Hepatoma Research

Review

Mechanisms and clinical behavior of hepatocellular carcinoma in HBV and HCV infection and alcoholic and non-alcoholic fatty liver disease

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Abstract

Hepatocellular carcinoma (HCC) is the main tumor of the liver and is the sixth most frequently diagnosed tumor in the world. It is the evolution of chronic hepatic injury secondary to different etiologies. Chronic hepatitis B virus and hepatitis C virus infection, chronic alcoholic hepatitis, as well as non-alcoholic fatty liver disease are the most common causes behind the development of HCC. The introduction of effective prophylaxis and treatment against hepatitis B, the recent use of highly effective hepatitis C treatments, as well as lifestyle changes observed in recent decades in the general population causing an increase in obesity and metabolic syndrome have led to significant epidemiological change in HCC in relation to the changed etiologic prevalence of liver injury. Increasing evidence was emerging, emphasizing how the development of HCC is a complex and multifactorial process. The knowledge of the molecular mechanisms involved is important for the understanding of the basic factors of the development of hepatocarcinogenesis and of possible therapeutic approaches. Several pathogenic mechanisms and clinical expression of HCC occur in relation to the different etiologies of the underlying liver disease. The different clinical behavior of HCC often makes diagnosis difficult at an early stage, that is necessary for an effective therapeutic approach. This review analyzes the possible different pathogenic mechanisms involved in the development of HCC and emphasizes the different epidemiological and clinical aspects of HCC observed in the most common forms of liver diseases of viral and non-viral origin.

Keywords: Hepatocellular carcinoma, hepatitis C virus, hepatitis B virus, alcohol hepatitis, non-alcoholic fatty liver disease

INTRODUCTION

Liver cancer is the fourth leading cause of cancer-related death in the world, with about 810,000 deaths annually[1]. It has a high incidence rate and is the fifth most commonly diagnosed cancer in males and the ninth in females[2]. In addition, unlike other more common neoplasms that have a downward trend in incidence, the rate of incidence of liver cancer appears to be increasing[3]. Hepatocellular carcinoma (HCC) is the most common liver cancer histotype, accounting for 80% of the liver cancers[4].

At present the main causes of HCC are viral infections such as hepatitis B virus (HBV) and hepatitis C virus (HCV) and alcohol abuse, but the obesity and the metabolic syndrome epidemic that is occurring in Western countries is leading to a significant increase in HCC secondary to non-alcoholic fatty liver disease (NAFLD)[1,5]. Chronic HBV infection is associated with about 33% of total deaths observed for HCC, while 30% is associated with alcohol abuse, 21% with chronic HCV infection and 16% with the remaining etiologies, including the ever-increasing metabolic etiology[1]. In fact, widespread lifestyle changes and the pandemic of the metabolic syndrome are causing a significant increase of about 9% per year in the incidence rates of NAFLD-associated HCC. Looking to the future, particularly in industrialized countries, this last condition could become the main factor of HCC causing an important epoch-making change between metabolic and viral forms[6].

The pathogenic mechanisms underlying the development of HCC, as well as the epidemiology, the clinics and the underlying diseases from each etiology are extremely dissimilar and explain the heterogeneous clinical impact of HCC. The different clinical behavior of HCC often makes diagnosis difficult at an early stage that is necessary for an effective therapeutic approach. Increasing evidence is emerging, emphasizing how the development of HCC is a complex and multifactorial process. The comprehension of the molecular mechanisms involved is important for the understanding of the basic factors of the development of hepatocarcinogenesis and of possible therapeutic approaches.

This review aims to define an updated clinical picture of HCC, its epidemiological changes and, above all, to highlight the differences in the pathogenic mechanisms related to each single etiology associated with HCC. Table 1 summarizes the main characteristics of HCC in relation to the different etiological association.

The common denominator in the pathogenesis of HCC

Regardless of etiology, any chronic hepatitis can alter the balance of the immune system causing a low-grade chronic inflammation that leads to the creation of reactive oxygen species (ROS), as well as the induction of cell proliferation and the onset and progression of liver fibrosis. A high turnover of hepatocytes exposes the patient to a higher rate of genetic alteration, such as point mutations, chromosomal abnormalities or epigenetic alterations, whose accumulation represents the first phase of hepatocarginogenesis. In addition, there are specific risk factors of the host such as diabetes mellitus and the male sex, and factors related to the etiological agent that increase the oncogenic potential of the inflammatory liver disease, thus causing the development of HCC, either in the presence or absence of significant hepatic fibrosis[7].

Irrespective of etiology, cirrhosis of the liver is an already pre-malignant condition that promotes the development of genetic aberrations and cellular transformations. In fact, the chronic hepatic inflammatory state and the accelerate hepatocyte turnover observed in cirrhosis promote the accumulation of genic mutations. The subsequent uncontrolled proliferation and the high rate of genetic errors will lead to the development of HCC.

HCC and HBV chronic infection

Clinical and epidemiological factors affecting development of HBV-related HCC

Currently, chronic HBV infection is responsible for about half of all observed HCC cases[8-11]. It has been estimated that HCC occurs 10-25 times more frequently in patients with positivity for HBV than uninfected

Table 1. Main features of HCC in relation to different etiologies

HCC	Related to			
Features	**HBV**	**HCV**	**ALCOHOL**	**NAFLD**
Annual Incidence	Non-Cirrhotic: 0.3%-0.6%	3%-7%	1.2%-5%	NAFLD: 0.044%
	Cirrhotic: 2.2%-3.7%	Increasing	Stable	NASH: 0.529%
	Low Increasing			Increasing
Gender Prevalent	Male	Male	Female	Indifferent
Onset stage of liver disease	Chronic hepatitis Cirrhosis	Cirrhosis	Cirrhosis	Chronic Hepatitis Cirrhosis
Carcinogenic factors	Viral factors	Viral factors (Genotype-related)	Direct (dose-related)	Inflammation
	Inflammation	Inflammation	Inflammation	Oxidative stress
	Oxidative stress	Oxidative stress	Oxidative stress	Lipid peroxidation
	Cirrhosis	Cirrhosis	Endotoxinemia	Mitochondrial damage
	Immunologic		Low Vitamin A levels	Endotoxinemia
	Genetic polymorphisms (DCL1, TGF-β1, STAT4, TPTE2, CTL-4, MDM2, among Asians)		Cirrhosis	Cirrhosis
Co-factors	Viral co-infection (HIV, HDV, HCV)		Genotoxicity	
	Diabetes	Viral co-infection (HBV, HIV)	Diabetes	Diabetes
	Alcohol	Diabetes	Obesity	Metabolic syndrome
	Tobacco	Metabolic syndrome	Genetic polymorphisms (PNLA3, TM6SF2)	Leptin/adiponectin imbalance
		Obesity		Genetic polymorphism (PNLA3)
		Alcohol		
		Iron overload		
Related-death of total	33%	21%	30%	16%
Effect of anti-viral treatment on incidence	Reduced	Reduced		

individuals[12]. HBV-related HCC is predominantly observed among males (203,000 new cases in 2015 versus 70,000 in females), with a male-to-female ratio of about 3 to 1 and is also responsible for one-third of HCC-related deaths[1]. In addition to epidemiological factors, the significant disparity in incidence between the two sexes is also determined by the different hormonal pathways. Androgens stimulate virus replication and transcription in males causing a higher viral load which is associated with an increased risk of occurrence of HCC[13]. In fact, high HBV-DNA serum levels have been reported to be associated with a nonlinear dose-response to a higher incidence and recurrence of HCC[14]. Furthermore, it has been reported that estrogens appear to act as protectors towards the development of HCC[15].

HBV-related HCC shows a tendency to occur at all stages of the natural history of chronic HBV hepatitis and not only in cirrhosis as in most cases seen during chronic HCV and alcoholic hepatitis. Accordingly, up to a third of patients develop HCC on a non-cirrhotic liver[12].

An additional factor associated with an increased risk of developing HCC is the duration of the disease[16]. The chronic inflammatory state and hepatic oxidative stress induced by chronic HBV infection accelerate cell senescence processes. The expression of aging processes is expressed at the genomic level in the form of shortening of telomeres, whose length is inversely proportional to the degree of fibrosis and reaches the lowest values in HCC[17]. Although cellular senescence is a protective mechanism itself, since it limits proliferation and reduces the risk of carcinogenic transformation, it has been shown that telomerase activity persists at high levels in 80%-90% of HCC cases thus emphasizing the development of escape mechanisms from the protective phenomena of cellular aging[12,18].

It has been shown that some co-factors associated with chronic HBV infection such as diabetes mellitus, alcohol consumption or tobacco use, as well as exposure to carcinogens (e.g., aflatoxin) may act in synergy with the virus in determining an early onset and a more rapid progression of HCC[19,20]. In particular, the

risk of developing HCC has been reported to be 6-, 5- and 4-fold higher in the case of concomitant use of alcohol, tobacco and in the presence of obesity, respectively[21].

The presence of HBV coinfection with HIV or HDV or HCV causes a more rapid progression of liver disease and a significant higher occurrence of HCC even in the early stages of the disease[22,23].

HBV genotype appears to significantly influence the appearance of HCC. In particular, it has been reported that HBV genotype C, which has a high prevalence in Southeast Asia, is associated with a higher risk of HCC compared to other genotypes[24,25]. It has been shown that HBV genotype C most frequently causes double helix breaks in the host genome, induces a greater stress of the endoplasmic reticulum through the accumulation of ROS and causes a greater number of chromosomal rearrangements that can promote carcinogenesis as better described below[26].

As mentioned above, high viral loads, as well as a seropositive status of HBe antigen (HBeAg) are associated with a higher risk of developing HCC[14]. It has been reported that HBeAg seropositive patients has a relative risk of HCC of 60.2 compared to 9.6 of those seronegative[27]. It has also been shown that high levels of HBV-DNA are closely linked to a high probability of developing HCC[28].

A genetic predisposition to the development of HCC during HBV infection has been reported among South-Asians. Single nucleotide polymorphisms (SNPs) at the level of several genes, e.g. DCL1, TGF-β1, STAT4, TPTE2, CTL-4, MDM2 have been associated with the development of HCC[12,29-33]. It is unknown, however, if these data may be extended to other ethnicities.

Pathogenic mechanisms of HBV-related HCC

HBV can cause the onset of HCC through direct and indirect mechanisms. The direct carcinogenic effect of HBV derives from its ability to integrate its own genome into that of the host, altering chromosomal stability and triggering various oncogenic mechanisms. In the early stages of the natural history of infection, HBV-DNA is converted into a covalently closed circular DNA form (cccDNA)[34] that allows the virus to persist in the infected cell nucleus and acts as a reserve for viral genome replication[12]. Although viral integration is more likely to occur randomly, whenever it occurs at the level of specific sites in the host genome, which are either close to the genes involved in cell cycle regulation and proliferation or those involved in cell survival mechanisms, can allow the clonal expansion of cells[35,36]. This mechanism is also responsible for the constant expression of viral oncogenic proteins such as HBx or preS/S polypeptides, which over time may lead to alterations in the control of cell transcription and proliferation[12]. The fact that such integration is more commonly seen in cancer tissue than in adjacent liver tissue (86.4% and 30.7%, respectively) seems to be an evidence of the central role played by viral genome integration in the development of HCC[37]. In this context, the transcription of a chimeric gene (viral/human) called HBxLINE1 has recently been identified and has been found in about a quarter of patients with HCC and associated with a worse prognosis[38].

In addition to the integration mechanisms of the viral genome, specific mutations in the *X regions, pre-core, core-promoter* and *pre-S* may increase the risk of developing HCC[39,40]. Among the most frequent mutations at the nuclear promoter level, the double mutation *A1762T/G1764A* is closely related to the probability of developing HCC. The presence of this mutation may represent a potentially risk-predictive biomarker for HCC as its presence may be evident many years before the development of HCC[41]. Several other potentially oncogenic mutations have been identified in the *pre-S* region. The combination of *pre-S C1653T, C1653T + T1753V* mutations and the aforementioned *A1762T/G1764A* have a specificity greater than 80% in the prediction of HCC development[42]. HBV-infected patients with mutations in the *pre-S* region have a 3.8-fold higher risk of developing HCC than non-mutated virus infection. These mutations are present in about 60% of cases of HCC and may alter the protein expression of the viral envelope. The accumulation of surface

proteins mutated at the endoplasmic reticulum may be able to induce the formation of ROS, with consequent oxidative stress to the host DNA and induction of the hepatocyte transformation[12,43-45]. Mutations in the *S region* can also contribute to the development of HCC. In this regard, it has recently been shown that a non-sense mutation at position 172 or 182 of viral genome can contribute significantly to the progression of liver disease, and in particular the sW182 mutation was found to be related to the development of HCC[46].

In addition to the structural proteins, the viral genome also codes for HBx proteins that is involved in the transcription mechanisms of cccDNA and viral replication and seems closely related to the oncogenicity of HBV[35,47,48]. HBx appears to be able to cause chromosomal instability affecting the mitotic checkpoints, cell proliferation through stimulation of CREB genes, inhibition of apoptosis through interaction with p53, promotion of neoangiogenesis through stimulation of vascular endothelial growth factor and angiopoietin 2 (ANG2) and induction of cell migration phenomena inducing the matrix metalloproteinase 3 and 9 expression[49,50]. Accordingly, HBx appears to be a crucial point of the oncogenic power of the virus, as well as in promoting invasiveness and the ability to metastasize of HCC[35]. HBx, among other things, inhibits senescence mechanisms through inhibition of p53 and inactivation of the suppression factors of cancer[51].

A great research interest is growing on the effects of viral protein expression such as wild type and HBx mutant, envelope and core proteins on different transcription and signaling pathways such as Wnt/β-catenin, TGF-β, NFkB, Raf/MAPK, P53 and ROS involved in the pathogenesis of HCC related to HBV. The beta-catenin pathway regulates multiple cellular processes and plays an important role in hepatocarcinogenesis and in progression from chronic inflammation to HCC[52]. Mutations in the *CTNNB1* gene (catenin beta 1) may activate the Wnt/β-catenin pathway and lead to the accumulation of β-catenin in HCC. The Wnt/Î²-catenin pathway is a potential promising target for future molecular HCC therapies[53].

Effect of Immune-tolerance phase of HBV infection and occult HBV on development of HCC

The immune tolerance phase of HBV infection is characterized by a high level of viral replication in the absence of significant cytolytic activity. These patients have been defined to be at low risk of disease progression, so, at present, there is no indication for antiviral treatment[54,55]. However, recent studies have questioned this principle by demonstrating high levels of chromosomal integration and clonal expansion of the viral genome and hepatocytes, emphasizing that carcinogenesis may also occur at this stage and in the absence of cytolytic activity[56]. In addition, a prospective study showed that the estimated cumulative incidence of HCC over a 10-year follow-up period is significantly higher in the immune tolerant group than the active immune group (12.7% *vs.* 6.1%, respectively)[57]. Furthermore, data from a recent study have demonstrated the benefits in terms of clinical outcomes such as the development of cirrhosis and HCC of antiviral treatment even during the immune tolerance phase[58]. Therefore, it was hypothesized that HBV positive patients not treated in the immune tolerance phase may be at a higher long-term risk of HCC[57]. On this basis it was suggested that the immune tolerance phase should not be more considered a "benign" condition and that the levels of HBV-DNA rather than alanine aminotransferase (ALT) values should be considered when estimating the risk of occurrence of HCC[58,59]. Further study and consensus will be needed to define this important aspect.

A special mention must be made for the so-called occult HBV infection (OBI), a condition in which HBV-DNA is detectable in the liver and possibly in the serum at low levels in the absence of HBsAg in the serum. Several studies point out that the OBI can be a hazardous condition for the development of HCC[60,61]. In these patients many of the above-described oncogenic mechanisms associated with HBV remain active. Recent evidence has shown that an OBI condition was present in 75% of HBsAg negative HCC cases, underlining the possible OBI role in HCC genesis[62]. Furthermore, a condition of occult infection that increases the risk of developing HCC seems to persist longer in the neoplastic tissue itself than in adjacent tissue[61,63]. It has also been shown that the risk of developing HCC is significantly higher in patients with

HCV-related cirrhosis and concomitant OBI carrier status than in negative OBI patients[64]. Therefore, regardless of the etiology, in patients with chronic hepatitis the presence of OBI may represent a significant co-factor for the development of HCC.

Effect of HBV treatment on development of HCC

It is well known that the long-term suppression of viral replication through the nucleos(t)ide analogues reduces but does not eliminate the risk of HCC. Several large patient case studies have shown that HCC incidence rates have significantly decreased in patients undergoing treatment[65,66]. It has recently been confirmed that the reduced incidence of HCC in patients receiving antiviral treatment is independent of age, sex, HBeAg status, cytolysis level and the presence of cirrhosis[67]. Treatment does not appear to have a significant clinical impact on patients with low levels of viremia (HBV-DNA < 2000 IU/mL)[67]. A large retrospective study of non-cirrhotic positive HBV patients showed that the incidence of HCC is significantly lower in patients receiving antiviral therapy regardless of the levels of ALT[68]. In addition, the required number of patients to be treated (NNT) to prevent 1 case of HCC 10 years after initiation of treatment was found to be similar both in the group of patients with ALT < 2 ULN (NNT = 14) and in those with ALT ≥ 2 ULN (NNT = 15)[68]. These data appear to confirm that hyper-ALT should not be considered a necessary requirement for antiviral treatment in patients with HBV-DNA > 2000 IU/mL[69].

HCC and HCV chronic infection

Clinical and epidemiological factors affecting development of HCV-related HCC

Chronic HCV infection is the third leading cause of HCC and accounts for about one-third of total incidence rates and one-fifth of HCC-related deaths[1]. In recent years, the incidence of HCV- related HCC has undergone the greatest increase compared to that associated with other etiologies[1]. The risk of developing HCC in the course of chronic HCV infection increases in proportion to the degree of hepatic fibrosis. In fact, most cases of HCV-related HCC occur during an established cirrhosis, suggesting that cirrhosis-mediated carcinogenesis may play a primary role in the development of HCC[70]. In patients with HCV-associated cirrhosis it is estimated that the annual incidence rate of HCC is between 3% and 7%[71,72]. The incidence of HCC is significantly higher among elderly patients (> 60 years) perhaps also due to the fact that the progression of fibrosis is related to the duration of the disease[73,74]. Compared to HBV infection or NAFLD, HCC related to HCV infection shows a tendency to appear in a more advanced phase of liver disease[75]. In addition to cirrhosis, other factors such as diabetes mellitus, metabolic syndrome, fatty liver disease and obesity are associated with a higher risk of developing HCC[76-78]. The presence of HBV or HIV coinfection, alcohol abuse or iron overload are additional risk factors for hepatocarcinogenesis in HCV-induced cirrhosis[9,22,79].

The role played by serum HCV-RNA levels in the development of HCC is controversial[80-82]. Lee *et al.*[80] in a large series reported that elevated serum HCV RNA levels were associated with a significant increase in the incidence of HCC. Furthermore, they demonstrated that the presence of elevated levels of hepatic cytolysis and HCV genotype 1 (12.6% *vs.* 4.5% for non-genotype 1) were associated with a higher HCC rate[80]. Other studies have confirmed the oncogenic potential of the HCV genotype 1[74,83]. A meta-analysis of 57 papers showed that patients with HCV genotype 1b have twice the risk of developing HCC compared to patients with non-1 genotype[83]. Therefore, in patients with HCV infection the presence of high viremia, high levels of ALT and genotype 1 appear to be risk factors for HCC.

Pathogenic mechanisms of HCV-related HCC

HCV is unable to integrate its genome into host cells and requires a constant replication process to maintain chronic infection[84]. Therefore, its oncogenic potential appears to be mostly indirect and mediated by the development of significant hepatic fibrosis. HCV infection causes a chronic inflammatory state, induces hepatocyte necrosis, as well as collagen production and accumulation, which will eventually lead to an

alteration of the structure of the hepatic parenchyma. The increase in hepatocyte turnover due to the continuous processes of cell death and regeneration, as well as the progression of fibrosis, lead to a high probability of genetic alterations, whose accumulation leads to the formation and proliferation of cell clones that favour the development of HCC[85]. Furthermore, apoptosis of hepatocytes can amplify the fibrogenic signal, thus stimulating the activation of stellate cells and causing the progression of hepatic fibrosis towards cirrhosis which is a pre-malignant condition[86]. HCV infection is also able to modify the intracellular signalling pathways of transforming growth factor beta (TGF-β) signalling, thus accelerating the progression of liver injury and increasing the risk of HCC[87].

Although the pathogenesis of HCV-related HCC is mostly due to the development of cirrhosis and cell regeneration mechanisms, different alterations in gene expression and signal transduction pathways involved in cell proliferation and in the neoplastic transformation of hepatocytes have been described in chronic HCV infection[88]. In this regard, there are various demonstrations, mostly obtained on animal models, which suggest that different viral proteins may play a direct role in hepatocarcinogenesis[85,89-92]. The NS3 non-structural protein is a serine protease that appears to be involved in the neoplastic transformation process by inducing the acquisition by the hepatocyte clones of a proliferative condition, as well as the escape from the host cell surveillance mechanisms[89]. In combination with the NS4A factor, it interacts with the ATM kinase and alters DNA cell repair mechanisms[93]. Similarly, the NS5A phosphoprotein appears to be able to alter the cell growth mechanisms and the physiological replication cycle of the host cell through interaction with the CDK1/2-cyclin kinase-dependent complex[90]. The HCV core protein and the E2 envelope protein have been shown to stimulate cell growth and heteroplastic degeneration[91,92]. The HCV core protein in particular seems to play a key role in the pathogenesis of HCC. Its oncogenic potential appears to be considerably high, as it causes oxidative stress on one side and alters the intracellular signalling cascade of the protein kinase on the other, resulting in a dysregulation of cell growth control[85]. In particular, the HCV core-protein is able to provoke an overproduction of ROS by increasing the lipid peroxidation and a mitochondrial dysfunction through the rearrangement of the lipoprotein double layer of the mitochondrial membrane[94,95]. The oxidative stress induced by the HCV core-protein leads to damages in the genome of the host cell with accumulation of genetic aberrations that predispose the evolution towards cancer[85,95,96]. Furthermore, the presence of insulin resistance and hepatic steatosis, which are associated with a high frequency to HCV infection, exacerbates the production of ROS[95]. In addition, the HCV core-protein is able to inhibit DNA repair mechanisms damaged by oxidative stress and alter various intracellular antioxidant systems[95,97]. At the same time, this protein is able to directly alter gene expression and intracellular regulation mechanisms. In this regard, a greater expression of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) was observed, together with an higher activity of the relative downstream effectors c-Jun N-terminal kinase and activator protein-1, and a stimulation in the mitogen-activated protein kinase (MAPK) cascade[98,99]. HCV core-protein is also capable of inhibiting the tumor suppression genes RB1, TP53 and TP73 as well as cell-cycle modulators such as CDKN1A[98,100]. Cytokinesis overexpression and gene expression alterations may represent the mechanisms through which HCV core-proteins modulate the apoptotic signalling pathways and mechanisms of defence and proliferation of the hepatocytes. Histologically, transgenic mice carrying the core gene develop an early hepatic steatosis, similarly to what happens in men during chronic HCV infection. These mice show progressively the onset of hepatocellular adenomas characterised by the presence of numerous intracytoplasmic fat drops, which then evolve towards the formation of HCC more or less rich in lipid drops, depending on the stage of differentiation[101]. These data highlight the key role played by HCV core-protein in the process of carcinogenesis.

HCV is able to cause alterations in the glucose and lipid metabolism, another important factor in the development of HCC[85]. HCV, in fact, stimulates the activation of insulin-like growth factor (IGF), a cell growth regulator, through the induction of proliferative and anti-apoptotic mechanisms[102]. Through the degradation of insulin receptor substrate 1 and 2 (IRS-1 and IRS-2)[103], the virus is also able to interfere with

insulin signalling and induce insulin-resistance, which in turn is responsible for the activation of hepatic stellate cells and subsequent fibrosis[104,105]. The presence of insulin resistance or diabetes mellitus represent independent risk factors for the progression of the liver disease and the development of HCC in patients with chronic HCV infection[106]. Some evidence suggests that somatic mutations of the leptin receptor (LEPR) gene may increase the susceptibility to hepatocyte cancer transformation[107].

Finally, virus-induced immune alterations can also help create an ideal environment for HCC development. The HCV, in fact, is able to inhibit the production of interferon type 1 and to alter the immune response of both T cells CD8+ and natural killer[105,108]. In combination with the aforementioned cytokine alterations and oxidative stress, these immune alterations contribute to the persistence of chronic inflammatory hepatic disease, which provides fertile soil for malignant degeneration.

Effect of treatment on development of HCV-related HCC

The recent introduction of direct-action antivirals (DAAs) for the treatment of HCV infection that causes a sustained virologic response (SVR) in more than 95% of cases appears to induce a significant decrease in HCC cases associated with this infection. As already demonstrated for interferon-based therapeutic regimens[109,110], several studies seem to demonstrate that achieving SVR using DAAs reduces the risk of HCC[111]. The incidence rate of HCC in cirrhotic patients with SVR may decrease up to 1% per year, although a lower rate of reduction is observed in patients with concomitant metabolic syndrome. However, because the risk of HCC in patients with cirrhosis persists even after HCV elimination, a reasonable time frame will be required before significant epidemiological changes can be observed[109]. Although it is quite clear that the achievement of SVR reduces the long-term risk of HCC, the possible role of DAAs in increasing the risk of HCC de novo and recurrence of HCC successfully treated is still a matter of debate[112]. In a meta-analysis of 26 studies including 11,523 patients[113] it was found that there were no significant differences in incidence rates of de novo HCC between treatment regimens based on DAAs and IFN. Ioannou *et al.*[114] identified a 71% HCC risk reduction after achieving SVR through DAAs on a cohort of approximately 62,000 patients and confirming that there are no substantial differences in the HCC rate between patients treated with DAA and those treated with IFN. Kanwal *et al.*[115] show that achieving SVR in patients treated with DAA is associated with a 76% reduction in the risk of HCC.

For what concerns the impact of DAAs on the recurrence of HCC previously treated with curative intent, the data available are still controversial and further studies may be necessary for a correct evaluation of the impact of DAAs therapeutic regimens on HCC recurrence risk.

HCC and alcohol abuse

Clinical and epidemiological factors affecting development of HCC secondary to alcohol abuse

Consumption of alcohol is the second leading cause of HCC worldwide, as it is responsible for around a third of cases[1]. Europe and Latin America are the areas with the highest incidence rates of HCC secondary to alcohol abuse, accounting for about half of the total. It has been estimated that chronic alcohol consumption is associated with an approximately 2-fold increase in the odds ratio for the development of HCC, but this risk increases up to 5-7 times if consumption exceeds 80 g/day for a time period of more than 10 years, thus underlining the close dose/risk correlation[116,117]. Although there is no absolute "threshold" dose that can be applied as a parameter to all people, as the risk of alcohol-related damage is individual, an average chronic consumption of ≥ 2 drink/day in females and ≥ 3 drink/day in males for longer than a 10-year time span is associated to the onset of alcoholic liver disease (ALD), which encompasses a wide spectrum of clinical pictures ranging from steatosis to steatohepatitis to the development of liver cirrhosis. From the time when cirrhosis is established, the risk of occurrence of HCC is 1%-2.5% per year[118,119]. Compared to other etiologies, the risk of neoplastic transformation appears to be lower among ALD patients. In fact, a recent observational study showed that the cumulative incidence rates of HCC after 10 years of observation were lower in ALD cases (8.4%) than in cases of chronic HCV infection (22%) and NAFLD (23,7%), with an annual incidence

rate of 1.1%, 2.9% and 3.1% respectively[119]. In this regard, however, the data in the literature seem to be conflicting.

The amount and duration of the alcohol consumption are directly related to the stage of the liver disease and the risk of HCC[116]. In particular, the cumulative lifetime amount of alcohol assumed acts as a major determinant of oncologic risk[120]. A ≥ 3 drink/day consumption is strongly associated with the incidence of HCC and liver-related death[121]. Furthermore, alcohol consumption is also closely related to the more rapid increase in cancer growth once it has developed[122]. This highlights the importance of the dose-response relation between alcohol consumption and HCC.

The female gender has an approximately 5-fold higher risk of developing liver cirrhosis and/or HCC for lower doses of alcohol than males[121]. In addition, females appear to show a faster progression of the damage towards cirrhosis in comparison with males[123].

A meta-analysis[124] evaluated the occurrence of HCC after cessation of alcohol use. An annual reduction of 6%-7% of the risk of developing HCC after cessation of alcohol consumption was estimated and an average period of 23 years because the risk is comparable to that of an ever ethylist.

Although the cumulative amount of alcohol during the lifetime is the main predictor of the risk of HCC, not all alcohol users are destined to develop cirrhosis and/or HCC. Indeed, a number of both genetic and clinical cofactors are also implicated in modulating the risk of ALD evolution to HCC[125]. Several SNPs have been reported to increase the risk of HCC, in particular those able to interfere with the metabolism of ethanol and lipids (PNPLA3, TM6SF2), as well as hepatic iron accumulation[126-128].

Different co-morbidities can modulate cancer risk. Obesity is an important co-factor in the development of alcohol-induced HCC[129,130]. The risk of developing HCC is three times higher among alcohol users with a BMI ≥ 30 kg/m^2 compared to those not taking alcohol and with a lower BMI[131]. This synergy is also recognized to exist between alcohol and other co-factors of liver injury[132]. The coexistence of diabetes mellitus and chronic alcohol consumption leads to a significant increase in the risk of developing HCC[129,133,134].

A study of patients with alcoholic cirrhosis showed that the incidence of HCC among patients with and without diabetes mellitus was 32.7% and 3.2% after 5 years, 32.7% and 20.2% at 10 years, 66.3% and 20.2% at 15 years, respectively[135]. It has been reported that the risk of HCC among patients with diabetes mellitus who consume more than 4 drinks/day has increased by 4.2 times[136].

The high consumption of alcohol in cirrhotic patients with concomitant HBV infection increases the risk of HCC by about 10% per year, apart from the progression of its onset at an earlier age[132,137,138]. Similarly, data are reported for OBI or previous HBV infection[139,140]. Several studies conducted on alcoholic subjects show the synergistic effect with chronic HCV infection[141,142] or hemochromatosis[143] on the incidence of HCC. Simultaneous exposure to alcohol and tobacco also appears to increase the risk of HCC[129].

Pathogenic mechanisms of HCC secondary to alcohol abuse
The presence of cirrhosis is the major mechanism related to the development of HCC. However, alcohol is able to directly induce carcinogenesis causing oxidative stress, inflammation and endotoxinemia.

Ethanol is first converted to acetaldehyde and then to acetate by alcohol-dehidrogenase (ADH) and acetaldehyde-dehidrogenase (ALDH) respectively, within a process that increases the $NADH/NAD^+$ ratio[144]. This condition, in turn, causes a drastic change in the mitochondrial redox balance, which leads

to an increase in the oxidation of fatty acids, as well as of lipogenesis, thus inducing the development of steatosis[145]. Ethanol is able to inhibit hepatocyte β-oxidation, increasing the synthesis and the uptake of fatty acids as well as promoting liver steatosis and inflammation[146,147]. Acetaldehyde, in addition to being toxic, is also highly oncogenic. The highest levels of ADH activity in tumor cells, when compared to ALDH, indicate that they have a high oxidation capacity of ethanol but a low ability to remove acetaldehyde[148]. Chronic alcohol consumption acts as an activator of cytochrome CYP2E1, which in turn increases the hepatic production of acetaldehyde. Its accumulation is responsible for the production of ROS that gives rise to oxidative stress induced by alcohol. The latter is responsible for mitochondrial damage, which in turn increases the production of ROS, thus creating a vicious circle that maximizes oxidative stress in the hepatocytes[149]. The accumulation of iron in the liver[150] and the low oxygen tension of the tissue induced by alcohol are also responsible for the production of ROS. Furthermore, accumulation of intracytoplasmic lipid droplets in ethanol-induced steatosis (as well as in NAFLD) can make hepatocytes more susceptible to toxic or other insults[151]. It follows that the subsequent generation of ROS, in combination with the accumulation of damaged proteins and the increased susceptibility acquired by hepatocytes to damage of other nature, is able to induce lipid peroxidation, enzymatic inactivation and mutations of DNA, which can cause cell damage and inhibit apoptosis[149]. In particular, alterations in cellular DNA methylation processes (especially at levels of the oncosuppressor genes such as *RASSF1A* and/or *DOK1*) represent one of the most frequent genotoxic effects of chronic alcohol consumption[152]. All this causes serious abnormalities in the proliferation of hepatocytes, which may eventually lead to the development of HCC.

Alcohol induces alteration of the microbiota and may contribute to the development of liver injury and HCC. Damage to the tight junctions of the intestinal epithelium following the chronic abuse of ethanol increases the permeability of the intestinal barrier and promotes the migration of bacteria and endotoxins from the intestine to the portal system, thus fuelling the previously caused liver inflammatory status[153]. Bacterial endotoxin interacts with the toll-like receptors (TLR) present at the Kupffer's cells, stimulating the production of pro-inflammatory cytokines that contribute to the progression of alcohol-induced liver injury[153]. The alteration of TLR4 following the translocation of intestinal bacteria is able to induce carcinogenesis by interacting with cancer-initiating stem-like cells[154-156]. The TLR4/intestinal microbiota interaction through processes of cellular proliferation stimulation and apoptosis inhibition play a role in the progression of HCC but it is not required for the induction of HCC[156].

As for other etiologies, alteration in the length of the chromosomal telomere occurs also in the course of ALD. It has been shown that the telomeres of individuals taking> 4 drinks/day are shorter than those of subjects who take ≤ 4 drinks/day[157] and that telomerase reactivation is closely related to the mechanisms of induction of hepatocarcinogenesis through uncontrolled hepatocyte replication[148,158].

A further mechanism of induction of HCC secondary to ALD is represented by the impact of alcohol on the homeostasis of vitamin A, whose hepatic level decreases in chronic alcohol consumption[159]. Alcohol acts as a competitive inhibitor of vitamin A, inducer of its catabolism, through CYP2E1 and is a promoter of its mobilization from the liver to peripheral tissues[160]. Reduced levels of vitamin A in the liver can contribute to the development of HCC by altering the mechanisms of cell proliferation and apoptosis[149,161].

From the above it is clear that ethanol abuse plays an important role as a promoter rather than an inducer of cancer development through a process in which oxidative stress is the basis of alcohol-induced cytotoxicity.

HCC and NAFLD
Clinical and epidemiological factors affecting development of NAFLD-related HCC
Due to the recent obesity and metabolic syndrome epidemic, NAFLD is currently the fastest growing chronic liver disease worldwide. The overage prevalence of NAFLD is currently estimated at 25%[5]. It is

characterized by the intrahepatic accumulation of triglycerides and includes a spectrum of diseases ranging from simple steatosis to steatohepatitis (NASH) and to cirrhosis of the liver. The annual incidence of HCC in patients with NAFLD is reported to be 0.44 in 1000 patients, while the annual incidence in patients who have already developed NASH is 5.29 cases in 1,000 patients[5]. It has been estimated that NAFLD is responsible for about 14% of HCC cases in the United States, with an annual rate of increase of 9%[6].

A recent meta-analysis of cases of liver cancer diagnosed in the United States between 2004 and 2009 showed that the prevalence of HCC secondary to NAFLD is about 14%[6]. The estimated annual cumulative incidence of HCC in cirrhosis by NAFLD is 2.6%[5,73,160]. A recent study of our group found an annual rate of incidence of 3.5% of HCC in patients with cirrhosis from NAFLD, this incidence is slightly lower than that observed in cirrhosis secondary to HCV (4.5%)[75]. Similar annual incidence rates of HCC were also observed by Ascha *et al.*[162] (2.6% and 4.0% in patients with metabolic cirrhosis and HCV-related cirrhosis, respectively).

However, as already mentioned, increasing evidence suggests that NAFLD may cause the development of HCC even in non-cirrhotic patients with mild or absent fibrosis[133,163-168]. There are conflicting data on the true prevalence of HCC on non-cirrhotic steatotic liver[169]. A recent review of data on 61 studies published between 1992 and 2011 shows that the risk of HCC in non-cirrhotic patients with NAFLD appears to be extremely low[166]. On the other hand, there are several studies that support the opposite hypotheses is[163,164,167,168,170]. In a group of 31 patients with NAFLD and HCC, Paradis *et al.*[164] observed that, 65% of cases were in a F0-F2 fibrosis stage, whereas in the control group with liver disease of another etiology, only 26% of HCC were in the F0-F2 fibrosis stage. Mittal *et al.*[167], in a cohort of 107 patients with HCC and NAFLD, 34.6% of liver cancer cases occurred in the absence of cirrhosis. Piscaglia *et al.*[168] have recently observed a high incidence rate (70%) of HCC in non-cirrhotic patients with NAFLD, although histology was only available for one third of patients.

The evaluation of further co-factors appears to be fundamental for the individual assessment of the risk of HCC. Obesity and diabetes mellitus in particular are by now well-known independent risk factors for HCC. Calle *et al.*[78] have shown, in a large cohort of patients, how obesity increases the risk of HCC by 2-4 times. In comparison with individuals with normal weight, Larsson *et al.*[171] estimated the risk of HCC in normal weight and obese subjects by establishing a relative risk of 1.17 and 1.89, respectively.

It has been shown that the presence of diabetes mellitus increases the risk of HCC in patients with NAFLD[19,172,173]. A recent study of 480 patients with NAFLD or ALD showed that the prevalence of HCC among diabetic patients was statistically higher compared to normoglycemic patients (8% and 3%, respectively) and the incidence rate of HCC during 3 years follow-up was almost three times higher (27% and 10% respectively)[172]. Davila *et al.*[173] confirm that the risk of HCC is three times greater in the presence of diabetes mellitus. Furthermore, diabetes mellitus and obesity can act in synergy. An Italian study has observed that the presence of one of the two factors leads to 3.5 odds ratio (OR) of HCC, while the OR increases to 11.8 in the presence of both, compared to normal weight and normo-glycemic subjects[174]. Therefore, an obese and diabetic patient with NAFLD is the most classic patient phenotype that shows a high probability of developing HCC, particularly when co-factors are associated with an existing hepatic damage. In fact, when obesity is accompanied by chronic alcohol consumption or by HCV or HBV infection, the risk of developing HCC shows a tendency to increase exponentially, thus observing the synergistic action of these co-factors of hepatic injury[130,175,176].

The general clinical picture of HCC occurring in NAFLD shows peculiar characteristics. In this regard, Younossi *et al.*[6] showed how the development of HCC on NAFLD involves an older average popula-tion with a higher prevalence of cardiovascular disease. Weinmann *et al.*[177] confirm a high average age (67.6 years) of patients with HCC in NAFLD, a higher prevalence among males, a higher incidence of

myocardial infarction and ischemic stroke, as well as a higher prevalence of obesity and diabetes mellitus. NAFLD-HCC patients show lower mean ALT levels and a higher platelet count than HCV-related HCC patients[75,178].

The onset of HCC in NAFLD is generally an early event in the natural history of liver disease[75,177]. In fact, the incidence of HCC on metabolic cirrhosis in a Child-Pugh A score appears to be 1.8 times higher than that observed in HCV-related cirrhosis[75]. In a recent Italian study the diagnosis of HCC was placed at an early stage (Child-Pugh A) in 82.3% of patients with NAFLD, compared to 68.1% of patients with chronic HCV infection[168]. The model for end-stage liver disease scoring (MELD) is also significantly lower for HCC in NAFLD than in other etiologies[177].

Contrary to the stage of cirrhosis of the liver, the stage of the diagnosis of the neoplasia is generally more advanced for the HCC related to the NAFLD than for other etiologies. Compared to patients with HCV infection, HCC in patients with NAFLD often presents greater dimensions at diagnosis and more frequently shows infiltration (21% in patients with NAFLD versus 4% in patients with HCV) or multifocal lesions[168,170,178].

The diagnosis in the advanced stage of neoplasia is not due only to the pathogenic mechanisms and epidemiological factors mentioned above but is mainly due to the lower attention to follow-up and screening of NAFLD[179,180]. The diagnosis of HCC in patients with NAFLD is often incidental, outside the surveillance protocols and in any case late, as it is dependent on the appearance of symptoms[168,178]. Patients with NAFLD seem to have the highest rate of cirrhosis undiagnosed before evidence of HCC compared to other etiologies, resulting in a decrease in attention to ultrasound surveillance and subsequent delay in the diagnosis of HCC. Furthermore, as another condition of difficulty in early diagnosis, it must be emphasized that the sensitivity of ultrasound in detecting small cancer is low in patients with NAFLD[181,182].

Along with a generally more advanced stage of cancer diagnosis, there are additional reasons why the prognosis of patients with HCC related to NAFLD appears to be worse than patients with HCC of different etiology[6,177]. In fact, the prognosis is negatively affected by a greater number of comorbidities, especially cardiovascular, to which this subgroup of patients is exposed. The highest rate of co-morbidities such as obesity, the highest mean age of patients with NAFLD and often delayed diagnosis lead to fewer liver transplants for these patients[6,168,170,177]. Finally, the advanced stage of the disease is often a limitation for the applicability of radical treatments (resection or ablation) in favour of purely palliative interventions (chemoembolization or pharmacological therapy). In fact, liver resection and transplantation are only practiced in 17.8% and 4.4% of cases, respectively[177]. As a result, the death rate in HCC secondary to NAFLD (61% of patients die within one year of diagnosis) is higher than in HCC secondary to viral hepatitis (50% of deaths within one year of diagnosis), with a shorter average life expectancy of 5 months[6]. Piscaglia *et al.*[168] observed an average survival of 25.5 months from diagnosis in patients with NAFLD-HCC, versus an average 33.7 months of patients with HCV-related HCC. However, when the patient is eligible for curative treatment, survival does not appear to vary between HCC related to NAFLD and other etiologies[181].

The rs738409 polymorphism of phospholipase domain similar to the patatine containing 3 (*PNPLA3*) has been reported to be an independent risk factor of HCC in patients with metabolic cirrhosis (odds ratio 1.40)[183]. In particular, homozygosity GG was associated with the onset of HCC at a younger age, in patients with a shorter history of cirrhosis. Furthermore, it is associated with a worse prognosis[181]. The rs738409 polymorphism seems to alter the export of lipoproteins and lipogenic activity, thus causing the hepatic accumulation of fatty acids with consequent increase of lipid β-oxidation, as well as the production of ROS, increasing the risk of progression of fibrosis hepatic and HCC development[183].

An alteration of the intestinal microbiota is often associated with NAFLD and this condition contributes to exacerbating the inflammatory liver[184]. In particular, there is the appearance of endotoxinemia that interacts with TLR receptors on Kupffer cells and hepatic stellate cells triggering a cascade of inflammatory signaling causing the production of TNF-α, IL-1β, IL-6 and ROS[185]. This condition contributes to the progression of liver injury and to carcinogenesis as reported above for ALF.

Pathogenic mechanisms of NAFLD-related HCC

Most of the HCC secondary to NAFLD occurs in the cirrhotic liver following the mechanism shown above for other etiologies. However, the literature shows a consistent set of evidence on how the development of HCC may also occur in patients without evidence of significant hepatic fibrosis[133,168]. As shown in mouse models[186], hepatic steatosis secondary to the metabolic syndrome is a pre-malignant condition, long before cirrhosis. In this regard, obesity and type 2 diabetes mellitus can be considered as independent risk factors for the onset of HCC[78,172,187].

The pathogenic mechanisms involved in the carcinogenesis of liver cancer in NASH, with or without significant fibrosis, could be related to chronic low-grade inflammation induced by obesity and the metabolic syndrome and mediated by the crucial role of insulin resistance. The development of oxidative stress, lipid peroxidation and mitochondrial damage also play a fundamental pathogenic role. Moreover, it has been shown that alterations of the intestinal microbiota, the presence of gene polymorphism and IR induced hyperinsulinemia can be significant co-factors for the development of NASH and HCC. These data support the concept of "multiple hits hypothesis" in which several factors cooperate in the pathogenesis of NAFLD and HCC[188-190].

The IR, the cornerstone of the metabolic syndrome, is able to induce the onset of HCC by increasing the release of free fatty acids from the adipose tissue and subsequent accumulation in the hepatocytes on one hand and inducing the formation of ROS and subsequent oxidative stress with mitochondrial damage and endoplasmic reticulum dysfunction on the other hand[167]. Moreover, IR is able to alter the balance between pro-inflammatory cytokine production (IL-6, TNF-α, leptin, resistin) and those anti-inflammatory (adiponectin) with a significant increase in pro-inflammatory cytokine[191], leading to a chronic hepatic and systemic inflammatory state. The IR-induced TNF-α stimulation leads to the activation of the nuclear factor Kappa B (NF-κB) and of the N-terminal kinase c-Jun (JNK) on one side, as well as to the overexpression of tumor growth promotion genes[192] on the other side. The increase in free fatty acids and TNF-α and ROS production are all powerful activators of JNK, overexpressed in more than half of the cases of HCC, which in turn causes the phosphorylation of the substrate-1 of the insulin receptor (IRS-1). The signalling mediated by IRS1 can therefore act as a stimulus for cell survival, promoting the proliferation of hepatocytes through the mitogenated protein kinase and PI3K and inhibiting cell apoptosis by blocking the TGF-β1[193]. IR-induced hyperinsulinemia is also able to stimulate the production of growth factors such as IGF-1. All the previously mentioned pathways are able to cause liver inflammation and aberrant stimulation of several genes that are crucial in regulating cell growth and inhibition of apoptosis[175,194].

The cytokinetic imbalance associated with the release of unsaturated fatty acids also contributes to inhibition of tumor suppression factors (for example, phosphatase and homologue of the tensin, PTEN)[195] and inhibition of apoptotic cell abilities[175]. In this regard, IR-induced oxidative stress can increase lipid peroxidation, thus leading to the production of trans-4-hydroxy-2-nonenal, which in turn can interact with DNA and cause mutations in the oncosuppressor gene p53, thus favouring hepatocarcinogenesis and the progression of HCC[196].

The IR-induced increase in TNF-α and IL-6 also stimulates leptin production[197]. The latter acts as a growth factor activating the Janus-activated kinase (JAK), which in turn stimulates signal transducers and activators

of transcription 3 (STAT3) and extracellular signal-regulated kinases (ERK)[169,198,199]. Such leptin-induced pathways represent early events in the promotion of the survival and proliferation of pre-neoplastic cell clones, thus favoring the development of HCC and its invasion and metastasis[198,199]. Furthermore, higher levels of leptin are closely related to an increased risk of recurrent HCC after curative treatment[200]. On the other hand, IR inhibits the production of adiponectin, an adipokin with anti-inflammatory functions, as well as anti-atherogenic, anti-proliferative, pro-apoptotic, insulin-sensitizing and anti-angiogenic factors[169]. In fact, this cytokine is able to stimulate the activation of JNK and induce cell apoptosis[201]. Reductions in adiponectin levels appear to be closely associated with the risk of carcinogenesis[202].

Similar to what is observed in ALD, deficiency in the autophagy mechanism is also observable in NAFLD, causing reticuloendothelial damage and cellular oxidative stress and contributing to the formation of an environment suitable for the development of HCC[203].

The immune system may also participate in the complex multifactorial mechanisms of hepatocarcinogenesis. In fact, the metabolic stress promotes the migration of immune cells in the liver, while the T cells CD8 + and Natural Killer (NK), stimulated by the cell damage caused by NAFLD, interact with the hepatocytes activating the signaling cascades that feed the pre-existing state inflammatory[204]. In this way, they can establish a further vicious circle that worsens hepatocyte damage, promoting the progression of NAFLD towards the development of HCC.

DECLARATIONS

Authors' contributions
Designed the study: Adinolfi LE
Contributed equally to the search for literature and the writing of the text: Nevola R, Rinaldi L, Giordano M, Marrone A
Reviewed the manuscript: Adinolfi LE, Nevola R
Approved the final version: all authors

Availability of data and materials
Not applicable.

Financial support and sponsorship
There was no financial support or sponsorship for this study.

Conflicts of interest
All authors declare that there are no conflicts of interest.

Ethical approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

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