Research Article

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Alcohol Drinking Mediates the Association between Polymorphisms of *ADH1B* and *ALDH2* and Hepatitis B–Related Hepatocellular Carcinoma

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Abstract

Background: The role of polymorphisms on *ADH1B* and *ALDH2* in patients with chronic hepatitis B is unclear. This study aims to examine whether alcohol drinking mediates the association between two *ADH1B* and *ALDH2* polymorphisms and the risk of hepatocellular carcinoma among chronic hepatitis B patients.

Methods: A total of 3,824 individuals were enrolled in this study. Two SNPs, rs1229984 (*ADH1B*) and rs671 (*ALDH2*), were genotyped using the Affymetrix Axiom Genome-Wide CHB1 Array (Affymetrix, Inc). Multivariate unconditional logistic regression and mediation analyses were used, comparing CT or TT with CC for rs1229984 and GA and AA with GG for rs671.

Results: There were 602 cases of hepatocellular carcinoma and 3,222 controls. Frequencies of the rs1229984 (*ADH1B*) T allele and rs671 (*ALDH2*) A allele were 72.9% and 28.8%, respectively. Individuals who carried at least one deficient allele for both SNPs were significantly less likely to become habitual alcohol drinkers, with an OR and 95% confidence interval (CI) of 0.24 (0.15–0.40). Alleles for rs1229984 (*ADH1B*) and rs671 (*ALDH2*) were not associated with hepatocellular carcinoma in multivariate analyses. However, mediation analyses showed that the rs1229984 T allele, rs671 A allele, and two SNPs combined were significantly associated with decreased hepatocellular carcinoma risk, mediated through alcohol drinking, with an OR (95% CI) of 0.87 (0.79–0.96), 0.70 (0.61–0.82), and 0.73 (0.58–0.88), respectively.

Conclusions: Polymorphisms on *ADH1B* and *ALDH2* had significant indirect effects on hepatocellular carcinoma risk, mediated through alcohol drinking.

Impact: Future genetic studies of chronic hepatitis B and hepatocellular carcinoma must take mediation effects into consideration. *Cancer Epidemiol Biomarkers Prev; 25(4); 693–9. ©2016 AACR.*

Introduction

Chronic hepatitis B (HBV) infection is a serious public health burden, with an estimated 400 million people worldwide chronically infected (1). It also has the potential to cause serious clinical consequences and is a significant cause of cirrhosis and hepatocellular carcinoma. The burden of chronic HBV infection is uneven, with the most significant burden occurring in areas such as East Asia, the Pacific Islands, Sub-Saharan Africa, and Eastern Europe (1–3). In highly endemic countries, such as Taiwan, transmission primarily occurs perinatally, and the prevalence of infection in the adult population is approximately 8% and was at one time as high as 15% to 20% (1, 4).

Various host, viral, and environmental risk factors for HBV-related hepatocellular carcinoma have been identified, including family history, serum HBV DNA and HBsAg levels, viral genotype, environmental toxins, and alcohol consumption (5–14). Alcohol consumption has been previously established as a risk factor for hepatocellular carcinoma even among individuals with chronic HBV infection (6, 7, 15, 16). In one landmark study, chronically infected individuals who consumed alcohol had an adjusted HR (95% CI) of 1.6 (1.1–2.4) of developing hepatocellular carcinoma compared with those who did not consume alcohol even after adjustment for other strongly predictive factors, such as HBV DNA levels, alanine transaminase (ALT) levels, HBeAg serostatus, and liver cirrhosis (6).

Alcohol metabolism in the liver most commonly involves the enzymes alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH), metabolizing alcohol to acetaldehyde, a toxin and carcinogen, and then to acetate. These two enzymes are encoded by multiple genes, and alleles on these genes can encode variants of these enzymes (17, 18). Two single-nucleotide polymorphisms (SNP), rs1229984 (*ADH1B*, 4q23) and rs671 (*ALDH2*, 12q24.12), are highly prevalent in Asians and have been shown to encode different versions of ADH and ALDH (18). Although carrying the rs1229984 T allele encodes a

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particularly active ADH enzyme that causes rapid accumulation of acetaldehyde, the rs671 A allele encodes an ALDH enzyme that is almost inactive, causing further acetaldehyde accumulation. For the ease of presentation, we term the T allele of rs1229984 and A allele of rs671 the "deficient alleles." Individuals who carry these variants thus experience the toxic and unpleasant effects of acetaldehyde and tend to drink less and are protected against alcoholism or alcohol dependence (17, 19–22). However, when individuals with these variants do drink, they are at higher risk for developing certain cancers (14, 23, 24).

Only some studies have examined the association between these SNPs and the development of hepatocellular carcinoma, and results have been inconclusive, and either consisted of small sample sizes, did not control for confounding factors, or did not fully examine the interaction or mediation effects between these variants and alcohol drinking on hepatocellular carcinoma risk (25–27). Moreover, very few studies have studied these interactions among patients chronically infected with HBV (25). Therefore, this study aims to clarify two things. First, this study will examine the association of *ADH1B* and *ALDH2* polymorphisms with alcohol drinking among patients with chronic HBV, and second, this study will also look at the association and mediation effects between these polymorphisms and alcohol drinking on the risk of hepatocellular carcinoma among those with chronic HBV.

Materials and Methods

Study cohorts

A total of 3,824 individuals with chronic HBV were enrolled in this study, including 3,416 participants from the REVEAL-HBV study and 408 patients with HBV-related hepatocellular carcinoma obtained from the Taiwan Liver Cancer Network (TLCN). The entire combined study cohort thus consisted of 602 patients with hepatocellular carcinoma taken from both the REVEAL-HBV and TLCN cohort and 3,222 controls taken from the REVEAL-HBV cohort.

The REVEAL-HBV study is a community-based cohort study consisting of individuals with ages 30 to 65 years at baseline who were HBsAg positive, anti-HCV negative, and free of liver cirrhosis at recruitment during 1991 to 1992. Participants were followed-up every six to twelve months with examinations that included blood collection for testing of HBV DNA, HBsAg, HBeAg, and ALT. Detailed information on study procedures have been previously described (5, 6, 28). TLCN is comprised of five major medical centers across Taiwan and aims to recruit liver cancer patients from various socioeconomic and ethnic backgrounds. TLCN has a participation rate of over 90%. Newly diagnosed hepatocellular carcinoma patients are contacted as soon as they are admitted to the hospital, at which time they are asked to participate, and the questionnaire is administered. Participating centers follow standard protocol to collect tumor tissues and blood samples, as well as clinical, pathologic, and epidemiologic information from patients with liver cancer. All hepatocellular carcinoma patients older than 30 years of age and anti-HCV seronegative were eligible to be enrolled into the TLCN cohort. Samples were obtained from TLCN after application to the network and approval by the research committee. Patients from both cohorts were infected with HBV at birth, as in Taiwan, HBV is primarily transmitted perinatally from mother to infant. All participants from the REVEAL-HBV study or the TLCN provided written informed consent. This study was approved by the Institutional Review Board of the College of Public Health, National Taiwan University, Taipei, Taiwan.

Ascertainment of alcohol drinking and hepatocellular carcinoma

Alcohol drinking amount and frequency was determined in baseline questionnaires administered at enrollment of the REVEAL-HBV study and by questionnaires administered to hepatocellular carcinoma patients upon enrollment into TLCN. Habitual drinking was defined as consuming alcohol at least four times per week for over one year. Cases of hepatocellular carcinoma in the REVEAL cohort were detected through follow-up examinations, which included both ultrasound and AFP testing, and through computerized linkages with the National Cancer Registry and National Death Certification databases. Identified cases were confirmed through medical chart reviews by gastroenterologists according to the following criteria: histopathologic confirmation; positive lesions detected by at least two different imaging techniques (such as abdominal ultrasonography, angiogram, or computed tomography); or positive lesions detected by one imaging technique combined with a serum α-fetoprotein level greater than 400 ng/mL. In the TLCN cohort, cases were ascertained through diagnosis and testing at participating hospitals.

Selection and genotyping of *ADH1B* and *ALDH2* SNPs

Two SNPs, rs1229984 (*ADH1B*) and rs671 (*ALDH2*), were chosen according to previous studies that have demonstrated their roles in alcohol metabolism and the prediction of alcohol dependence among those of East Asian descent (18, 21). These two SNPs were genotyped as a part of a genome-wide association study (GWAS) of the 3,824 individuals in the combined cohort using the Affymetrix Axiom Genome-Wide CHB1 Array (Affymetrix, Inc), which maximizes genomic coverage of common alleles (MAF > 5%) of the Han Chinese genome. Early studies using Affymetrix microarrays to detect SNPs have been cited for genes including HIV-1, and this GWAS array has also been used in various studies in other diseases among the Han Chinese population (29, 30).

Statistical analysis

Host and virologic factors that were available in both REVEAL and TLCN cohorts were included as predictors in analyses. These factors included age, gender, serum ALT and HBV DNA levels, alcohol drinking, and genotypes for rs1229984 and rs671. Including ALT in this study is important. A signal of liver necroinflammation or damage, it is a marker of the immune response of the body to the virus, as well as the inflammation status of each patient at study entry, and also considers the effects of other seromarkers, independent of liver inflammation. Differences in the distributions of host and virologic characteristics between cases and controls were compared with the χ^2 test. The association with alcohol drinking was done to confirm the association between SNPs and alcohol drinking and included factors such as age and gender, which are important in Asians (21). As detailed time-to-event data was not available in the TLCN cohort, this study was analyzed as a case–control study, using unconditional logistic regression to estimate ORs [with 95% confidence intervals (CI)], adjusting for age, gender, serum ALT and HBV DNA levels, habitual alcohol drinking, and the SNPs rs1229984 and rs671. The direct and indirect effects of the two SNPs on hepatocellular

Table 1. Baseline characteristics of composite REVEAL-TLCN cohort

	Cases (HCC; *n* = 602)	Controls (non-HCC; *n* = 3,222)	*P*
Age (years)			
30–39	97 (16)	1,129 (35)	<.001
40–49	169 (28)	921 (29)	
50–59	196 (33)	886 (28)	
>60	132 (22)	286 (9)	
Gender			
Female	179 (30)	1,322 (41)	<.001
Male	415 (70)	1,900 (59)	
ALT (U/L)			
<45	388 (64)	3,064 (95)	<.001
>45	214 (36)	158 (5)	
HBV DNA level (copies/mL)			
<300	36 (6)	782 (26)	<.001
300–9,999	99 (17)	1,003 (33)	
10,000–999,999	247 (42)	819 (27)	
>1 million	200 (34)	454 (15)	
Alcohol drinking			
No	424 (72)	2,854 (89)	<.001
Yes	167 (28)	363 (11)	
rs1229984 *(ADH1B)*			
CC	48 (8)	236 (7)	<.001
CT	262 (44)	1,229 (38)	
TT	283 (47)	1,748 (54)	
rs671 *(ALDH2)*			
GG	303 (50)	1,617 (50)	0.11
GA	248 (41)	1,354 (42)	
AA	49 (8)	250 (8)	

Abbreviation: HCC, hepatocellular carcinoma.

carcinoma risk were examined using mediation analyses incorporating statistical interaction between the SNPs and alcohol drinking (31). Specifically, the indirect effect is the effect of the two SNPs on hepatocellular carcinoma risk mediated through alcohol drinking, and the direct effect is the alternative effect independent of alcohol drinking but perhaps through other biologic mechanisms. CIs of direct and indirect effects were estimated from 1,000 bootstrapping repetitions, and *P* values were calculated by bootstrapping with normal approximation. Further details behind the methodology of the mediation analyses can be found elsewhere (32–34). Finally, a principal component analysis (PCA) was performed to ensure that there was no population stratification within the cohort. All analyses were performed with SAS Software (version 9.3; SAS Institute, Cary, NC) and R 3.2.0, and statistical significance was determined using two-tailed tests ($P < 0.05$).

Results

Patient characteristics

Table 1 lists the baseline characteristics of hepatocellular carcinoma cases and controls. Among 602 cases of hepatocellular carcinoma and 3,222 non-hepatocellular carcinoma controls, there was a higher percentage of males in cases than controls (70% vs. 59%), higher ALT levels among cases (36% vs. 5%), and higher HBV DNA levels among cases as well (76% greater than 10,000 copies/mL compared with 42% among controls). The frequency of the deficient rs1229984 (*ADH1B*) T allele was 72.9% in the overall cohort, as well as 69.8% in cases and 75.6% in controls, whereas the frequency of the deficient rs671 (*ALDH2*) A allele was 28.8% in the overall cohort, while also 28.8% in cases and 28.8% in controls. After performing a PCA, there was no apparent population stratification, even when stratified by hepatocellular carcinoma status, *ADH1B* or *ALDH2* genotype, or cohort (Supplementary Fig. S1).

Association with habitual alcohol drinking

Additional analyses to predict alcohol drinking were performed to confirm the association between rs1229984 and rs671 and habitual alcohol drinking. Carriers of the T allele for rs1229984 or of the A allele for rs671 were significantly less likely to become habitual alcohol drinkers. Compared with those with rs1229984 CC and rs671 GG genotypes, only individuals who carried at least one deficient allele for both SNPs were significantly less likely to become alcohol drinkers, with an age-adjusted OR (95% CI) of 0.24 (0.15–0.40).

Association with hepatocellular carcinoma incidence

Table 2 shows the analyses of factors associated with hepatocellular carcinoma. Univariate analysis did not show significant differences in the distribution of rs1229984 CC and CT/TT genotypes or rs671 GG and GA/AA genotypes between hepatocellular carcinoma cases and non–hepatocellular carcinoma controls. Similarly, when the two SNPs were combined, no difference in distributions was seen for combinations of genotypes between hepatocellular carcinoma cases and controls in univariate analyses (data not shown).

Multivariate models examined the direct effect of rs1229984 and rs671 genotypes on hepatocellular carcinoma risk, as shown in Table 2. Model 1 looked at alcohol drinking, rs1229984 and rs671 independently, and found that after adjustment for each other and important confounders, alcohol drinkers had a 2.6-fold higher risk for hepatocellular carcinoma, although the two SNPs did not seem to significantly affect hepatocellular carcinoma risk

Table 2. Multivariate analyses of factors associated with HCC

	Adjusted OR (95% CI)	*P*
Model 1		
Age (years; vs. 30–39)		
40–49	2.09 (1.55–2.82)	<.001
50–59	3.36 (2.51–4.51)	<.001
>60	7.65 (5.42–10.79)	<.001
Gender (vs. female)		
Male	1.09 (0.87–1.38)	0.46
ALT (U/L; vs. <45)		
>45	7.64 (5.86–9.95)	<.001
HBV DNA level (copies/mL; vs. <300)		
300–9,999	1.93 (1.32–2.81)	<.001
10,000–999,999	5.53 (3.91–7.80)	<.001
>1 million	6.78 (4.68–9.83)	<.001
Alcohol drinking (vs. No)		
Yes	2.55 (1.94–3.36)	<.001
rs1229984 *(ADH1B*; vs. CC)		
CT/TT	0.97 (0.67–1.40)	0.86
rs671 *(ALDH2*; vs. GG)		
GA/AA	1.19 (0.96–1.47)	0.11
Model 2		
rs1229984/rs671 genotype[a] (vs. CC/GG)		
TC or TT/GG	1.44 (0.82–2.55)	0.21
CC/GA or AA	2.34 (1.14–4.83)	0.02
TC or TT/GA or AA	1.61 (0.91–2.85)	0.10
Model 3		
rs1229984/rs671 genotype[a] (vs. wild type or one mutant)		
TC or TT/GA or AA	1.10 (0.89–1.36)	0.38

Abbreviation: HCC, hepatocellular carcinoma.
[a]Also adjusted for age, gender, ALT, HBV DNA, and alcohol drinking.

Table 3. Mediation analyses for *ADH1B*, *ALDH2*, alcohol consumption, and HCC risk

	ADH1B		*ALDH2*	
Effect type	**OR (95% CI)[a,b]**	***P***	**OR (95% CI)[b,c]**	***P***
Marginal	0.93 (0.64–1.35)	0.70	1.02 (0.84–1.25)	0.83
Without SNP by alcohol interaction				
Direct	0.97 (0.67–1.50)	0.89	1.19 (0.97–1.52)	0.10
Indirect	0.87 (0.79–0.96)	.006	0.74 (0.67–0.82)	<.001
Proportion of mediation (%)	81.3		—[d]	
With SNP by alcohol interaction				
Direct	0.98 (0.58–1.67)	0.95	1.38 (0.98–1.95)	0.07
Indirect	0.87 (0.79–0.96)	.006	0.70 (0.61–0.82)	<.001
Proportion of mediation (%)	87.6		—[d]	

Abbreviation: HCC, hepatocellular carcinoma.
[a]CT/TT vs. CC.
[b]Adjusted for age, gender, HBV DNA, ALT, and the other SNPs.
[c]GA/AA vs. GG.
[d]Not valid as effects are in opposite directions.

[OR (95% CI) = 0.97 (0.67–1.40) and 1.19 (0.96–1.47), respectively]. In model 2, alcohol drinking habit was examined independently, while the two SNPs were combined for a composite variable. Although alcohol drinkers still had a 2.5-fold increased risk of hepatocellular carcinoma (not shown), carrying deficient alleles for one or both SNPs did not show a direct effect on hepatocellular carcinoma risk. Model 3 combined all individuals with one or less deficient genotypes as a referent and found that carriers of both deficient alleles still did not show a direct effect on hepatocellular carcinoma risk [OR (95% CI = 1.10 (0.89–1.36)]. These models show that when direct effects of alcohol drinking and rs1229984 and rs671 SNPs were analyzed, after considering other important risk predictors, alcohol drinking showed a strong direct effect on hepatocellular carcinoma risk, while carriers of one or both deficient alleles did not show visibly increased effects on developing hepatocellular carcinoma, suggesting that these SNPs may not have direct effects, independent of alcohol drinking, on hepatocellular carcinoma incidence (Table 2).

Mediation analyses of SNPs, alcohol drinking habit, and hepatocellular carcinoma

To further clarify the roles of rs1229984, rs671, and habitual alcohol drinking, mediation analyses were performed to determine whether or not the effect of rs1229984 and rs671 on hepatocellular carcinoma was mediated through alcohol drinking, rather than focusing on the marginal effect. Table 3 shows that indeed, both SNPs have highly significant effects on hepatocellular carcinoma risk, mediated through alcohol consumption. *ADH1B* indirectly affects hepatocellular carcinoma risk through alcohol drinking [OR (95% CI) = 0.87 (0.79–0.96)] and did not have any direct effect on hepatocellular carcinoma risk, independent of alcohol drinking [OR (95% CI) = 0.98 (0.58–1.68)]. Under the risk difference scale, more than 80% of the *ADH1B* effect on hepatocellular carcinoma risk is mediated through alcohol drinking (Table 3). *ALDH2*, on the other hand, also had significant indirect effects on hepatocellular carcinoma risk mediated through alcohol drinking [OR (95% CI) = 0.70 (0.61–0.82)], while also having a marginally significant direct effect of the opposite direction on hepatocellular carcinoma risk [OR; (95% CI) = 1.38 (0.98–1.95)]. When both SNPs were examined together, as shown in Table 4, significant indirect effects on hepatocellular carcinoma were seen only in individuals who carried both deficient alleles (CT or TT/GA or AA), with an adjusted OR (95% CI) of 0.73 (0.58–0.88). Similarly, no direct effects on hepatocellular carcinoma risk were seen. Analyses did not find any significant SNP by alcohol interaction.

Discussion

Although previous studies have at times examined the statistical interaction between alcohol drinking, and SNPs on *ADH1B* and *ALDH2*, they did not examine the mechanistic coordination of genetic factors and alcohol consumption on hepatocellular carcinoma risk. We utilize mediation analyses to investigate the indirect and direct effects of these SNPs on hepatocellular carcinoma risk with respect to alcohol drinking. To our knowledge, this is the largest study to examine the relationship between the SNPs rs1229984 and rs671, alcohol drinking, and future risk for hepatocellular carcinoma among individuals chronically infected with HBV. Whereas other studies have focused on simple adjustment for confounding factors, this study instead analyzed mediation effects using a mechanistic approach, which enables the examination of both direct and indirect effects, of these SNPs, while also accounting for SNP-by-alcohol interaction.

This study has two main findings. First, this study confirmed the protective effect of rs1229984 and rs671 against alcohol drinking among a large cohort of individuals with chronic HBV in Taiwan. Interestingly, we found that only individuals who carried at least

Table 4. Combination mediation analyses for *ADH1B*, *ALDH2*, alcohol consumption, and HCC risk

	Direct effect		Indirect effect	
***ADH1B/ALDH2* genotype**	**OR (95% CI)[a]**	***P***	**OR (95% CI)[a]**	***P***
CC/GG	1.0		1.0	
CT or TT/GG	1.34 (0.64–2.66)	0.41	1.03 (0.93–1.17)	0.57
CC/GA or AA	1.76 (0.69–5.77)	0.31	1.02 (0.87–1.24)	0.79
CT or TT/GA or AA	1.67 (0.74–2.54)	0.20	0.73 (0.58–0.88)	0.005

[a]Adjusted for age, gender, HBV DNA, and ALT.

one deficient allele for both polymorphisms were significantly protected against alcohol drinking, with a 4-fold decrease in risk.

Second, this study also found that SNPs on *ADH1B* and *ALDH2* had significant effects on hepatocellular carcinoma risk, mediated through habitual alcohol drinking. Traditional multivariate analyses in this study found significant association between alcohol drinking and hepatocellular carcinoma, with a greater than 2.5-fold OR, but did not show any direct association between hepatocellular carcinoma and the SNPs rs1229984 and rs671. Past studies examining the association between alcohol drinking and *ADH1B*/*ALDH2* also did not find significantly increased risk for hepatocellular carcinomaamong drinkers with chronic liver disease or chronic HBV who carried at least one polymorphism (25–27).

However, this does not mean that these SNPs did not play a significant role in hepatocarcinogenesis, as mediation effects were not considered. The seemingly null overall effect of ALDH2 is the consequence that direct and indirect effects mask each other. Further mediation analyses showed that carrying deficient alleles for both of these polymorphisms did in fact have a strong association with hepatocellular carcinoma risk and that this effect was highly mediated through the environmental risk factor of habitual alcohol consumption. The SNPs rs1229984 and rs671 were protective against the development of hepatocellular carcinoma through their protective effect against habitual alcohol drinking (indirect effect). Put in another perspective, it can be said that carriers of normal genotypes for these two SNPs are more likely to drink alcohol, thus increasing their risk for hepatocellular carcinoma. When examined separately, although *ADH1B* (rs1229984) did not have any significant direct effect on hepatocellular carcinoma incidence, *ALDH2* (rs671) did have a marginally significant direct effect on increasing hepatocellular carcinoma risk, independent of habitual alcohol drinking (Table 3). It is interesting to note that we also did not see any significant marginal effect of the two SNPs, as their effect was largely masked by the downstream heterogeneity of habitual alcohol drinking. The interesting findings revealed by mediation analyses demonstrate its advantage compared with the conventional main effect or statistical interaction approach. Another example of effect cancellation by a positive direct effect and a negative indirect effect can be found in the study by Huang and colleagues (2015; ref. 32).

In addition, we did not find any significant SNP-by-alcohol interaction, meaning that the effects seen did not vary according to alcohol drinking habit. Had there been significant interaction, it would suggest that the SNPs perhaps also altered the susceptibility of patients to the effects of alcohol. Such gene–environment interactions have been seen in other studies of mediation effects between genetic factors, smoking, and lung cancer (35). However, this was not seen in the current study; it can be inferred that rs1229984 and rs671 significantly affects alcohol consumption behavior itself, rather than susceptibility to the effects of alcohol, which in turn subsequently significantly reduces risk for developing hepatocellular carcinoma. However, future studies should examine whether this is mechanistically the case.

In addition, the marginal increase in hepatocellular carcinoma risk that was seen for carriers of the rs671-deficient allele is consistent with previous studies, which suggested that increased acetaldehyde accumulation among drinkers who carry these SNPs can lead to increased risk for certain cancers, including liver cancer (14, 23). However, the true effect of *ALDH2* on hepatocellular carcinoma risk remains to be clarified. It is known that other sources of acetaldehyde exposure exist, including cigarette smoke and indoor/outdoor air. Clarification of the direct effects of *ALDH2* would require a cohort of nondrinkers who are exposed to these secondary sources. However, this was not possible due to a lack of sample size, and future studies should investigate other acetaldehyde sources as well.

The results of this study also emphasize the importance of alcohol consumption as a nongenetic risk factor for hepatocellular carcinoma. As seen in Table 2, habitual alcohol consumption still confers significantly higher risk for hepatocellular carcinoma, again confirming the significant role of alcohol consumption, even after adjustment for other well-known risk factors.

Unfortunately, despite strong evidence supporting alcohol as an environmental risk factor for hepatocellular carcinoma, the exact pathways by which alcohol causes hepatocellular carcinoma are still unknown. Many mechanisms of alcohol-induced hepatocarcinogenesis have been suggested. One commonly hypothesized pathway is through oxidative stress; the metabolism of ethanol by ADH leads to an accumulation of acetaldehyde, as well as the production of free radicals (36). Acetaldehyde has been shown to affect DNA replication and repair mechanisms. In addition, consuming large amounts of ethanol induces microsomal ethanol metabolism by CYP2E1, which leads to additional production of acetaldehyde, as well as an increase in free radicals that can lead to cell death, DNA damage, and even production of other carcinogenic substances (13, 14, 36–38).

Other hypothesized pathways have included the decreased DNA methylation of tumor promoter genes (39, 40). It has also been suggested that alcohol inhibits natural killer (NK) immune surveillance; studies have shown decreased numbers of NK cells in humans with alcoholic cirrhosis, and alcohol administration has also caused increases in lung cancer metastasis in NK cell–controlled cancer, implying that alcohol use can affect the ability of the body to detect and eliminate early cancer (41).

Finally, strong synergistic interaction also exists between alcohol consumption and chronic hepatitis virus infection. In one study, compared with nondrinkers uninfected with chronic hepatitis, heavy drinkers with chronic hepatitis had an adjusted OR (95% CI) of 53.9 (7.0–415.7) for developing hepatocellular carcinoma, compared with 2.4 (1.3–4.4) and 19.1 (4.1–89.1) for alcohol drinking alone and chronic hepatitis alone, respectively (42). These results suggest a possible common pathway for hepatocarcinogenesis, through both cirrhosis and/or oxidative stress, although as discussed above, the exact pathways are still unclear and need to be elucidated.

This study also highlights the importance of taking mediation effects into consideration in future genetic studies. As seen in this study, traditional analyses showed no significant effect of rs1229984 and rs671 on hepatocellular carcinoma risk. However, this study has shown that results of these direct analyses can be misleading if mediation effects are not considered. Moreover, in diseases such as hepatocellular carcinoma that have well-established nongenetic risk factors, future studies should focus more carefully on biologically plausible and mechanistic models that take into account gene–environment interaction, rather than on purely GWAS-based studies, which would have missed the SNPs discussed in this study entirely. As these SNPs did not have a significant direct effect on hepatocellular carcinoma, traditional GWAS studies would be unable to detect them, and, in addition,

would have been unable to determine that the joint effect of both genes was needed to see significant mediation effects. Future genetic studies must also consider the possibility that certain genes may act to determine environmental exposure instead of the disease itself and should also be sure to take variant-by-mediator interaction into effect, so as to clearly delineate the different effects of gene–environment interaction as true interaction versus mediation.

For causal inference, mediation analyses assume that after adjustment for covariates, there is no confounding of the association between (i) SNPs and hepatocellular carcinoma, (ii) alcohol drinking and hepatocellular carcinoma, (iii) SNPs and alcohol drinking, and (iv) no common cause of alcohol drinking and hepatocellular carcinoma that are affected by the SNPs (33). Because PCAs showed no population stratification within the current cohort (Supplementary Fig. S1), assumptions (i) and (ii) hold. Assumption (ii) has been shown to be true in previous studies (43), and assumption (iv) holds based on specific functions of these two SNPs as discussed above.

This study was conducted in a homogeneous cohort of patients of Han Chinese descent, which has been shown in the 1000 Genomes Project to have much higher population frequencies of the rs1229984 "T" allele and the rs671 "A" allele than other populations. For example, frequencies of the rs1229984 "T" allele were 0%, 6%, and 3% in African, American, and European populations, respectively, compared with 72.9% in the REVEAL-TLCN cohort. More strikingly, reported frequencies of the rs671 "A" allele were 0% for African, American, and European populations compared with 28.8% in the REVEAL-TLCN cohort (44). Therefore, the results of this study would only apply to East Asian populations, and mediation effects of these two SNPs would not be able to be measured in other populations. However, the mediation effects of other reported SNPs affecting alcohol metabolism should be explored in non–East Asian populations.

This study has several limitations. Detailed information on exact drinking volume was not available for all participants, and therefore, drinking was classified as habitual drinking or not, defined as drinking four or more times per week or for a duration of at least one year. We are confident that our definition of drinking adequately separates heavy drinkers from light-to-moderate drinkers. Moreover, alcohol use was self-reported via questionnaire and for the TLCN cohort was reported after diagnosis. Therefore, the definition of alcohol use in this study may be subject to biases such as recall bias, resulting in possible misclassification of alcohol drinking status. Although we also did not have information on other environmental risk factors for alcohol dependence, we did include age and gender. Participants were from 30 to 65 years old and mostly infected perinatally with HBV genotypes B and/or C. These results need to be clarified in individuals infected with other HBV genotypes, such as A and D. Data on some predictors, such as serum HBsAg levels and family history, were not available in the TLCN patients. Therefore, these predictors could not be considered in this analysis.

In conclusion, the association between *ADH1B* and *ALDH2* polymorphisms and hepatocellular carcinoma risk is significantly mediated by habitual alcohol consumption. Therefore, management of patients with chronic HBV should, in addition to focusing on viral suppression, also particularly in patients with wild-type *ADH1B* and *ALDH2* polymorphisms, reduce the harmful effects of important environmental risk factors such as alcohol consumption.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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