

Non-invasive Biomarkers in Non-Alcoholic Steatohepatitis-induced Hepatocellular Carcinoma

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is by far the most common form of chronic liver disease worldwide, affecting adults as well as children. Under the term of NAFLD there is a wide spectrum of diseases ranging from simple steatosis to the non-alcoholic steatohepatitis (NASH), which can progress to cirrhosis and hepatocellular carcinoma (HCC). Several mechanisms have been described to influence the progression of the disease from the benign NAFL to the aggressive NASH. The imbalance between pro- and anti-oxidant mechanisms and between pro- and anti-inflammatory cytokines is thought to play a pivotal role in the pathogenesis of NAFLD and disease progression toward NASH and fibrosis. The present review intends to look at some of the mechanistic biomarkers to be employed in establishing an early diagnosis in HCC derived from NASH.

Key words: non-alcoholic fatty liver disease – non-alcoholic steatohepatitis – hepatocellular carcinoma – biomarkers – non-invasive.

Abbreviations: ANGPT: angiotensinogen-converting enzyme; AFP: α -fetoprotein; ALT: alanine aminotransferase; AST: aspartate aminotransferase; CI: confidence interval; COL: collagen; DCP: des-carboxyprothrombin; γ GT: gamma glutamyl transpeptidase; HBV: hepatitis B virus; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; HR: hazard ratio; ITG: integrin; LAM: laminin collagen genes; MMP: matrix metalloproteinase; MS: metabolic syndrome; NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis; PDGFR α : platelet derived growth factor receptor- α

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer in the world and the only increasing in incidence digestive tract carcinoma. Until recently, the incidence of HCC paralleled that of the various types of viral hepatitis around the world. Additionally, cirrhosis is the most important risk factor for HCC, regardless of etiology [1]. In North America, non-alcoholic fatty liver disease (NAFLD)/non-alcoholic steatohepatitis (NASH) is the most common cause of HCC after hepatitis C virus (HCV), hepatitis B virus (HBV) and alcohol [2], and one

of the most important factors responsible for the increasing incidence of HCC.

The incidence of HCC not related to viral hepatitis is on the rise. It is related to lifestyle factors, including obesity and diabetes. In particular, a higher body mass index (BMI), especially among females, is a risk factor for NAFLD-HCC. Furthermore, the presence of diabetes is higher in individuals with NAFLD-HCC than in patients with HCC of other etiologies, while the presence of cirrhosis was lower in NAFLD-HCC patients. A high BMI is a poor predictor of survival, while the presence of diabetes does not influence survival in NAFLD-HCC to any considerable degree [3]. Cirrhosis, steatosis and metabolic abnormalities play a role in the pathogenesis of NAFLD-HCC. The presence of cirrhosis usually predicts a less favorable outcome in NAFLD-HCC patients, partly owing to the fact that cirrhotic individuals also tend to be older [4].

The prevalence of NAFLD-HCC was assessed in a large sample of patient records belonging to the Veteran Affairs population. NAFLD was identified as the third most common cause of HCC in this population, with an incidence of 8.0% (120

cases of NAFLD-HCC among 1,500 cases of HCC). The annual incidence rate was found to be stable. Cirrhosis was present in only 58.3% of the NAFLD-HCC sample, which was lower than the 72.4% incidence of cirrhosis in patients with alcoholic disease-HCC and 85.6% in HCV-HCC. Hepatocellular carcinoma surveillance and treatment were less common among patients with NAFLD-HCC, although the survival rate was similar regardless of etiology [5]. A steady increase in the proportion of liver transplant recipients with NASH-HCC has been observed in a large retrospective analysis. Indeed, NASH has become the second leading cause of liver transplant among HCC patients in the USA behind only HCV, and more worrisome is the fact that NASH-HCC appears to be an ever-increasing indication for liver transplant [6].

GENOMICS, TRANSCRIPTOMICS AND METABOLOMICS

Up to 25% of NASH cases can progress to HCC [7]. A recent analysis of transcriptomic and metabolomic datasets revealed different genes expression in HCC evolved from NASH. No significant differences were observed between samples belonging to patients with NASH with fatty liver and NASH without fatty liver. Extracellular matrix/angiogenesis genes were up-regulated in NASH patients compared to healthy patients. Among these were 5 of 24 matrix metalloproteinase genes (especially *MMP14*), 14 of 23 integrin genes (especially *ITGA3*), 7 of 12 laminin genes (especially *LAMA2*), and 10 of 19 collagen genes (especially *COL1A2*), as well as angiopoietin-2 (*ANGPT2*) and platelet derived growth factor receptor- α (*PDGFRA*). These genes are known to be up-regulated in HCC as well, and these findings suggest that their up-regulation occurs early during NASH and they are therefore unlikely to be associated with the progression to HCC [7].

Iron homeostasis genes were down-regulated in NASH, including hepcidin antimicrobial peptide (*HAMP*), the master regulator of iron homeostasis. Also down-regulated were iron transporters solute carrier family 11, member 2 (*SLC11A2*, commonly called *DMT1*) (proton-coupled divalent metal ion transporters) and solute carrier family 39, member 14 (*SLC39A14*, commonly called *ZIP14*) (zinc transporter). On the other hand, solute carrier family 40 (iron-regulated transporter) member 1 (*SLC40A1*) (commonly called ferroportin1) was normal in NASH. Ferroreductases [*STEAP3* and cytochrome b reductase 1 (*CYBRD1*)] and ferroxidases [hephaestin (*HEPH*)] were further dysregulated in NASH. However, the influence of perturbed iron homeostasis on development of HCC from NASH was unclear based on these findings [7].

Genes belonging to the Wnt signaling pathway were either up-regulated or down-regulated in NASH compared to control. Specifically, Wnt pathway genes were generally down-regulated, while Wnt pathway inhibitor genes were up-regulated in NASH compared to control [7]. In NASH, the expression of Wnt frizzled receptors (*FZD3*, *FZD5*, *FZD7*) and Wnt signaling inhibitors (*FRZB*, *SFRP5*, *DKK3*, *PRIKL1*, *PRIKL2*, *DACT1*) is increased while the expression of Wnt ligands (*WNT3*, *WNT2*) and Wnt activators (*FRAT1*, *PIN1*) is decreased. While downstream genes in the Wnt pathway

were generally not affected in NASH, signalling through this pathway was shown by microarray to be largely inhibited [7].

Several differences were also found in terms of metabolite profiles between NASH and HCC, when comparing the findings of Clarke et al [7] with those of two previously published reports [8, 9]. Based on the cumulative data of these reports, lyso-phosphatidyl-choline metabolites were decreased in both NASH and HCC. Lysine, phenylalanine, citrulline and creatinine in plasma were decreased in HCC but were increased in NASH. In contrast, plasma glycodeoxycholic acid, creatine, inosine and α -ketoglutarate were increased in HCC and were decreased in NASH [7-9].

The status of the cell-cycle regulator p27 was recently investigated in resected liver samples of NASH-HCC patients by Western blotting and by phosphorylation at threonine 157 (T157) and serine 10 (S10) through immunohistochemical analysis [10]. Decreased p27 expression was noted in 13 (59.1%) of 22 HCC samples, and correlated with larger tumor size ($p=0.01$) and increased cell proliferation ($p<0.01$). Phospho-p27 at T157 was noted in 4 (18.2%) of 22 patients and at S10 in 7 (31.8%) of 22 patients. p27 phosphorylation was associated with decreased disease-free survival. These findings suggest that p27 may be a useful biomarker of HCC recurrence in NASH-HCC [10].

The c.444C > G single nucleotide polymorphism (rs738409) in patatin-like phospholipase domain-containing 3 (*PNPLA3*) gene, which encodes the p.I148M (isoleucine to methionine substitution at residue 148) variant is well recognized as a modifier of hepatic triacylglycerol accumulation and NAFLD progression [11]. *PNPLA3* was genotyped in peripheral blood lymphocytes. The *PNPLA3* rs738409 C > G polymorphism was associated with a risk of HCC development. Male gender ($p<0.0001$) and the presence of cirrhosis ($p<0.0001$) were also associated with NAFLD-HCC [11]. The presence of the G allele was associated with NAFLD-HCC at a younger age. Furthermore, the G allele was present predominantly in younger NAFLD-HCC patients. Despite a strong association between the G allele and cirrhosis, the presence of this allele was associated with HCC regardless of the degree of fibrosis in a subanalysis of cirrhotic NAFLD patients with and without HCC [11]. In another study, genotypes of *PNPLA3* single nucleotide polymorphism (SNP) did not differ significantly in a small sample of NAFLD patients between those with HCC and those without HCC [12].

Amplification of chr.6p21.1 was observed in metabolic syndrome (MS)-related HCC compared to HCV-HCC, with an increased expression of cullin7 (*CUL7*), a gene located at this locus. This amplification is believed to influence cellular proliferation [13].

SERUM BIOMARKERS (α -FETOPROTEIN AND DES-CARBOXYPROTHROMBIN)

Serum biomarkers of HCC include α -fetoprotein (AFP, cut-off 20 ng/mL), des-carboxyprothrombin (DCP, cut-off 40 mAU/mL), lectin-bound AFP, tyrosine kinase with Ig and EGF homology domains 2, IL-6, squamous cell carcinoma antigen. However, these biomarkers often differentiate between HCC and healthy controls, or between HCC and chronic

liver disease. They do not predict development of HCC from NASH [14].

Alpha-fetoprotein and DCP were elevated in HCC patients compared to controls with non-malignant liver diseases [34 (20.7%) of 164 patients had NASH]. Alpha-fetoprotein was generally positive in patients with HCV-HCC or in patients with advanced stages of HCC [15]. Mean serum AFP levels measured by enzyme-linked immunosorbent assay were significantly higher in patients with HCV-HCC than in patients with NASH-HCC, although a large degree of within-group variability was observed in each group [16]. A trend towards higher AFP levels and a higher proportion of patients with abnormal (>10 ng/mL) AFP levels was observed at HCC diagnosis compared to NASH diagnosis in a small sample of Japanese patients with histologically-proven NASH [17]. Lower AFP levels (<20 ng/mL) were more common in NAFLD-HCC compared to alcoholic disease-HCC or HCV-HCC [5].

In contrast, DCP was generally positive in NASH-HCC (15). Its levels were significantly higher at the time of HCC diagnosis compared to the time of NASH diagnosis in a small sample of Japanese patients with histologically-proven NASH (mean 34 vs. 18 mAU/mL, $p=0.004$). Likewise, a higher proportion of patients had abnormal (>40 mAU/mL) DCP levels at the time of HCC diagnosis [17]. Mean serum DCP levels measured by enzyme-linked immunosorbent assay were not different between HCV-HCC and NASH-HCC, although a large degree of within-group variability was observed in each group [16].

LIPIDS AND ADIPOKINES

Lipid accumulation in hepatocytes induces cancer-related molecular signalling through nuclear factor- κ B, c-Jun N-terminal kinase/activator protein-1 activity, and overexpression of tumor growth-promoting genes [18]. Additional components include phosphatase and tensin homologue (PTEN) in hepatocytes, and the nuclear factor- κ B/mammalian target of rapamycin (mTOR) complex, as well as sterol regulatory element binding proteins (SREBP), which act as master regulators of hepatic lipogenesis. Mitochondrial dysfunction and increased free fatty oxidation in peroxisomes and microsomes may lead to an excess of free reactive oxygen species and lipid peroxides that lead to oxidative stress. Oxidative stress may form adducts with DNA, leading to malignancy. Hepatic iron overload may contribute to oxidative stress. Based on these findings, fatty liver may be a risk factor for HCC independent of MS [18]. Extrahepatic tissue, especially adipose tissue, may lead to changes in the expression and secretion of adipokines such as leptin and adiponectin, which may influence the development of MS-associated HCC [18].

Adiponectin increases disease-free survival in HCC, whereas leptin is significantly associated with oncogenic effects based on the immunohistochemistry of human HCC tissue microarrays [19]. In obese individuals with MS, leptin is the main adipokine produced by the adipose tissue. Leptin has fibrinogenic effects on the liver, while deregulation of the expression of leptin and its receptor were associated with metabolic disorders that can result in human cancers [20]. While

some studies found leptin to be elevated in NASH compared to controls or patients with viral hepatitis, other studies failed to find this association. At most, leptin is associated with the degree of steatosis but not with necroinflammation or fibrosis, which casts doubt over its association with HCC in NAFLD patients [17, 20].

Serum leptin levels were elevated in cirrhotic patients compared to control individuals, yet no differences were observed between cirrhotic patients with and without HCC [21]. Leptin promotes pro-inflammatory and pro-fibrogenic effects in NAFLD. On the other hand, adiponectin inhibits hepatic carcinogenesis. Studies have shown that leptin is up-regulated and adiponectin is down-regulated in MS-related NAFLD, thereby promoting HCC. The study by Tian et al further details the proposed mechanism of action of leptin and adiponectin [22].

INCIDENCE RATES

The incidence of NAFLD-HCC was 2.0% in a nation-wide survey in Japan (292 of 14,530 cases of HCC) [23]. Among cases of NAFLD-HCC, the median age was 72 ± 8.4 years, gender distribution showed a proportion of 38% females, obesity, diabetes, dyslipidemia, hypertension and MS were risk factors, cirrhosis was present in 68% of the sample, while glycated hemoglobin (hemoglobin A1C) and fasting blood glucose levels were high [23]. NAFLD-HCC was more common among older white individuals, and those with cardiovascular co-morbidities such as hypertension. Lower AFP levels (<20 ng/mL) were more common among NAFLD-HCC compared to alcoholic disease-HCC or HCV-HCC [5].

Hepatocellular carcinoma was not one of the predominant malignancies in a large sample of NAFLD patients (age ≥ 60 years). It occurred in 10 individuals (6.0% of 167 individuals with malignancies and 0.6% of 1,600 NAFLD patients). Predictive factors for malignancies in NAFLD patients were older age (≥ 70 years), smoking, low platelet count ($<150\times 10^3/\mu\text{L}$) and higher glucose levels. On the other hand, liver enzyme levels [alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ -glutamyl transpeptidase (γGT)] were not risk factors [24].

A low HCC incidence of 0.25% was observed in a retrospective cohort study in a large sample of NAFLD patients diagnosed by ultrasound (16 of 6,508 NAFLD patients) [25]. Aspartate aminotransferase ≥ 40 IU/L [hazard ratio (HR) 8.20, 95% confidence interval (CI) 2.56-26.26, $p<0.001$], platelet count $<150\times 10^3/\mu\text{L}$ (HR 8.20, 95% CI 2.26-23.26, $p=0.001$), age ≥ 60 years (HR 8.20, 95% CI 1.30-14.01, $p=0.017$) and diabetes (HR 8.20, 95% CI 1.09-9.50, $p=0.035$) were independent risk factors for HCC. Hepatocellular carcinoma was associated with significant fibrosis assessed by the AST to platelet ratio index (APRI) (equivalent to stage 3-4 NASH, HR 25.03, 95% CI 9.02-69.52, $p<0.001$) but not by the BARD score [25].

Hepatocellular carcinoma occurred in comparable numbers of males and females in a small sample of Japanese patients with histologically-proven NASH ($n=19$, 52.6% female) [17]. In this sample, HCC was diagnosed after a median follow-up of 3.8 years since NASH diagnosis, during which time the degree of fibrosis became more advanced in all patients, with

no significant change in the degree of steatosis. BMI, platelet count and ALT levels were significantly lower at the time of HCC diagnosis compared to the time of NASH diagnosis, with no differences in AST and gGT levels.

The incidence of NAFLD-HCC was 2.0% in a national survey investigating the etiology of HCC in Japan. Older age, male gender, advanced liver fibrosis, lower activity of liver histology, lower ALT and higher γ GT levels were detected as risk factors for HCC in NAFLD patients. However, advanced fibrosis remains one of the predominant risk factors for HCC development [26]. The probability of having bridging fibrosis or cirrhosis on liver biopsy is approximately 66% in patients with cryptogenic cirrhosis who are older, overweight/obese and diabetic. This may reflect referral bias to hepatologists in tertiary referral centers. NAFLD has a much higher prevalence in the elderly population. A prevalence rate of 46% for NAFLD detected by ultrasound was reported in a cohort of healthy octogenarians admitted to the rehabilitation wards of a geriatric hospital [27]. This rate was significantly higher than that found in a younger population [28].

In addition, new prognostic biomarkers include microRNAs both in NAFLD and alcoholic liver disease during the transition from simple non-inflamed steatosis to hepatocarcinoma [28, 29]. NAFLD is a common cause of cirrhosis and end-stage liver disease; it is more common in the population of elderly people than in younger groups. The challenge is to understand this paradox and the natural history of patients with NAFLD before the disease leads to HCC.

CONCLUSIONS

In people diagnosed with NAFLD/NASH, a better medical and laboratory surveillance is necessary to treat the disease in time and to avoid HCC development. There is a need for a multidisciplinary team and a more active approach to ensure specific and sensitive understanding of the clinical evolution of the disease and interpretation of the results. Monitoring the patients is the only solution to ensure an early detection and a better prognosis for NAFLD-HCC patients.

Conflicts of interest: No conflict to declare.

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