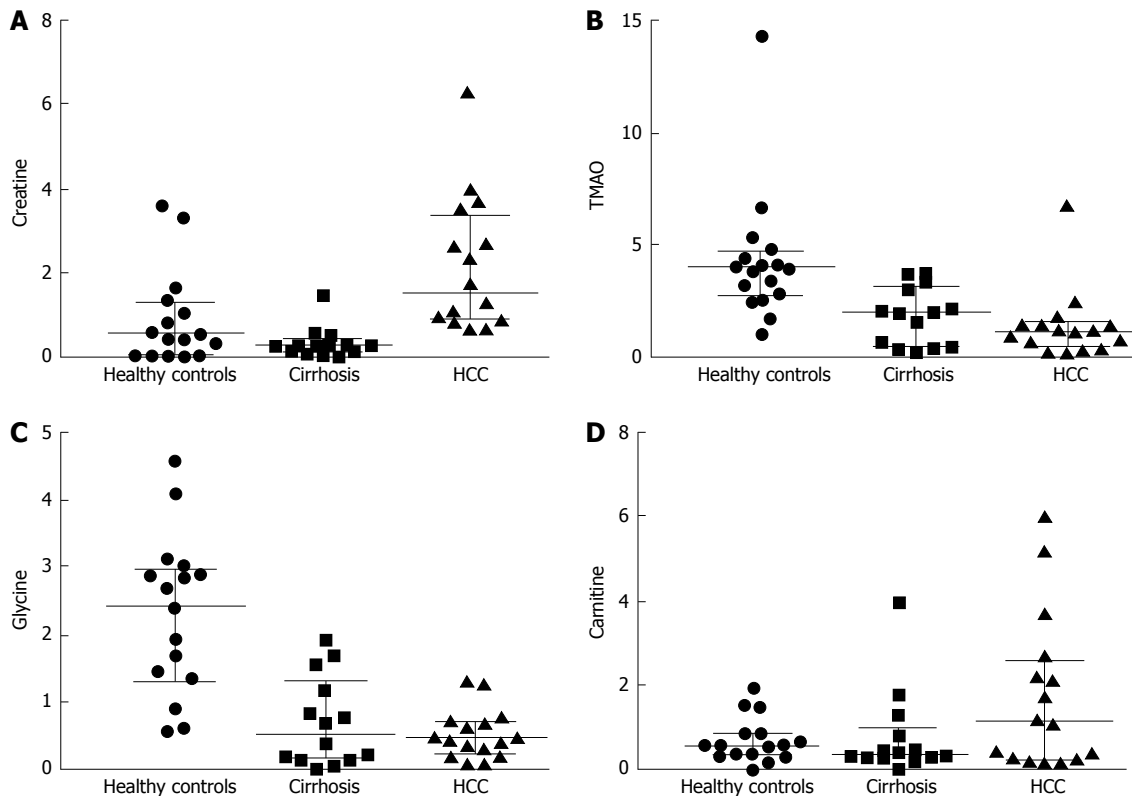
Figure 5 Univariate analysis of discriminatory urinary variables from an Egyptian cohort, comparing values from healthy controls, cirrhosis group and hepatocellular carcinoma group. Discriminatory variable A: Creatine; B: Trimethylamine N-oxide (TMAO); C: Glycine; D: Carnitine. Adapted from Shariff *et al*[125]. HCC: Hepatocellular carcinoma.

Urinary trypsin inhibitor

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

Soluble urinary metabolites

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

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

were altered between groups.

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

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

Urinary creatine levels were significantly elevated in

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

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

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

CONCLUSION

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

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

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

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

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