Fat and hepatocellular carcinoma

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Abstract

Obesity and diabetes are associated with the onset of hepatocellular carcinoma (HCC). These two illnesses correlate also with the development of non-alcoholic fatty liver disease (NAFLD). Currently, NAFL is considered the leading form of chronic liver disease in the Western industrialized countries. Insulin resistance is the common pathogenic factor among these three pathologies. NAFL is characterized by fat accumulation in the liver that involves greater than 5% of the liver parenchyma with no evidence of hepatocyte injury. However, NAFL may progress toward non-alcoholic steatohepatitis that in turn may lead to advanced fibrosis, cirrhosis and HCC. It is alarming that NAFLD related HCC has been, at present, considered as a growing burden worldwide, and its prevalence is tending to further increase together with the increasing incidence of obesity and diabetes. Worthy of note is that in the presence of chronic accumulation of fat in the liver it has been reported the emergence of HCC during chronic liver disease in absence of liver cirrhosis, usually the major risk factor for the development of HCC. Thus, in the future NAFLD related HCCs will place a growing strain on health-care systems from the need for their management. Unfortunately, most of the NAFLD related HCC patients are diagnosed at advanced stages and are characterized by a poor prognosis, because they are ineligible to radical treatments. Thus, it is urgent to boost up new screening policies to make early diagnoses, as well as to develop preventive-therapeutic strategies.

Keywords: Hepatocellular carcinoma, obesity, non-alcoholic fatty liver, non-alcoholic steatohepatitis, copper

INTRODUCTION

In Western countries the growing epidemics of obesity and type 2 diabetes are associated with increasing incidence of hepatocellular carcinoma (HCC)⁴. These two conditions are strictly associated with the development of non-alcoholic fatty liver disease (NAFLD) and considered the leading forms of chronic liver
disease in the Western industrialized countries[^2]. NAFLD has a wide geographic distribution to take on epidemic proportions: prevalence of NAFLD has been reported to be 25% worldwide. The highest prevalence is reported from South America (31%) and Middle East (32%), followed by Asia (27%) and USA (24%), while the prevalence is lowest in Africa (14%). In Europe the median prevalence is 23%-26% with variations in different European populations[^3].

NAFLD is caused by an insulin resistance and, as reported above, often occurs with the presence of diabetes, obesity, and metabolic syndrome; therefore, liver can be considered the alarm bell of all of these pathologies[^4]. The progressive form of NAFLD is non-alcoholic steatohepatitis (NASH) that can lead to advanced fibrosis, cirrhosis and HCC. It is worrying that NAFLD related HCC has been at present recognized as a growing burden worldwide, and its impact is expected to further grow together with the increasing incidence of obesity and diabetes[^4].

Of particular interest is the emergence of HCC during chronic liver disease in absence of liver cirrhosis, that is known as the major risk factor for HCC development[^5,^6].

HCC development requires decades and is characterized by a gradual transition through a dysplastic to transformed liver tissue[^7]. Liver transformation is the result of uncontrolled cell growth that results in the accumulation of genomic alterations occurring during cell division thus becoming the driving force for tumorigenesis. Five mechanisms are involved in maintaining genomic stability during cell division: (1) high-fidelity of DNA replication in S-phase; (2) precise distribution of chromosomes in daughter cells during mitosis; (3) DNA repair throughout the cell cycle; (4) cell cycle checkpoints; and (5) induction of apoptosis or senescence in case of genomic instability[^8]. On the other hand, there are multiple oncogenic mechanisms that participate in genomic instability: alterations in the DNA-damage-response pathways, telomere erosion, chromosome segregation defects[^9].

Even if several pathogenic mechanisms, such as obesity-mediated chronic inflammation and diabetes, have been described to be involved in NAFLD related HCC till now, as extensively reported below, we do not have clear ideas on the pathogenic mechanisms driving transformation of the cell during NAFLD[^10,^11]. In this context, low grading chronic inflammation has indubitably a crucial role in NAFLD disease progression toward HCC[^12]. During low grade inflammation, the overproduction of reactive oxygen species (ROS) induces the output of advanced glycation end-products (AGEs), advanced lipoxidation end-products (ALEs) and protein oxidation products (PrOPs) in tissues[^13,^14], inducing pro-inflammatory cascades and increasing the risk of liver tissue transformation. Thus, to better understand the pathogenic mechanisms underlying NAFLD related HCC, first of all, we should better know all the biological factors involved in promoting inflammation that consequently participate in hepatocarcinogenesis.

Further studies should be performed to highlight new insights in the pathogenesis of HCC during NAFLD. However, scientific consensus exists a on the concept that the progression of NAFLD toward HCC is surely linked not only to environmental but also genetic factors. Accordingly, genome-wide association studies highlighted several single nucleotide polymorphisms (SNPs) associated with the pathology of NAFLD [Table 1]. Furthermore, induction of epigenetic alterations due to unhealthy diet and/or other environmental factors are surely involved in NAFLD related HCC.

Perspective studies are needed to implement screening strategies and preventive approaches for NAFLD-related HCC development, particularly in the non-cirrhotic population. The notions reported in this review, describing several NAFLD-related molecular target pathways, will be useful to clinicians to outline diagnostic and prognostic profiles of these complex and heterogeneous patients.
Table 1. Single nucleotide polymorphisms associated with the pathology of nonalcoholic fatty liver disease

<table>
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<tr>
<th>Gene</th>
<th>SNP</th>
<th>Region</th>
<th>Location</th>
<th>Functional class</th>
<th>Total allele frequency (Gnomad)</th>
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**EPIDEMIOLOGY**

HCC causes more than 700,000 deaths/year worldwide and accounts for 70%-85% of cases of liver cancers. HCC is the fourth most often diagnosed cancer in males (70% occur over age 50) and the seventh in females; moreover, it represents the overall second cause of cancer deaths. These statistics reflect the poor prognosis of liver cancer worldwide.

About 80% of HCC cases occur in less developed countries and are typically associated with alcohol, chronic hepatitis B (HBV) and C (HCV) infections: importantly, the incidence in these countries is decreasing. On the other hand, in western countries the HCC incidence is increasing, ranging from 2.4% over 7 years to 12.8% over 3.2 years of median follow-up period, following the geographic distribution of obesity. In particular, 10 year annual cumulative risks of HCC in alcohol, HCV or NAFLD are 1.1%, 2.9% and 3.1%, respectively.
respectively. Accordingly, an increasing number of HCC has been reported in the setting of obesity and diabetes and it has been associated with an increased relative risk of dying for HCC.

Unfortunately, even if consistent epidemiological data concerning viral and alcoholic hepatitis have been reported, there is a lack of strong epidemiological results regarding the incidence and prevalence of NAFLD-related HCC. The problem is mainly due to the absence of a correct and clear definition of NAFL/NAFLD/NASH. Thus, so far we cannot evaluate the real dimension of NAFLD-related HCC and how to lower and prevent its appearance.

A few longitudinal outcome studies reveal that the cumulative mortality in NAFL/NASH, in a follow-up period between 5.6 and 21 years, vary from 0% to 3%, but we have to take into account that there are 400,000 and 40,000-80,000 new cases/year of NAFL and NASH, respectively.

Finally, the unquestionable evidence showing the increased risk of HCC in patients with NAFLD, and mainly its appearance in non-cirrhotic patients, is in close association with the alarming and more rapidly increasing indication for liver transplantation in respect to any other liver disease.

PATHOGENESIS

The aberrant activation of immune response and inflammation signaling observed in NAFLD have a key role in the pathogenesis and progression of this liver disease.

The accumulation of lipids in patients with NAFLD may induce an intracellular chronic status of oxidative stress that, in turn, leads to the activation of low-grade inflammation. The enlargement of adipocytes may lead over time to the rupture of these cells. As a consequence, macrophages are recruited in the site of inflammation and M1/M2 macrophage polarization is induced. The activation of macrophages stimulates the production of adipose tissue related adipocytokines, that, once released in the systemic circulation, reach different organs, including liver. The inversion of M1/M2 ratio is due to the increase of M1 macrophages and reduction of M2 macrophages. The higher number of M1 cells cause an over production of several pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8, IL-12, and TNF-α.

Consequently, the serum of NAFLD patients is characterized by the presence of high levels of TNF-α and IL6, that in turn are correlated with a higher risk of progression to NASH. Moreover, higher levels of TNF-α induce insulin resistance and contribute to exacerbate the liver damage through the activation of nuclear factor-kappa-B (NFkB) inflammatory pathways. Furthermore, NFkB protein has been recently found to be involved in the regulatory feedback of two important chemokine receptors: C-X-C chemokine receptor type 4 and 7 (CXCR4/7). The NFkB-CXCR4/7 axis mediates the signaling of toll-like receptors, TLR3 and TLR4, promote, in this way, the progression of NASH towards HCC.

Thus, deeply understanding the role of chronic inflammation as underlying the cause of liver transformation will improve the prevention and cure of this cancer.

An incorrect lifestyle is currently considered the main predisposing factor of NAFLD-related HCC. In fact, the development of HCC in NAFLD includes low-grade chronic inflammatory response (NASH) associated with genetic alterations, oxidative stress, obesity, insulin resistance and alteration of gut microbiota. The pathogenic mechanisms involved in the progression of NAFL toward NASH are characterized by two hits: excess accumulation of triglyceride (TG) in the hepatocyte and, in a second moment, induction of oxidative stress and inflammation by several factors, such as free radicals. In line with these findings, more and more researchers are recognizing the central role of low-grade inflammation in inducing all of the...
NAFLD-related comorbidities, such as insulin resistance, diabetes, and cardiovascular disease. Accordingly, NASH is a recognized cause of cirrhosis and is associated with development of HCC \[38\].

In this context, it is worth mentioning that multiple additional mechanisms may be implicated in the progression from NAFLD to NASH and HCC. In fact, keeping in mind that there are a growing number of patients who can progress from NAFLD to advanced fibrosis in the absence of significant inflammation, the alterations in immunologic, endocrine and metabolic pathways have a key role in the progression of NASH toward HCC.

Accordingly, despite the few data on NAFLD-related hepatocarcinogenesis, it has been highlighted that the phosphoinositide 3-kinase (PI3K)-AKT-mTOR pathway, implicated in the control of cellular energetic homeostasis, is deregulated in over 50% of NAFLD-related HCCs \[39\].

The β-catenin/WNT signaling, that has a crucial role in cell proliferation, stem cell self-renewal and cell migration, was found affected by somatic mutation in > 37% of NAFLD-related HCC \[39\].

Below we reported a detailed description of some factors involved in HCC development in patients with NAFLD.

Regarding the genetic factors involved in the progression from NAFLD to HCC, recent genome-wide studies have highlighted genetic heterogeneity of liver cancers. Of note, some SNPs, such as Patatin-like phospholipase domain-containing 3 (PNPLA3) gene variant I148M, have been related to the development and progression of NAFLD, NASH and NAFLD-related HCC, whereas others, such as the transmembrane 6 superfamily member 2 (TM6SF2) gene variant E167K, have been mainly correlated with the development of cardiovascular diseases \[5,40,41\]. In this context, the most recent findings from genomic profiling let us better understand that different pathways are involved in the initiation and progression of liver cancer \[42\], as shown in Figure 1.

In addition, altered transcriptional gene expression might be linked to inappropriate microRNAs (miRNAs)-guided transcriptional control. The human genome is envisaged to encode approximately 1000 miRNAs \[43\], which are a perfect class of blood-based biomarkers for cancer detection \[44\]. MiRNAs are endogenous 19-24 nucleotides noncoding single-stranded RNAs, which control, at post-transcriptional level, many complementary target mRNAs implicated in several pathophysiological processes, such as cell proliferation, differentiation, metabolism, apoptosis and cancer \[45\]. Lack of miRNA processing enzymes in cancer cells promotes tumor invasiveness and more aggressive phenotypes, revealing their main role in controlling tumor- and metastasis-initiating events \[46-48\]. Accordingly, different sets of miRNAs have been specifically correlated with NAFLD, NASH and HCC [Table 2] \[49-67\]. Among the miRNAs recently identified in NAFLD patients, it is worth mentioning the up-regulation of miR-146b-5p, miR-181b and miR-375, and the down-regulation of miR-29a, miR30b-5p, miR-122-5p, miR-139-5p, miR-155 and miR-422a \[45,53-56\]. In addition, in NASH it has

### Table 2. Summary of miRNAs significantly associated with NAFLD, NASH and HCC patients

<table>
<thead>
<tr>
<th>Disease</th>
<th>Upregulated miRNAs</th>
<th>Downregulated miRNAs</th>
<th>References</th>
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<td>NAFLD</td>
<td>miR-21, miR-34a, miR-122 (serum), miR-146b-5p (tissue), miR-181b, miR-451</td>
<td>miR-29a, miR-139-5p, miR-30b-5p, miR-122-5p (tissue), miR-155, miR-422a, miR-181d, miR-99a, miR-197, miR-146b (serum)</td>
<td>[49-56]</td>
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<td>NASH</td>
<td>miR-21, miR-33a, miR-34a, miR-122, miR-144, miR-192, pri-miR-26a-1/2</td>
<td>miR-125b, miR-451</td>
<td>[50,51,57-60]</td>
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<td>HCC</td>
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<td>let-7f, miR-16, miR-21 (serum), miR-24, miR-30e, miR-99a, miR-106b, miR-125b, miR-145, miR-146a, miR-148a, miR-155, miR-183, miR-199a, miR-199a3p, miR-200c, miR-215, miR-223, miR-229, miR-7706</td>
<td>[50,61-67]</td>
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</table>

HCC: hepatocellular carcinoma; NAFLD: nonalcoholic fatty liver disease; NASH: nonalcoholic steatohepatitis
been reported the up-regulation of miR-33a and miR-144 and the down-regulation of miR-451. Finally, in NAFLD-related HCC the up-regulation of miR-10a, miR-33a, miR-144, miR-155, miR-183, miR-375 and miR-423 and the down-regulation of miR-229 and miR-7706 were found.

MiRNA analyses, in combination with other clinical parameters and standard liver examinations, may be extremely useful to predict the possible progression of NAFLD toward HCC, and for monitoring the response to treatments. However, despite the association between definite miRNA signatures and pathogenesis of NAFLD-related HCC, the expression levels of specific hepatic miRNAs during liver tissue transformation are still controversial. Further studies are needed to shed light on their function in the context of NAFLD-related HCC.

Regarding oxidative stress, reactive oxygen species (ROS) have a central role in HCC onset. ROS, in fact, are noticeable factors playing an essential role in regulating cell homeostasis. In this regard, our group has recently highlighted the role of altered systemic biometals distribution in NAFL/NASH patients and the associated increasing levels of ROS. Accordingly, toxic biometal accumulation is a common feature in many cancers. Moreover, perturbations of mechanisms that control transcripts encoding proteins that regulate biometals have been described in cancer cells, including differences in epigenetic control (methylation and acetylation), miRNAs expression and protein activities.
In particular, biometals have the ability to catalyze oxidation-reduction reactions, which can lead to the production of ROS, thus their tight homeostatic regulation should be always present in the body. Accordingly, although the mechanisms are at present still unclear, a contribution of iron (Fe), copper (Cu) and zinc (Zn), in the development of HCC, has been often suggested. In fact, dysregulation of Fe, Cu or Zn homeostasis stimulate proliferation, modulate the expression of epithelial mesenchimal transition (EMT) related proteins, glycolysis, and antioxidant molecules, such as SOD1 and 2, HIF1, GSH, in various cancer cells and human tumors.

Accordingly, NASH and NAFL patients display higher iron absorption after the administration of an oral iron absorption test and its deposition has been related to HCC development in NAFLD-cirrhosis. The underlying mechanisms are not already clear, but might be related to oxidative DNA damage. Interestingly, a recent meta-analysis highlighted that HFE mutations C282Y and H63D, associated in homozygosity with hemochromatosis, were characterized by a higher risk of HCC in NAFLD patients. In addition, our group highlighted that a statistical significant enhancement of serum copper levels has been reported in NAFLD-cirrhotic patients and the altered homeostasis of this biometal was even more evident in HCC patients. In the presence of higher concentrations of extracellular copper liver cells are sensitized to transformation. The pathogenic copper-related pro-oncogenic mechanism seems to be, at least in part, managed by MYC, which is able to directly bind a specific region of the CTR1 promoter, regulating its transcription. In this regard, it is really interesting the recent study reporting that Golgi protein 73 (GP73) is an effective and reliable serological marker for the diagnosis of advanced fibrosis and prediction of appearance of cirrhosis. The awareness that copper serves as a limiting factor for multiple aspects of tumor progression, including growth, angiogenesis and metastasis suggests more attention to be paid to the potential and undiscovered role of copper-specific chelators as effective therapeutic agents against HCC.

The prevalence of obesity is increasing worldwide as well as the link between obesity and cancer, becoming an important and accepted risk factor for the development of HCC. As reported above, it is currently accepted that NAFLD is caused by an insulin resistance and often appears in the presence of obesity. The relationship between obesity and HCC was supported by a cohort study in Italy. In this work the odds ratio progressively increased in the patients who have associated metabolic syndrome factors. Obesity is characterized by the excess of adipose tissue and the altered secretion of adipocytokines that correlate with the occurrence of HCC and liver-related death in patients with cirrhosis. In the last decade, it has become evident that obesity-related metabolic inflammation is involved in different aspects of HCC progression and metastatic dissemination, among which: neural regulation, innate immune responses, intestinal immune system and endocrinal regulation. Unfortunately, only few studies have been focusing on long-term mechanisms involved in obesity related HCC development, thus prospective studies are needed.

Finally, alterations in intestinal microbiota (or dysbiosis, defined as any change in the composition of the microbiota commonly found in healthy conditions), creating a pro-inflammatory microenvironment in the liver, seem to play a main role in the development of NAFLD-related HCC. Dysbiosis, beyond the known risk factors for NAFLD, promotes the development of chronic liver diseases and HCC, independent of body mass index (BMI) and insulin resistance, producing a large amount of bioactive molecules, which deeply affect physiological and pathological body status. Interestingly, in a mouse model, drugs able to modify the microbiome (e.g., rifaximin) may prevent HCC development. Rifaximin may additionally improve portal hypertension, spontaneous bacterial infection (SBP) risk, liver fibrosis and hepatic encephalopathy. Actually, metabolic alterations have been associated with dysbiosis: ob-ob mice (homozygous for the obese mutation) have an imbalance of the intestinal microbiota with a decrease of Bacteroides and an increase in Firmicutes. The pattern of intestinal bacteria has the increased capacity to harvest energy from diet, as well as the microbiota composition described in NAFLD. The altered microbiota (the genetic information genomes of gut microbiota) is characterized by the ability to produce alcohol, which in turn will be increased in the
blood promoting hepatic oxidative stress and liver inflammation. As demonstrated in obese patients, the equilibrium can be restored in case of a fat restriction diet.

Target/biomarker discovery and “Omic” approaches will help in finding new pro-oncogenic and oncosuppressor to be used as novel biomarkers. The new knowledge on HCC pathogenesis will open new avenues in the diagnosis and design of patient-tailored therapies.

HCC IN NAFLD CHRONIC HEPATITIS

NAFLD has a proportion of HCC, occurring in the absence of cirrhosis, higher than other chronic liver diseases. HCC in NAFLD generally lacks encapsulation and is well differentiated and characterized by large dimensions. Multiple studies described a significant proportion of HCC (from 51% to 65%) that have stage 0-2 fibrosis, highlighting a specific dangerous behavior of NAFLD chronic hepatitis. Given the high number of patients with non cirrhotic NAFLD, screening for HCC in this population is not practicable. Interestingly, the features of NAFLD-related HCC are similar to those of HCC of obese patients and of non-cirrhotic HCC, independently of the etiology. Accordingly, it has been reported that obese patients have a relative risk of liver cancer of 189% relative to the 117% of overweight subjects. Thus, the pathogenic mechanisms of hepatocarcinogenesis in steatosis might be different from the classic mechanisms involved in cirrhosis. In fact, all the NAFLD-related HCC pathogenic mechanisms are independent from fibrosis and this might explain the particular epidemiology of HCC in NASH, where non-cirrhotic HCC is quite frequent relative to other etiological factors.

In the light of what has been reported above, pathophysiological studies are needed to better understand the underlying mechanisms involved in NAFLD-related HCC development. In this context, it is important to note that the EASL evidence based clinical practice guidelines should be improved because the up-to-date version does not exhaustively represent this specific problem.

HCC IN NAFLD CIRRHOSIS

Cirrhosis in NAFLD modifies prognosis and management. Increasing age, obesity and diabetes are considered as risk factors for the progression of NAFLD to cirrhosis. Thus, it is well known that a subset of individuals with NAFLD may progress to liver cirrhosis, which in turn could be complicated by liver failure or even HCC, requiring liver transplantation (LT), resection, or loco-regional therapies.

However, although NAFLD has begun the most common cause of chronic liver disease worldwide, even today, a significant amount of patients with NAFLD are already incidentally diagnosed with cirrhotic. Unfortunately, NAFLD patients are asymptomatic, thus, the diagnosis of cirrhosis often occurs incidentally because it is done during clinical assessments for the investigation of different medical conditions unrelated to liver disease or an unexpected surgical finding. Accordingly, about the 15% of NAFLD patients selected for biopsy have cirrhosis, confirming that the prevalence of cirrhosis in patients with NAFLD is higher than expected. In the presence of liver cirrhosis, the main problem is the occurrence of important complications, such as: liver decompensation, thrombocytopenia, splenomegaly or, sometimes, HCC related with a poor survival. Late diagnosis increases the risk to find a late stage HCC, no longer curable with the available treatments, whereas the diagnosis of HCC, if done at the early stage, is associated with better results.

Cirrhosis has to be seen as a prognostic factor predicting negative outcomes in patients. Accordingly, in recent studies, it has been reported in NAFLD cirrhotic patients an overall mortality of 80% and a liver-related mortality of 55%, after 12 years.
Early recognition of NAFLD patients with cirrhosis, who have a higher risk of progression toward HCC, is the first crucial aim to reduce NAFLD-related morbidity and mortality. Thus, in patients with NAFLD, an improvement of diagnostic approach alertness is required for underrating the prevalence and the important clinical condition of NAFLD. Clinicians have developed adequate screening. Finally, it is important to underline that ultrasonography (US) is likely inadequate in several subgroups of patients (obese, Child Pugh B or C, alcohol and NASH related cirrhotic) and does not permit the exclusion of the presence of HCC.

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Authors’ contributions
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