REVIEW

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Hepatic Oncology

Genetic risk markers for hepatocellular carcinoma in patients with alcoholic liver disease

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Practice Points

- The study of genetic predisposition to alcohol-related liver cancer aims to identify among patients with alcoholic cirrhosis those particularly prone to develop hepatocellular carcinoma (HCC).
- Although large cohorts of patients with alcoholic liver disease are still scarce, several variants modulating various biological pathways involved in alcoholic hepatocarcinogenesis have been reported as associated with a higher risk of HCC in these patients.
- These genetic traits can affect ethanol metabolism, oxidative stress, inflammation process, and iron and lipid homeostasis.
- The challenge is to understand how the combination of several variants might refine stratification of alcoholic cirrhotic patients according to different HCC risk classes.
- These advances will enable implementation of personalized medicine in these patients, that is, the adaptation of screening and preventive measures, criteria of early diagnosis and the choice of therapeutic procedures based on inter-individual susceptibility.
- Large prospective cohorts are urgently needed to validate and integrate predisposing genetic traits into individual HCC risk assessment models.

SUMMARY Various single nucleotide polymorphisms have been reported to be associated with a higher risk of hepatocellular carcinoma in alcoholic cirrhotic patients. Until now, only common variants conferring a small increase in liver cancer risk have been identified. These inherited factors are able to modulate several biological pathways involved in alcohol-induced hepatocarcinogenesis, such as ethanol metabolism, inflammation, oxidative stress, or iron and lipid homeostasis. How the combination of these variants might collectively define an individual genomic risk prediction is currently being investigated. The other challenge in clinical practice lies in defining how to integrate this genetic information with other clinical parameters so as to refine selection of alcoholic cirrhotic patients according to various classes of hepatocellular carcinoma risk.

KEYWORDS

- alcoholic liver disease
- cirrhosis
- liver cancer
- single nucleotide polymorphisms

Context of genetic predisposition to liver cancer in patients with alcoholic liver disease

Although chronic infections by hepatitis B virus (HBV) and hepatitis C virus (HCV) represent the main causes of hepatocellular carcinoma worldwide, the percentage of cases attributable to excessive chronic alcohol consumption in Europe is high [1] and, as a whole, is increasing [2,3]. This trend will

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be confirmed with the decline in viral-induced hepatocellular carcinoma (HCC) expected in forthcoming years in Western countries on the heels of more effective viral eradication and control [4], and by the increase in alcohol consumption observed in developing countries [5]. It is therefore pivotal to identify, among millions of excessive drinkers, those susceptible to developing HCC, that is, primarily cirrhotic patients, and, among the latter, to select those especially prone to developing liver cancer [6].

• **Particularities of HCC in patients with alcoholic cirrhosis**

Longitudinal studies dealing with the natural history of alcoholic cirrhosis are scarce [7–9]. Moreover, major selection biases in studies conducted in decompensated patients or based on registry data hamper their interpretation in terms of HCC occurrence, as they mainly report the incidence of other liver-related complications that constitute risks of death competing with liver cancer occurrence in these populations [10,11]. In these patients, the annual incidence of HCC has been estimated between 2 and 4% [12,13], thus justifying periodic screening. Risk factors for HCC include classical features such as older age, components of metabolic syndrome and severity of underlying cirrhosis [8,12–13]. Interestingly, by combining these features, cirrhotic patients can be stratified into various HCC risk classes and thus define a specific phenotype of patients at risk [12,13]. However, all alcoholic cirrhotic patients are subjected to the same management, namely 6-month periodic HCC screening, with no preventive measures undertaken [14]. Genetic risk markers of HCC in patients with alcoholic cirrhosis have also been described and might at least partly explain the wide inter-individual susceptibility to HCC in these patients [15].

• **Genetic predisposition to HCC in patients with alcoholic cirrhosis**

Genetic heterogeneity is the result of a combination of single nucleotide polymorphisms (SNPs). These variations in a DNA sequence due to the change in a single nucleotide are scattered throughout the human genome, every 1000 pairs of bases, and define the phenotype of a given individual. Some of these variants are situated in coding regions and can modify gene product expression and/or function, which may result in biological consequences. If the latter are implicated in alcoholic liver disease

(ALD) progression or liver carcinogenesis, then these modifications might affect individual susceptibility to HCC in the case of excessive alcohol consumption.

Various lines of evidence suggest a role for genetic traits in the emergence of liver cancer in patients with alcoholic cirrhosis. First, several variants are reported to be associated with a higher risk of evolution towards cirrhosis in individuals with chronic alcohol abuse [16], suggesting that these markers might influence progression through all steps of ALD up to cancer. Secondly, several SNPs have been linked to development of other alcohol-related cancers such as that of the upper aerodigestive tract, in particular, those modulating the formation of highly carcinogenic acetaldehyde [17]. The third and strongest argument is derived from the example of other types of chronic liver diseases: mainly conducted in Asia, results from large cohorts of HBV- or HCV-infected patients have established that the presence of HCC in first-degree relatives is associated with a higher incidence of this tumor [18], and have also identified various SNPs through multiple candidate gene or genome-wide association (GWA) approaches [15]. The extent to which these findings are reproducible in Caucasian patients with alcoholic cirrhosis and exposed to risk of HCC occurrence warrants clarification.

• **Exploration of genetic risk markers of HCC in patients with ALD**

Conversely to HCV- or HBV-induced HCC, no reports from GWAs conducted in patients with ALD and HCC have been yet published. Only candidate-gene approaches have been developed thus far to explore genetic susceptibility to liver cancer in heavy drinkers. While GWAs compare the genotypic distributions of millions of SNPs between HCC patients and ‘controls’ in order to discover previously unsuspected variants associated with HCC, candidate gene studies usually select one or several SNPs with known functional consequences in a biological pathway that is implicated in hepatocarcinogenesis. **Table 1** describes the characteristics and specific biases of these methods. These studies propose comparing allelic or genotypic distributions of SNPs thought to influence liver carcinogenesis between ‘cases’ (ALD patients with HCC) and ‘controls’ (ALD patients without HCC). However, this case–control approach is subject to numerous biases. Indeed, most of the published studies

Table 1. Methodological quality criteria for critical analysis of genetic associations studies in alcoholic hepatocarcinogenesis.

	Candidate-gene studies	GWAs
Number of tested SNPs	Several, choice based on literature data	Millions, unsupervised
Type of studies	Case-control or longitudinal	Case-control
Number of published studies in the setting of alcohol-induced HCC	Several decades	None
Choice of control group for case-control studies	Patients with alcoholic cirrhosis and without HCC	Patients with alcoholic cirrhosis and without HCC
Selection of patients/controls	Context should be precise (compensated outpatients vs decompensated hospitalized patients)	Should precise the context (compensated outpatients vs decompensated hospitalized patients)
Statistical differences	According to Bonferroni correction	Usually below 10^{-8}
Power calculation and number of patients	<ul style="list-style-type: none"> Depending on frequency of genetic variation, HCC incidence and length of follow-up in prospective cohorts Usually several hundreds of individuals 	<ul style="list-style-type: none"> Depending on frequency of genetic variation Usually several thousands of individuals
Confirmation of identified SNPs in discovery cohort	<ul style="list-style-type: none"> Replication in independent case-control cohorts Validation in prospective cohorts 	<ul style="list-style-type: none"> Replication in independent case-control cohorts Validation in prospective cohorts
Main biases	<ul style="list-style-type: none"> False-positive report probability Selection biases of cirrhotic population Confounding factors degree of liver failure, comorbidities, competing risks of death 	<ul style="list-style-type: none"> Non selection of possible variants of interest due to prerequired high levels of p values for difference Selection biases of cirrhotic population Confounding factors degree of liver failure, comorbidities, competitive risks of death

GWA: Genome-wide association; HCC: Hepatocellular carcinoma; SNP: Single nucleotide polymorphism.

were conducted in small samples from single centers and did not systematically take into account the stage of ALD in included patients. As a consequence, cirrhotic patients exposed to risk of liver cancer, a major prerequisite for the study of risk factors in HCC development, were not always included as controls: such selection biases do not allow the conclusion that a given variant is clearly implicated in alcoholic carcinogenesis or if it simply favors the progression of ALD towards end-stage liver disease. More importantly, the criteria of selection of these control populations is rarely precise. Some cohorts include or even mix outpatients with a long-standing uncomplicated liver disease or patients with decompensated cirrhosis; it has, however, been shown that these patients could bear a strikingly different genetic profile [19], another selection bias that can lead to major changes in genotypic distributions and risks of misinterpretation. Moreover, power calculation according to the expected prevalence of genotype distribution should always be performed prior to analyses: it has been indeed shown that less than one-third of reported associations between genetic variants and cancer diseases are actually

statistically significant [20], due to high risk of false-positive report probability. Finally, in order to validate these associations, there is an urgent need for prospective cohorts of alcoholic cirrhotic patients with long follow-up, allowing the occurrence of numerous events, to allow multivariate analyses to be performed that, along with genetic traits, take into account competing risks of death as well as clinical and environmental factors known to influence HCC occurrence.

• Pathophysiology of alcohol-induced liver carcinogenesis

Figure 1 shows the major pathways thought to be implicated in alcohol-related hepatocarcinogenesis [21]. Ethanol is metabolized by alcohol dehydrogenase (ADH) into acetaldehyde, which acts as a carcinogen, by directly damaging DNA and proteins. In addition, ethanol is oxidized by CYP2E1, enhancing acetaldehyde, but also reactive oxygen species (ROS) formation. Oxidative stress plays a pivotal role in cancer promotion during ALD, in particular through mitochondrial dysfunction. ROS accumulation triggers lipid peroxidation and DNA damage through the formation of

Figure 1. Major biological pathways implicated in alcohol-induced hepatocarcinogenesis and their genetic heterogeneity, thought to influence liver cancer risk in patients with alcohol liver disease. The main variants reported as possible genetic markers of liver cancer in these patients are also depicted, according to their specific interaction with these various biological pathways. ROS: Reactive oxygen species; SNP: Single nucleotide polymorphism.

mutagenic adducts. The effects of iron and lipid metabolism also interfere with oxidative stress, creating a complex interplay that further influences the progression of the carcinogenic process during ALD. Furthermore, acetaldehyde alters methyl transfer, leading to DNA hypo-methylation that is able to modify the expression of oncogenes and tumor suppressor genes. Finally, ethanol decreases the level of retinoic acid, leading to generation of toxic metabolites associated with changes in cell cycle regulation and cellular proliferation.

• Challenges for research programs focusing on liver cancer susceptibility in ALD patients

Studying genetic predisposition to alcohol-induced HCC might provide the possibility for individualized management of patients according to specific genetic backgrounds. Various SNPs have been reported to be associated with a higher risk of HCC in alcoholic cirrhotic patients, but up until now, only common

variants conferring a small increase in liver cancer risk (usually with a relative risk below 3) have been identified [15]. How the combination of several variants might collectively define a ‘genomic risk prediction’ for these individuals is a current challenge in this field. The other challenge in clinical practice lies in defining how to integrate this genetic information in order to refine stratification of alcoholic cirrhotic patients according to risk classes. The questions addressed in the case of HCC surveillance are the identification of individuals at risk, modalities and periodicity of HCC screening, and the ability to adapt preventive or curative management according to a specific genetic background [22]. Finally, these findings might change the decision-making process in clinical practice and could be translated into more global perspectives that would modify the approach to liver cancer at the population level [3].

This review provides an overview of genetic traits that modulate the risk of HCC development in alcoholic patients with cirrhosis and, when

necessary, highlights the influence of these variants on other spectra of ALD progression and their role in the liver cancer process due to other etiologies.

Genetic variants associated with HCC risk in patients with alcoholic cirrhosis

Table 2 shows the most reliable and robust results based on previous methodological considerations. Far from being exhaustive, this selection highlights the quality of these studies and classifies them according to the specific biological pathways explored through their genetic modulation. Differences or similarities with viral hepatocarcinogenesis are highlighted, as well as limitations or quality criteria allowing a critical assessment of these studies.

• Inherited variations in alcohol-metabolizing enzymes: first steps in tumor promotion

As previously mentioned, acetaldehyde is generated and then metabolized by the successive effects of alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) families [41]. This complex enzymatic system is subjected to genetic heterogeneity, possibly providing an attractive explanation for individual susceptibility to alcohol-related malignancies. Consequently, genetic variants involved in alcohol-metabolizing enzymes that modulate subsequent amounts of carcinogenic acetaldehyde were explored as potential candidates, suggesting a possible interaction between genetic susceptibility and alcohol exposure in cancer diseases, including those of the liver [17].

The study of Caucasian patients with ALD highlighted functional polymorphism in the ADH1C isoenzyme as a possible inherited factor implicated in the emergence of various alcohol-related cancers, particularly HCC [23]. Indeed, individuals harboring ADH1C*1, thought to confer higher enzymatic activity, may be hypothetically exposed to stronger acetaldehyde formation and accumulation. By comparing genotype distribution in ALD patients with cirrhosis complicated or not by HCC, Homann *et al.* concluded that homozygosity for allele ADH1C*1 was a risk factor in liver cancer development. This association is further supported by the fact that excessive drinkers carrying the same genotype are more likely to develop cirrhosis [42], thus suggesting genetic modulation of this variant throughout the entire ALD spectrum. Furthermore, the same study reported a higher prevalence

of alcohol-related malignancies of the upper gastrointestinal tract in the same individuals, a fact that strengthens the role of the carcinogenic effects of genetically influenced production of acetaldehyde in chronic alcohol abuse.

A large proportion of individuals of Asian descent are unable to sufficiently detoxify acetaldehyde in case of ethanol consumption due to frequent genetic variations in ALDH2 resulting in weak enzymatic activity [43]. This explains their poor tolerance for even small amounts of alcohol ingestion, resulting in nausea, vomiting and facial flushing. These observations led several teams from Japan to explore this genetic heterogeneity as a possible modifier of liver cancer risk [24–25]. As expected from the acetaldehyde accumulation hypothesis, individuals carrying the weakly active ALDH2*2-conferring allele were more prone to developing HCC in case of excessive alcohol intake. However, it is important to note that those studies, conducted in Asia, primarily included patients with liver disease of viral etiology in whom alcohol consumption was recorded, a consideration to take into account for interpreting these data as the same genotype could also lead patients to refrain from consuming large amounts of alcohol.

• Genetic modulation of oxidative stress & iron metabolism: pivotal role in alcohol-induced liver carcinogenesis

ROS promote damage to cellular macromolecules and participate in liver carcinogenesis progression [44]. The combination of DNA damage with continuous DNA replication causes gene mutations that will accumulate over the years, some of them may eventually hamper the control of the cell cycle and/or apoptosis, to produce a cancerous clone. Given the importance of oxidative stress (particularly inside mitochondria) in the pathogenesis of ALD, genetic variants affecting enzymes regulating the production and detoxification of ROS have been shown to modulate its outcome. In particular, MPO is expressed in neutrophils and Kupffer cells, both of which are involved in the pathogenesis of ALD, and catalyzes the reaction of H_2O_2 with Cl^- to form the highly reactive hypochlorous acid (HOCl) and anion (OCl^-) [45]. A G to A base exchange at position -463 affects its promoter [46]. The GG-MPO genotype is associated with higher protein expression and favors cirrhosis constitution in patients with hemochromatosis [47] as well as various malignancies [48].

Biological pathway	Study (year)	SNP (rs number)	Other HCC etiologies	Progression of ALD	Type of study (n = number of included publications if meta-analysis)	Cases/controls or n if a prospective cohort of cirrhotic patients	OR or HR	Ethnicity	Limitations according to quality criteria exposed in Table 1	Ref.
Ethanol metabolism enzymatic systems	Homann <i>et al.</i> (2006)	ADH1C 1/2 (rs283413)	No	Yes	Case-control	86/217 [†]	OR = 3.6	Caucasian	<ul style="list-style-type: none"> • No replication • Low number of cases 	[23]
	Sakamoto <i>et al.</i> (2006)	ALDH2 1/2 (rs 886205)	No	No	Case-control	275/481	OR = 4.5	Asian	<ul style="list-style-type: none"> • No replication • Choice of controls • Selection of patients not precised 	[24]
	Munaka <i>et al.</i> (2003)	ALDH2 1/2 (rs 886205)	No	No	Case-control	78/138	OR = 2.53	Asian	<ul style="list-style-type: none"> • No replication • Low number of cases and controls • Choice of controls • Selection of patients not precised 	[25]
	Nahon <i>et al.</i> (2009)	SOD2 A16V (rs4880)	No	Controversial	Prospective	n = 190	HR = 1.7	Caucasian	<ul style="list-style-type: none"> • No replication 	[26]
Oxidative stress / iron metabolism	Nahon <i>et al.</i> (2009)	MPO G-463A (rs2333227)	HCV	No	Prospective	n = 190	HR = 3.8	Caucasian	<ul style="list-style-type: none"> • No replication 	[26]
	Nahon <i>et al.</i> (2008)	HFE C282Y (rs1800562)	Controversial	Controversial	Prospective	n = 165	HR = 2.7	Caucasian	<ul style="list-style-type: none"> • No replication 	[27]
	Jin <i>et al.</i> (2010)	HFE C282Y(rs1800562)	Controversial	Controversial	Meta-analysis (n = 9)	1102/3766	OR = 4.06	Caucasian	<ul style="list-style-type: none"> • Limitations of meta-analyses • Not performed on individual participant data 	[28]
	Charni <i>et al.</i> (2011)	RANTES G-403A (rs2107538)	No	No	Prospective	n = 253	HR = 2.7	Caucasian	<ul style="list-style-type: none"> • No replication 	[29]
Inflammation	Falletti <i>et al.</i> (2009)	IL6 C-174G(rs1800795)	HBV/HCV	Yes	Case-control	66/153 [†]	OR = 7.5	Caucasian	<ul style="list-style-type: none"> • No replication • Low number of cases and controls 	[30]

Note: OR or HR takes into account the highest reported value according to allelic or genotypic expression of results and, when available, after multivariate analysis.

[†]Case-control studies conducted in alcoholic cirrhotic patients with or without HCC (i.e., appropriate control group).

ALD: Alcoholic liver disease; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HR: Hazard ratio; NASH: Non-alcoholic steato-hepatitis; OR: Odds ratio; SNP: Single nucleotide polymorphism.

Table 2. Published single nucleotide polymorphisms associated with risk of hepatocellular carcinoma in patients with alcoholic liver disease (cont.).

Biological pathway	Study (year)	SNP (rs number)	Other HCC etiologies	Progression of ALD	Type of study (n = number of included publications if meta-analysis)	Cases/controls or n if a prospective cohort of cirrhotic patients	OR or HR	Ethnicity	Limitations according to quality criteria exposed in Table 1	Ref.
DNA synthesis and repair mechanisms	Saffroy <i>et al.</i> (2004)	MTHFR C-677T (rs1801133)	HCV/HBV	No	Case-control	72/122	OR = 2.03	Caucasian	<ul style="list-style-type: none"> No replication Low number of cases and controls 	[31]
	Fabris <i>et al.</i> (2009)	MTHFR C-677T (rs1801133)	HCV/HBV	No	Case-control	17/46	OR = 4.47	Caucasian	<ul style="list-style-type: none"> Choice of controls No replication Low number of cases and controls Choice of controls Selection of patients not precised 	[32]
Growth factors	Tanabe <i>et al.</i> (2008)	EGF A61G (rs4444903)	HBV/HCV	No	Case-control	44/121 [†]	OR = 2.9	Caucasian	<ul style="list-style-type: none"> Low number of cases and controls (subgroup analyses) 	[33]
	Zhong <i>et al.</i> (2012)	EGF A61G (rs4444903)	HBV/HCV	No	Meta-analysis (n = 8)	1304/2613	OR = 1.79	Mixed	<ul style="list-style-type: none"> Limitations of meta-analyses Not performed on individual participant data 	[34]
Lipid metabolism	Falletti <i>et al.</i> (2011)	PNPLA3 I148M (rs738409)	HCV/NASH	Yes	Case-control	66/132 [†]	OR = 1.45	Caucasian	<ul style="list-style-type: none"> No replication Low number of cases and controls 	[35]
	Nischalke <i>et al.</i> (2011)	PNPLA3 I148M (rs738409)	HCV/NASH	Yes	Case-control	80/80 [†]	OR = 2.8	Caucasian	<ul style="list-style-type: none"> No replication Low number of cases and controls 	[36]
	Trépo <i>et al.</i> (2012)	PNPLA3 I148M (rs738409)	HCV/NASH	Yes	Case-control	145/425 [†]	OR = 4.7	Caucasian	<ul style="list-style-type: none"> No replication 	[37]
	Guyot <i>et al.</i> (2013)	PNPLA3 I148M (rs738409)	HCV/NASH	Yes	Prospective	n = 279	HR = 1.72	Caucasian	<ul style="list-style-type: none"> No replication 	[38]

Note: OR or HR takes into account the highest reported value according to allelic or genotypic expression of results and, when available, after multivariate analysis.

[†]Case-control studies conducted in alcoholic cirrhotic patients with or without HCC (i.e., appropriate control group).

ALD: Alcoholic liver disease; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HR: Hazard ratio; NASH: Non-alcoholic steato-hepatitis; OR: Odds ratio; SNP: Single nucleotide polymorphism.

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Biological pathway	Study (year)	SNP (rs number)	Other HCC etiologies	Progression of ALD	Type of study (n = number of included publications if meta-analysis)	Cases/controls or n if a prospective cohort of cirrhotic patients	OR or HR	Ethnicity	Limitations according to quality criteria exposed in Table 1	Ref.
Lipid metabolism	Trépo <i>et al.</i> (2013)	PNPLA3 I148M (rs738409)	HCV/NASH	Yes	Meta-analysis (n = 5)	442/932 [†]	OR = 2.13	Caucasian	• Limitations of meta-analyses	[39]
	Nischalke <i>et al.</i> (2014)	NCAN C274T (rs2228603)	No	No	Case-control (with replication)	126/356 [†] (83/146 [†])	OR = 1.84	Caucasian	• None	[40]

Note: OR or HR takes into account the highest reported value according to allelic or genotypic expression of results and, when available, after multivariate analysis.
[†]Case-control studies conducted in alcoholic cirrhotic patients with or without HCC (i.e., appropriate control group).
ALD: Alcoholic liver disease; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HR: Hazard ratio; NASH: Non-alcoholic steato-hepatitis; OR: Odds ratio; SNP: Single nucleotide polymorphism.

In addition, manganese superoxide dismutase (SOD2) generates H₂O₂ within mitochondria [49] that can form hypochlorous acid in the presence of MPO or the hydroxyl radical in the presence of iron. A genetic dimorphism substitutes either alanine (Ala) or valine (Val) in the mitochondrial targeting sequence of SOD2 [50] and results in higher mitochondrial activity for the Ala-SOD2 variant. Indeed, in acute mitochondrial import and transfection experiments, the Ala-SOD2 variant achieved higher mitochondrial activity than the Val-SOD2 variant [50,51]. However, MnSOD is inducible by ROS, cytokines, and ethanol [52], and is inactivated by peroxynitrite [53] or by the misincorporation of iron instead of manganese into the MnSOD active site [54]. The transfection of Ala-SOD2 variant in cell lines is associate with higher hydrogen peroxide formation [55], a finding that may explain the multiple reports linking this variant to numerous cancers.

It has been shown, in large prospective cohorts of alcoholic cirrhotic patients, that carriage of two G-MPO alleles, or possession of at least one Ala-SOD2 allele alone, were independent risk factors for HCC occurrence [26]. Furthermore, the occurrence of HCC increased dramatically in individuals who cumulated these two genetic traits, suggesting a synergic effect of these SNPs on ROS-mediated liver carcinogenesis. Interestingly, these variants also favor liver iron overload, possibly through enhanced mitochondrial hydrogen peroxide production [55]. Liver iron overload, as well as HFE gene mutations have also been reported as associated with the risk of HCC in Caucasians with alcoholic cirrhosis [27–28]. These findings suggest that hepatic iron accumulation might not only be a cause of enhanced oxidative stress in the course of ALD but also a consequence of mitochondrial dysfunction, thus establishing the basis of a vicious circle, an association that does not seem to be as relevant in HCV-related liver cancer development [27,56].

Interestingly, the comparison of these results with those obtained in patients with HCV-related cirrhosis suggests both similarities and differences in HCV- and alcohol-induced hepatocarcinogenesis. HCV-related liver cancer is also associated with increased ROS production and iron accumulation [57]. A possible explanation for the role of the Ala16Val-SOD2 and iron metabolism in alcohol-induced but not HCV-mediated carcinogenesis could reside in

the fact that both ROS and iron downregulate HCV replication [58,59]. Thus any genetic trait increasing ROS formation and/or iron accumulation within HCV-infected hepatocytes could also decrease HCV replication in these cells. These differences might also explain why liver iron accumulation seems to act as a strong carcinogen in alcohol-induced carcinogenesis, while its effect on HCV carcinogenesis seems to be less relevant [27].

Since the benefits of using antioxidant treatments or implementing iron depletion to prevent cancer remain controversial [60], individual variations influenced by the cause of liver disease and by individual characteristics as well, point out the need for development of targeted antioxidant chemopreventive strategies.

• Inflammation, cytokine & chemokine systems

Chronic alcohol consumption induces activation of macrophages and Kupffer cells, in particular, through the IL-1 pathway, which leads to production of pro-inflammatory cytokines such as IL-6 and TNF α . IL-6/STAT3 and TNF α /NF- κ B axes have been pointed out as being major biological pathways involved in hepatocarcinogenesis [61]. In the liver, NF- κ B activation in nonparenchymal cells is necessary for HCC tumor promotion, but also stimulates production of growth factors and cytokines such as EGF and IL-6, leading to an increase in ROS production [62]. Although the mechanisms by which pro-inflammatory cytokines promote liver cancer are not clear, their signals regulate gene expression through transcription factors STAT3 and NF- κ B. NF- κ B is the intracellular signaling effector for many pro-inflammatory cytokines, including TNF α , IL-1, and toll-like receptors (TLRs). In the liver, NF- κ B activation in nonparenchymal cells is necessary for HCC tumor promotion, but also stimulates production of growth factors and cytokines such as EGF and IL-6, leading to an increase in ROS production [62]. Interestingly, TNF α and IL-6 create a positive feedback loop and form a complex of regulatory networks that promote hepatocarcinogenesis. In this setting, downstream from TNF α and IL-1 production, IL-6 activates STAT3 and thereby enhances cell growth [63].

This pro-inflammatory cytokine signaling pathway can be further modulated by complex genetic heterogeneity. In this setting, variants influencing TNF α , IL1 β , IL6 or EGF

production were reported to be modifiers of liver cancer risk, particularly in patients infected by HBV or HCV [64]. These SNPs also seem to influence the course of ALD, in particular variants affecting TNF α and IL6 secretion, which have been shown to favor progression to cirrhosis in heavy drinkers [65,66]. In particular, the highly productive cytokine IL6–174G allele has been reported to be a possible genetic variation influencing the risk of liver cancer in Caucasians with alcoholic cirrhosis [30]. The influence of TNF α variants on alcoholic carcinogenesis, however, seems more controversial [67].

EGF promotes cancer growth and invasiveness [68] and triggers liver carcinogenesis in animal models [69]. This factor is subjected to functional polymorphism involving an A to G exchange in the 5' untranslated region of the *EGF* gene, resulting in stronger transcription and development of malignancies in individuals harboring 2 G-alleles [70]. Tanabe *et al.* [33] genotyped two distinct case–control sample cohorts of cirrhotic Caucasian patients (alcohol- or HCV-related), and reported a higher frequency of the at-risk homozygote GG genotype in both cohorts. This observation, consistent with a ubiquitous role for this variant in carcinogenesis progression in both viral and alcoholic disease, is further supported by a recent meta-analysis [34]. Furthermore, the impact of this genetic trait on the occurrence of liver cancer was validated in patients included in the HALT-C trial [71], indicating that this SNP is an essential candidate gene driving hepatocarcinogenesis.

Chemokines are chemotactic cytokines involved in the attraction and activation of specific inflammatory cells to sites of inflammation in the course of chronic liver disease [72]. The role of chemokines and their receptors in promotion of tumor growth and subsequent invasion has been reported, via lymphocyte recruitment in the livers of patients with HCC [73]. Chronic ethanol consumption is characterized by an increase in the expression of a number of inflammatory cytokines and chemokines. In particular, TNF α induces the expression of RANTES, a T-cell chemoattractant and immunoregulatory molecule that may play a pivotal role in migration of inflammatory cells to the liver in patients with ALD. Indeed, RANTES/CCL5 is secreted either from tumoral or stromal cells, and enhances motility and invasion of cancer cells [74]. A *RANTES* promoter polymorphism (*G-403A*) results in increased RANTES expression for

A-403 alleles (10, 11) and favors development of various malignancies (12–14). A prospective study conducted in Caucasian patients with cirrhosis revealed the influence of genetic heterogeneity modulating RANTES chemokine expression upon the risk of HCC. In particular, alcoholic cirrhotic patients bearing two *G-403* alleles had higher incidences of HCC during follow-up. As described earlier, this association seemed to be specific to patients with alcohol-induced HCC and could not transposed to all patients with chronic liver diseases, as this observation was not replicated in HCV-infected individuals [29].

Taken together, these data highlight the importance of inflammation in initiation and progression of liver carcinogenesis, as well as its modulation by complex genetic heterogeneity, that might help to refine selection of alcoholic cirrhotic patients who may benefit from HCC chemoprevention. These approaches, by affecting anti-tumor-specific immune responses through antitumoral immunotherapy [75] or by blocking the EGF/EGFR pathway, for example [69], warrant further testing in the setting of specifically targeted therapies.

• Genetic modulation of folate metabolism

Variants in the *MTHFR* gene, leading to alterations in folate metabolism, an essential component of DNA synthesis and methylation, have been reported to be associated with HCC development [76], irrespective of the cause of liver disease or ethnic origin. The polymorphism of *MTHFR*, resulting in a substitution of C to T at nucleotide 677, which converts an alanine to a valine residue, seems to be a possible susceptibility locus for liver cancer in alcoholic patients [31–32]. Although based on the hypothesis that *MTHFR* activity and folate availability might affect gene expression (through DNA methylation) and genome integrity (through DNA synthesis and repair) [17], these studies nonetheless showed discrepancies, warranting further elucidation.

• Synergic effect of alcoholic consumption & genetic factors affecting risk of liver cancer in other chronic liver diseases

As mentioned earlier, most studies in the field of HCC predisposition are currently conducted in Asia, where the most prevalent causes of chronic liver disease are dominated by chronic viral infections. However, some reports have identified several interactions between various

SNPs influencing liver cancer emergence and the amount of chronic ethanol consumption in these individuals. This is for example the case of genetic modifiers of cytochrome CYP2E1, for which a recent meta-analysis performed in nearly 4000 individuals confirmed that while CYP2E1 Pst I/Rsa polymorphism was not associated with HCC risk, the interaction between this variant and alcohol consumption increased the risk of liver cancer [77]. Other emerging data focused on genetic modulation of miRNA genes, leading to changes in expression of mature miRNAs have been identified as genetic modifiers of HCC risk in HBV- or HCV-infected individuals from Asia such as miR-146a, miR-122 and miR-378 [15]. In the same way, some of these SNPs such as rs4938723 in the promoter region of pri-miR-34b/c, seem to modify liver cancer risk in these patients through specific interaction with ethanol consumption [78]. The extent to which these observations might be translated in patients with alcoholic cirrhosis, and thus to ethanol-related hepatocarcinogenesis, remains to be clarified and should be specifically addressed in populations with ALD.

• PNPLA3 rs738409: a robust association with alcohol-induced liver cancer, but as yet no clear explanation

Several GWAs conducted in NASH and ALD patients identified a single nucleotide polymorphism (rs738409 C>G) which encodes for an isoleucine to methionine substitution at position 148 in the PNPLA3 protein sequence [79–81], as a promoter of intracellular triglyceride accumulation [82]. Subsequently, the rs738409 (G) allele was reported to be associated with more pronounced steatosis, advanced fibrosis and a higher incidence of cirrhosis in the most prevalent causes of chronic liver diseases affecting Caucasians, namely NASH [83,84], HCV infection [85,86] and ALD [87,88]. It was thus tempting to speculate that the same genetic trait that influences development of initial steps common to all chronic liver disease might also promote the emergence of liver cancer. In this context, case–control studies were subsequently conducted in alcoholic and/or HCV-infected patients from Europe with cirrhosis, complicated or not by HCC, in order to test this hypothesis. These initial reports identified an association between the rs738409 (GG) genotype and HCC in these patients [35–37,85,88–89]. However, results seemed concordant for ALD, but remained

conflicting in HCV-infected patients. More recently, a prospective cohort study conducted in cirrhotic patients screened for HCC confirmed that the G-PNPLA3 allele was a risk factor for HCC in alcoholic patients, while the same variant did not influence the outcome of HCV-infected individuals [38].

All studies in this field were conducted in Europe and included a homogeneous population of more than 2500 Caucasian patients with cirrhosis, complicated or not by HCC. This led to a recent meta-analysis [39], which also had the advantage of using individual participant data. As a whole, this approach confirmed the influence of PNPLA3 genotypes on liver carcinogenesis in Caucasian cirrhotic patients, which was more pronounced in alcoholic patients, in whom a twofold HCC risk was observed for those bearing the G variant. A recent second meta-analysis confirmed this specific association [90].

The exact mechanisms by which PNPLA3 influences hepatocarcinogenesis in ALD patients remain to be determined. Indeed, the question of whether the rs738409 variant promotes liver carcinogenesis by creating a favorable micro-environment constituted by steatosis, inflammation and fibrosis, or by specifically triggering the carcinogenic process, is still unclear. In particular, these complex entities composing the tissue environment might interact and thereby drive cancer/host cross-talk, but the details of this interplay are not clear. However, it is noteworthy that the PNPLA3 variant seems to influence progression of chronic liver disease independently of the grade of steatosis [85–86,91], and that this histological feature has not been clearly associated with the risk of HCC occurrence in prospective cohorts of cirrhotic patients [92]. Mechanistic studies should unravel specific impaired biological pathways explaining the frequently reported associations between rs738409 and HCC in patients with ALD. Such results are currently emerging, with, for example, the recent report of an unsuspected interaction between PNPLA3 and retinoid metabolism [93]; indeed, these liposoluble micronutrients are known to influence cell proliferation and differentiation [94] and have been shown to be effective in liver cancer prevention trials [95].

Interestingly, other genetic modifiers of lipid metabolism seem to further influence HCC risk in ALD. A variant in extracellular matrix proteoglycan NCAN has been identified in a GWAS conducted in patients with NASH as

a polymorphism affecting hepatic steatosis [96]. Very recently, a case–control study reported a higher prevalence of the same *NCAN*-T variant with HCC developed in patients with alcoholic cirrhosis [40]. This particular study presented the advantage of meeting several methodological quality criteria. First, it was conducted in a large case–control series including an appropriate control group of patients with alcoholic cirrhosis. Second, the authors concomitantly explored the influence of the genetic trait in other clinical settings such as ALD progression or HCV-related HCC. Third, a validation set confirmed this association. Fourth, the authors also took into account variations in PNPLA3, and were thus able to observe that the proportion of HCC patients among carriers of both *NCAN*-T and *PNPLA3*-G risk variants were increased as compared with carriers of only one or none of these traits (OR: 1.891 and OR: 4.575, respectively). Finally, genotype–phenotype correlations were undertaken in liver samples, highlighting a possible involvement for this ECM protein in ALD progression, although mechanisms of cancerization process remain to be clarified.

Future perspective

Except for the involvement of PNPLA3 variants in alcohol-related HCC, most associations still suffer from a lack of robustness or remain inconclusive. Numerous methodological limitations hamper validation of these candidate genes as definitive risk markers of liver cancer in ALD patients. Structuring of this field of research is urgently needed, as recently demonstrated by genetic studies conducted in the field of lipid metabolism genetic modulation, but will only be feasible by using large, well-defined populations in order to overcome these pitfalls. **Figure 2** provides an example of optimal research programs in three steps that could lead to progress in discovering, exploring and then validating genetic traits associated with liver cancer risk in patients with alcoholic cirrhosis.

First, case–control studies should compare genotypic distribution between HCC patients and individuals exposed to liver cancer, namely cirrhotic patients. Whether performed in the setting of supervised exploration or GWASs, such a rigorous approach might limit selection bias. This is well illustrated by the converging results on PNPLA3 variants obtained in several homogeneous cohorts of patients with alcoholic cirrhosis complicated or not by HCC. In the

Figure 2. Challenges and perspectives in research programs. (A) Optimal design for case-control studies aimed at exploring HCC susceptibility through candidate gene or GWA approaches. (B) Translational studies should explore both germ-line and functional genetics of alcohol-induced liver cancer. (C) Longitudinal studies should validate and incorporate genetic data into complex HCC risk assessment models in order to refine selection of alcoholic cirrhotic patients according to prognosis. GWA: Genome-wide association; HCC: Hepatocellular carcinoma; SNP: Single nucleotide polymorphism.

absence of such an in-depth approach at both the genotypic and phenotypic level, the exact implications of these variants in liver cancer emergence cannot be correctly assessed, leading to a high risk of misinterpretation.

Second, the exploration of biological consequences of newly discovered variants should be assessed in liver samples in which exploration at the molecular level can be performed. The precise knowledge of effects of a given variant on tumor promotion is indeed a prerequisite for confirming its role in modulation of cancer risk, and may also pave the way for development of possible therapeutic targets. Furthermore, the question as to whether genetic factors at the individual level are associated with the occurrence of specific genomic subtypes of liver tumors remains to be elucidated. HCC is indeed a very heterogeneous tumor and can be classified

into various transcriptomic groups [97], a fact that can impact treatment response [98] and also differ according to underlying liver disease [99]. The combination and integration of these complex genetic signatures at the individual and tumor level in clinical decision-making is another exciting challenge in forthcoming years.

Third, all newly discovered SNPs should be validated in cohorts of compensated alcoholic cirrhotic patients prospectively followed up and screened for HCC, so as to include all confounding factors. The ultimate challenge will be to successfully integrate genetic data into individual HCC risk assessment models. The incremental value of this genetic information must be assessed in order to refine the prognosis in ALD patients, that is, to measure its relative weight in various phenotypes of patients with alcoholic cirrhosis encountered in clinical practice. This

approach is currently providing interesting results in both alcoholic- and HCV-induced cirrhotic cohorts [38,67,71]. For example, it has been shown that adding PNPLA3 genotypes to simple clinical scoring systems in prospectively followed-up patients with alcoholic cirrhosis led to a better prediction of liver cancer emergence at the individual level [38]. In this setting, numerous SNPs could be incorporated into complex HCC predictive scores combining genetic and clinical parameters, enabling selection of patients according to level of liver cancer risk. This integrative approach could distinguish specific sets of genes as new theranostic markers of HCC in alcoholic cirrhotic

patients, and might change our perception of the individual risk level of patients in clinical situations where pivotal therapeutic decisions could be made.

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