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ORIGINAL RESEARCH

Genetic variants of *ALDH2*-rs671 and *CYP2E1*-rs2031920 contributed to risk of hepatocellular carcinoma susceptibility in a Chinese population

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Objective: Acetaldehyde dehydrogenase 2 (*ALDH2*) and cytochrome P450 2E1 (*CYP2E1*) have been associated with hepatocellular carcinoma (HCC) susceptibility and prognosis. The polymorphisms *ALDH2* rs671 and *CYP2E1* rs2031920 are reportedly correlated with the prevalence of HCC in other countries. The aim of this study was to investigate associations between *ALDH2* and *CYP2E1*, and HCC susceptibility in a population of Guangxi, southern China, an area with a high incidence of HCC.

Patients and methods: The study cohort included 300 HCC cases, 292 healthy controls for HCC susceptibility analysis, and another 20 HCC cases and 10 healthy controls for ascertainment. Genotyping was performed using the polymerase chain reaction-restriction fragment length polymorphism method.

Results: The study results demonstrated that mutant genotypes of *ALDH2* (G/A and A/A) led to significant differences in HCC susceptibility, as compared with the wild genotype (G/G) with the same C1/C1 genotype in non-drinking individuals (adjusted P=0.010, OR=0.20, 95% CI=0.06–0.68). The mutant genotypes of *CYP2E1* (C1/C2 and C2/C2) brought about significant differences in HCC susceptibility, as compared with the wild genotype (C1/C1) and the same G/G genotype (adjusted P=0.025, OR=0.42, 95% CI=0.20–0.90). Drinking plays a role in HCC susceptibility in the same G/G genotype individuals (adjusted P=0.004, OR=0.32, 95% CI=0.15–0.69), but had no impact when combined with *CYP2E1* for analysis (all P>0.05).

Conclusion: These results suggest that the mutant genotypes of *ALDH2* and *CYP2E1* may be protective factors for HCC susceptibility in Guangxi province, China.

Keywords: hepatocellular carcinoma susceptibility, genetic polymorphism, *ALDH2*, *CYP2E1*, Chinese population

Introduction

Hepatocellular carcinoma (HCC), the most common histological subtype of primary liver cancer, is the third most common cause of cancer-related death, with an estimated 745,500 HCC-related deaths worldwide in 2012: about 50% of which occurred in China.[1] Etiologically, chronic infections of hepatitis B and C viruses, aflatoxin B1 exposure, and alcohol abuse are the primary risk factors for HCC occurrence.[2,3] Alcohol intake varies among cultures, but is prevalent around the world and likely to induce alcohol dependence in heavy drinkers, which account for approximately 14% of the general population,[4] approximately 10–15% of whom are at high risk of liver cirrhosis, including a small percentage at an exceptional risk for many types of cancers.[5,6] It is well documented that liver cirrhosis can eventually impel the occurrence of HCC. Therefore, identifying alcohol metabolizing genes may help to postpone

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and even prevent the manifestation of HCC. Although there are many treatment options for HCC, such as hepatectomy, liver transplantation, radiofrequency ablation, transcatheter arterial chemoembolization, percutaneous acid injection, immunotherapy, and percutaneous ethanol injection,[7,8] and prognosis has improved remarkably in recent years, prognosis of HCC remains dissatisfactory, especially when diagnosed at advanced stages. Specifically, the 5-year survival rate of HCC is less than 20% in the United States.[9] Consequently, the validation of alcohol metabolizing genes on HCC carcinogenesis and progression is of great significance.

Acetaldehyde dehydrogenase 2 (*ALDH2*) and cytochrome P450 2E1 (*CYP2E1*) are two principle enzymes involved in alcohol metabolism. Acetaldehyde (AA) is produced from alcohol by alcohol dehydrogenase and CYP2E1, and is then metabolized to acetate by *ALDH2*.[5] *CYP2E1*, a member of the cytochrome P450 superfamily and microsomal ethanol oxidizing system, is induced by greater amounts of ethanol though *CYP2E1* metabolism of ethanol, which is predominantly metabolized by alcohol dehydrogenase, and ranges from 10–30% via the non-alcohol dehydrogenase pathway.[10,11] CYP2E1 enzymatic activity is closely associated with the RsaI polymorphism of the *CYP2E1* gene, also known as rs2031920, which contains a wild type C1 allele and a variant C2 allele.[12–14] Besides, the mutant C2 allele is related to reduced CYP2E1 enzymatic activity.[12]

ALDH2, a mitochondrial enzyme, is not only primarily responsible for the oxidation of AA, but is also encoded by two alleles (G and A) that are closely correlated with AA metabolism.[5,15] Individuals heterozygous or homozygous with a mutated allele metabolize ethanol to AA normally, but metabolize AA poorly, while those homozygous for the variant A allele are completely devoid of ALDH2 activity, and those heterozygous with the G/A phenotype display only 17–50% of normal ALDH2 activity.[5,16] Thus, in the population carrying the variant G allele, the capacity to convert AA into acetate is influenced dramatically and leads to an abundance of AA in the circulation, even when consuming a moderate amount of alcohol.[17] Notably, about 40% or so of the Eastern Asian population carry the A/G phenotype.[15] It is well known that AA, rather than ethanol, is highly toxic, carcinogenic, and mutagenic, and has been identified as the cause of Asian Alcohol Flushing Syndrome, which is characterized by nausea, facial flushing, muscle weakness, tachycardia, palpitation, perspiration, headache, and sleepiness.[15,18] Hence, HCC susceptibility could be estimated in individuals with different genotypes by distinguishing the capacity of alcohol ingestion through analysis of drinking habits.

Therefore, the aim of this study was to investigate the role of the ethanol metabolism genes *ALDH2* and *CYP2E1* on HCC susceptibility in a Chinese population in Guangxi province in southern China, an area with a notably high incidence of HCC.

Patients and methods

Study population

The study protocol was approved by the Ethical Review Committee of the First Affiliated Hospital of Guangxi Medical University (Nanning, Guangxi, China), and written informed consent was obtained from all enrolled subjects. A total of 300 HCC patients and 292 healthy control subjects were enrolled from the First Affiliated Hospital of Guangxi Medical University from March 2000 to December 2004 for association analysis between HCC susceptibility and expression levels of the *ALDH2* and *CYP2E1* genes. In addition, 20 HCC patients and 10 healthy control subjects were enrolled from the First Affiliated Hospital of Guangxi Medical University in 2006 to complementary analyses to ascertain the role of ethanol in vitro in patients with different ALDH2 and CYP2E1 genotypes. All cases of HCC were confirmed by pathological diagnosis after hepatectomy. Data regarding the HCC patients were collected from medical records, while information of the control subjects was obtained by questionnaires. Drinking was defined in our research as drinking a high concentration of white wine (ethanol > 40%) at least once in a week, continuing for over half a year.

Collection of HCC tissues and blood samples

HCC tissues were collected within 30 min during surgery and immediately stored at −80°C (Thermo Fisher Scientific, Waltham, MA, USA) until assayed. A 5-mL peripheral venous blood sample was collected from 292 control subjects, mixed well, and then stored in EDTA-coated centrifugation tubes at 4°C (Qingdao Haier Co. Ltd., Qingdao, China). DNA was extracted within 1 week after storage of the blood samples at 4°C and then stored at −20°C (Qingdao Haier Co. Ltd.). A 20-mL peripheral venous blood sample was collected from 30 subjects, of which a 200-µL aliquot was stored in an EDTA-coated centrifugation tube at 4°C for DNA extraction, while the remaining volume was used for lymphocyte extraction (TBDLTS1077).

Lymphocyte identification and growth curve

The blood samples were stained with H&E, then lymphocyte counts were performed under a microscope. The lymphocyte

survival rate was determined by the trypan blue method at 3, 6, 12, 24, and 48 h, respectively, and growth curves were constructed.

Identification of ethanol concentration by the MTT method

Various ethanol concentrations (25, 50, 100, 200, 400, 800, and 1600 mmol/L) were used to assay samples from the experimental group. Using the MTT method (MTT xldT0793), optical density was measured at 490 nm (BioTek, Inc, Vernichi, Vermont, USA). Then, the half maximal inhibitory concentration (IC_{50}) was calculated using the Bliss method, and the ethanol concentration was determined. As the maximum ethanol concentration cut-off was 1/3–1/2 of the IC_{50} value, the ethanol concentration was reasonably set at 200, 100, and 50 mmol/L, respectively.

Identification of ethanol culture time

Using the MTT method, lymphocytes were cultured for various time periods (3, 6, 12, and 24 h, respectively, Gibco 31800-022, FBS, Hangzhou sjq02) in three ethanol gradients (200, 100, and 50 mmol/L, respectively). Accordingly, the cell inhibitory rate was calculated, and the ethanol culture time was determined according to the designed cut-off cell inhibitory rate.

Polymerase chain reaction (PCR) assay

Total RNA was isolated from lymphocytes with the alcohol precipitation method. cDNA was synthesized from mRNA by PCR in a 20.0-μL reaction mixture. Then, PCR amplification of another 20.0-μL reaction mixture was performed. The PCR cycle included a denaturation step at 94°C for 4 min, followed by 30 cycles of denaturing at 94°C for 1 min, annealing at the indicated temperature for 1 min, extension at 72°C for 1 min, and then a final extension step at 72°C for 10 min. The PCR products were identified by electrophoresis at 40 V for 40 min in a 2% agarose gel (xsq01; Biowest, Barcelona, Spain) stained with ethidium bromide (Sigma-Aldrich Co., St Louis, MO, USA) and viewed under ultraviolet light.

Genotyping

ALDH2 rs671 and *CYP2E1* rs2031920 polymorphisms were genotyped using PCR-restriction fragment length polymorphism after PCR amplification using the following primers: rs671, forward: 5′-CAA ATT ACA GGG TCA ACT GC-3′/reverse: 5′-CCA CAC TCA CAG TTT TCT CTT-3′ and rs2031920, forward: 5′-CCA GTC GAG TCT ACA TTG TCA-3′/reverse: 5′-TTC ATT CTG TCT TCT AAC TGG-3′.

Bioinformatic analysis

A variety of available bioinformatic methods were used to verify the expression patterns of the *ADLH2* and *CYP2E1* genes. The Genotype-Tissue Expression (GTEx) portal (https://gtexportal.org/home/; accessed July 1, 2017) was used to search for *ADLH2* and *CYP2E1* expression levels in different tissues and the MERAV website (http://merav.wi.mit.edu/; accessed July 1, 2017) was assessed to retrieve *ADLH2* and *CYP2E1* expression levels in normal and primary tumor tissues. The GeneMANIA prediction server of biological network integration for gene prioritization and predicting gene function (http://genemania.org/; accessed July 1, 2017) was employed to explore potential interactions between *ADLH2* and *CYP2E1*. The STRING database of known and predicted protein–protein interactions (https://string-db.org/; accessed July 1, 2017) was used to search for biological interactions between the ALDH2 and CYP2E1 proteins. The F-SNP integrated website of the functional effects of single nucleotide polymorphisms (SNPs) (http://compbio.cs.queensu.ca/F-SNP/; accessed July 1, 2017) was used to mimic concrete mechanisms of the two SNP sites. Finally, the SNP function prediction website was employed to explore changes in the two SNPs (https://snpinfo.niehs.nih.gov/snpinfo/snpfunc.html, accessed July 1, 2017).

Statistical analysis

The Hardy–Weinberg equilibrium was calculated by the goodness-of-fit χ^2 test. The χ^2 test was performed to analyze the association between polymorphisms of *ALDH2* and *CYP2E1* and HCC susceptibility. The odds ratio (OR) and 95% confidence interval (95% CI) were calculated to estimate relative risk with a binary logistic regression model. The Pearson correlation coefficient was calculated to analyze the correlation between the ethanol concentration and lymphocyte inhibitory rate. All statistical analyses were performed using SPSS version 16.0 software (SPSS, Inc., Chicago, IL, USA). A probability (P) value of <0.05 was considered statistically significant.

Results

Baseline subject characteristics

The genotype frequencies of *ALDH2* and *CYP2E1* met the Hardy-Weinberg equilibrium in both the HCC and control groups (all $P>0.05$). The baseline characteristics of the HCC

Table 1 Baseline characteristics of the HCC and control groups

Factors	HCC (%) (n=300)	Control (%) (n=292)	OR (95% CI)	*P*-value
Sex			1.11 (0.67–1.86)	0.685
Male	265 (88.3)	261 (87.0)		
Female	35 (11.7)	31 (27.0)		
Age (years)			1.62 (0.93–2.84)	0.091
≤60	265 (88.3)	270 (92.5)		
>60	35 (11.7)	22 (7.5)		
Race			0.44 (0.30–0.64)	<0.001
Han	245 (81.7)	193 (64.3)		
Minority	55 (18.3)	99 (35.7)		
Drinking status			1.03 (0.72–1.47)	0.875
Yes	214 (71.3)	210 (71.9)		
No	86 (28.7)	82 (28.1)		
Smoking status			0.92 (0.67–1.27)	0.623
Yes	154 (51.3)	144 (49.3)		
No	146 (48.7)	148 (50.7)		
Tumor family history			0.60 (0.34–1.04)	0.070
Yes	36 (11.6)	22 (7.5)		
No	264 (88.4)	270 (92.5)		
HBsAg status			0.02 (0.01–0.03)	<0.001
+	255 (85.0)	30 (10.3)		
–	45 (15.0)	262 (89.7)		

Abbreviations: HCC, hepatocellular carcinoma; OR, odds ratio.

patients and control group subjects are shown in Table 1. Overall, there were no significant differences in age, sex, drinking status, smoking status, and tumor family history between the HCC and control groups (all $P>0.05$), while there were significant differences in race and HBsAg status (both $P<0.001$).

Correlation between ALDH2 and CYP2E1 SNPs and HCC susceptibility

For the *ALDH2* rs671 polymorphism, neither the wild-type genotype, GG, nor mutant genotypes (G/A and A/A) gave rise to significant differences in HCC susceptibility (all $P>0.05$). For the *CYP2E1* rs2031920 polymorphism, both the wild-type (C1/C1) and mutant genotypes (C1/C2 and C2/C2) failed to produce significant differences in HCC susceptibility (all $P>0.05$). Detailed data are presented in Table 2.

Joint-effects analysis of combining the ALDH2 and CYP2E1 genes on HCC susceptibility

Joint-effects analysis of the ALDH2 and CYP2E1 genes combined on HCC susceptibility showed significant difference between group 2 (GG + C1C2/C2C2) and group 1 (GG + C1C1) (adjusted $P=0.025$), between the non-drinking populations of group 3 (G/A + A/A + C1/C1) and group 1 (G/G + C1/C1) (adjusted $P=0.010$), and between group 4 (GA/AA + C1C2/C2C2) and group 1 (adjusted $P=0.045$). Detailed data are shown in Table 2.

Joint-effects analysis of combining drinking status with ALDH2 or CYP2E1 on HCC susceptibility

Joint-effects analysis of combining drinking status with *ALDH2* or *CYP2E1* on HCC susceptibility showed significant differences between drinking (group 3) and non-drinking individuals with the same GG genotype (adjusted $P=0.004$). Among the non-drinking individuals, there were significant differences between mutant genotypes (group 2) and the wild genotype (adjusted $P=0.014$), while there were no significant differences between the other groups (all $P>0.05$).

For joint-effects analysis of combining drinking status with *CYP2E1* genotypes, there were no significant differences between any groups (all $P>0.05$). Specific results are presented in Table 3.

Lymphocyte growth curve and growth inhibitory curve, and identification of ethanol intervention time

Lymphocytes stained by the H&E method are presented in Figure 1A. A growth curve of lymphocytes is presented in Figure 1B. In order to eliminate hindrance from lymphocytes, the

Table 2 Joint-effects analysis between *ALDH2* or *CYP2E1* and drinking status on HCC susceptibility

Groups	Genotype		Whole		Drinking		Non-drinking		Crude OR	Crude	Adjusted OR	Adjusted
	ALDH2	*CYP2E1*	HCC	Control	HCC	Control	HCC	Control	(95% CI)	*P*-value	(95% CI)	*P*-value*
	G/G		149	152					1.00	0.469	1.00	0.999
	G/A		121	119					1.04 (0.74–1.46)	0.833	0.99 (0.59–1.65)	0.967
	A/A		30	21					1.46 (0.80–2.66)	0.220	1.00 (0.40–2.48)	0.996
	G/A+A/A		151	140					1.10 (0.80–1.52)	0.561	0.99 (0.61–1.62)	0.972
		C1/C1	203	196					1.00	0.546	1.00	0.381
		C1/C2	87	81					1.04 (0.72–1.49)	0.843	0.67 (0.38–1.19)	0.171
		C2/C2	10	15					0.64 (0.28–1.47)	0.295	0.77 (0.22–2.70)	0.684
		C1/C2+C2/C2	97	96					0.98 (0.69–1.38)	0.888	0.69 (0.40–1.18)	0.170
i	G/G	C1/C1	104	96					1.00	0.397	1.00	0.160
ii	G/G	C1/C2+C2/C2	45	56					0.74 (0.46–1.20)	0.223	0.42 (0.20–0.90)	0.025
iii	G/A+A/A	C1/C1	99	100					0.91 (0.62–1.35)	0.653	0.72 (0.40–1.31)	0.287
iiii	G/A+A/A	C1/C2+C2/C2	52	40					1.20 (0.73–1.97)	0.472	0.83 (0.39–1.77)	0.626
1	G/G	C1/C1			70	79			1.00	0.067	1.00	0.137
2	G/G	C1/C2+C2/C2			32	45			0.80 (0.46–1.40)	0.438	0.45 (0.19–1.06)	0.068
3	G/A+A/A	C1/C1			74	63			1.33 (0.83–2.11)	0.235	1.13 (0.56–2.26)	0.740
4	G/A+A/A	C1/C2+C2/C2			38	23			1.87 (1.01–3.43)	0.045	1.33 (0.54–3.28)	0.538
!	G/G	C1/C1					34	17	1.00	0.043	1.00	0.053
! !	G/G	C1/C2+C2/C2					13	11	0.59 (0.22–1.59)	0.299	0.35 (0.07–1.72)	0.197
! ! !	G/A+A/A	C1/C1					25	37	0.34 (0.16–0.73)	0.006	0.20 (0.06–0.68)	0.010
! ! ! !	G/A+A/A	C1/C2+C2/C2					14	17	0.41 (0.17–1.03)	0.058	0.20 (0.04–0.96)	0.045

Note: *Padjustment for race and HBsAg.

Abbreviations: HCC, hepatocellular carcinoma; OR, odds ratio; CI, confidence interval.

Table 3 Joint-effects analysis between *ALDH2* or *CYP2E1* and drinking status on HCC susceptibility

Groups	Drinking status	Genotype	HCC (%) (n=300)	Control (%) (n=292)	Crude OR (95% CI)	Crude *P*-value	Adjusted OR (95% CI)	Adjusted *P*-value*
I	–	G/G	47	28	1.00	0.006	1.00	0.022
2	–	G/A+A/A	39	54	0.43 (0.23–0.80)	0.008	0.32 (0.13–0.79)	0.014
3	+/++	G/G	102	124	0.49 (0.29–0.84)	0.009	0.32 (0.15–0.69)	0.004
4	+/++	G/A+A/A	112	86	0.78 (0.45–1.34)	0.362	0.49 (0.23–1.07)	0.075
I	+	G/G	55	77	1.00	0.004	1.00	0.360
II	+	G/A+A/A	69	68	1.42 (0.88–2.30)	0.153	1.75 (0.85–3.58)	0.127
III	++	G/G	47	47	1.40 (0.82–2.38)	0.215	1.53 (0.68–3.42)	0.306
IV	++	G/A+A/A	43	18	3.34 (1.75–6.41)	<0.001	2.00 (0.78–5.12)	0.148
a	–	C1/C1	59	54	1.00	0.982	1.00	0.338
b	–	C1/C2+C2/C2	27	28	0.88 (0.46–1.68)	0.704	0.69 (0.25–1.85)	0.456
c	+/++	C1/C1	144	142	0.93 (0.60–1.44)	0.737	0.71 (0.37–1.37)	0.307
d	+/++	C1/C2+C2/C2	70	68	0.94 (0.57–1.55)	0.815	0.49 (0.23–1.05)	0.067
A	+	C1/C1	88	98	1.00	0.094	1.00	0.534
B	+	C1/C2+C2/C2	36	47	0.85 (0.51–1.44)	0.550	0.61 (0.28–1.34)	0.215
C	++	C1/C1	56	44	1.42 (0.87–2.31)	0.162	1.14 (0.55–2.37)	0.728
D	++	C1/C2+C2/C2	34	21	1.80 (0.97–3.34)	0.060	1.00 (0.39–2.57)	0.995

Notes: **P* adjustment for race and HBsAg. –, Not drinking; +, drinking little; ++, drinking a lot.
Abbreviations: HCC, hepatocellular carcinoma; OR, odds ratio; CI, confidence interval.

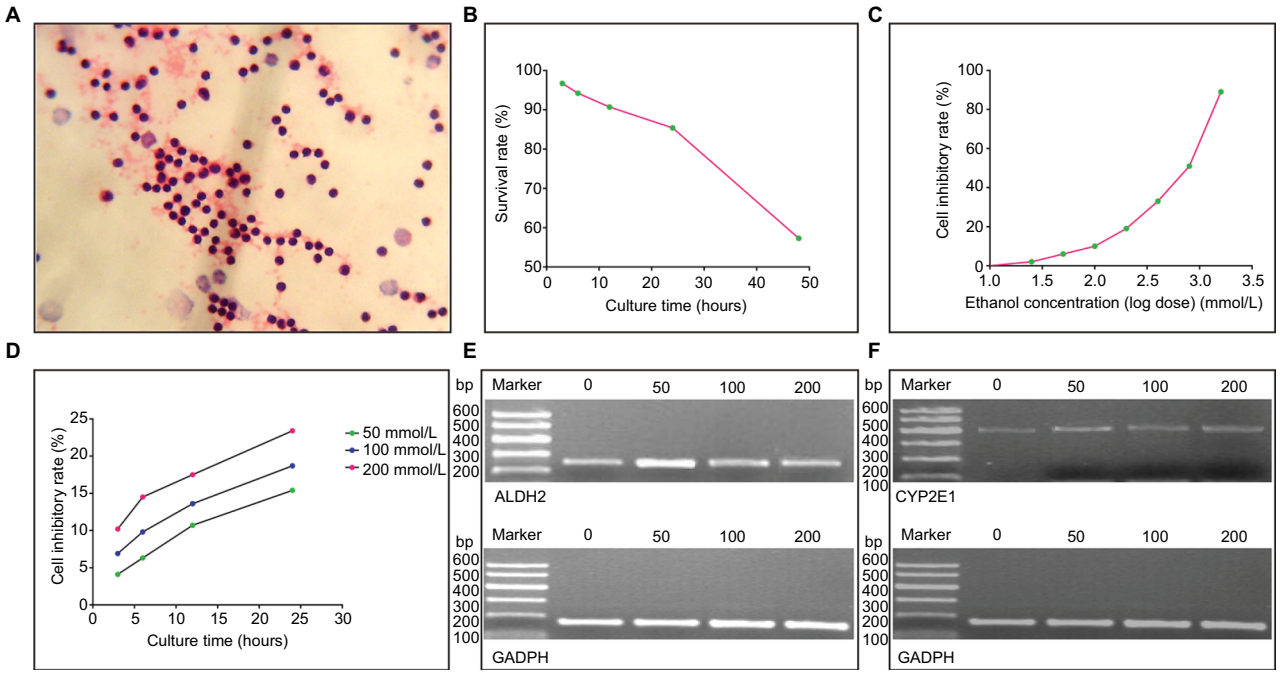

Figure 1 Lymphocytes and *ALDH2*/*CYP2E1* mRNA expression profiles. (**A**) Lymphocytes stained by the H&E method. (**B**) Curve of lymphocyte survival rate–culture time. (**C**) Curve of lymphocyte inhibitory rate–ethanol concentration. (**D**) Curves of lymphocyte inhibitory rate–culture time at different ethanol concentrations. (**E** and **F**) *ALDH2*, *CYP2E1*, and *GAPDH* mRNA expression levels at different ethanol concentrations.

lymphocyte intervention time was set at 4 h before ethanol intervention. Pearson correlation analysis was conducted to identify correlations between lymphocyte inhibitory rates and ethanol concentrations (r=0.993, $P<0.0001$). A lymphocyte inhibitory rate curve (logarithm dose conversion) is shown in Figure 1C.

The IC_{50} value was calculated to be 539.646. Accordingly, ethanol concentrations were set at 200, 100, and 50 mmol/L. A lymphocyte inhibitory curve after intervention for 3, 6, 12, and 24 h is depicted in Figure 1D. The ethanol intervention time was determined as 2 h.

ALDH2 and CYP2E1 mRNA expression levels of the HCC and control groups

The mRNA expression levels of *ALDH2* and *CYP2E1* after intervention with different ethanol concentrations for 2 h are shown in Figure 1E and F, respectively. *ALDH2* mRNA expression levels increased in both the HCC and control groups, as compared with non-ethanol intervention, but then decreased with increasing ethanol concentrations. Ethanol at 50 mmol/L showed a significant difference in the HCC group ($P<0.05$; Figure 2A), while no concentration led to a significant difference in the control group (all $P>0.05$; Figure 2B).

For *CYP2E1* in both the HCC and control groups, mRNA expression levels increased, as compared with non-ethanol intervention, but then decreased with increasing ethanol concentrations. Peculiarly, only 50 mmol/L ethanol intervention showed a significant difference in the HCC group ($P<0.05$; Figure 2C), and all control groups showed statistically significant *P*-values (all $P<0.05$; Figure 2D).

mRNA expression levels of the ALDH2 rs671 and CYP2E1 rs2031920 polymorphisms

For the *ALDH2* rs671 polymorphism, mRNA expression levels increased, as compared with non-ethanol intervention, but then decreased with increasing ethanol concentrations for all genotypes. For the G/G genotype, only 50 mmol/L ethanol intervention brought about a significant difference ($P<0.05$; Figure 2E). For the G/A + A/A genotypes, all three ethanol concentrations gave rise to significant differences (50 and 100 mmol/L, $P<0.01$; 200 mmol/L, $P<0.05$; Figure 2F).

For the *CYP2E1* rs2031920 polymorphism, mRNA expression levels increased, as compared with non-ethanol intervention, but then decreased with increasing ethanol concentrations for all genotypes. For the C1/C1 genotype, no concentration was associated with a significant difference (all $P>0.05$; Figure 2G). For the C1/C2 + C2/C2 genotypes, only 200 mmol/L ethanol intervention produced a significant difference ($P<0.05$; Figure 2H). Specific results are shown in Table 4.

Bioinformatic analysis

Expression levels of *ALDH2* and *CYP2E1* in different tissues were presented in Figure 3. Besides, Figure 4A and 4B showed the expression levels of ALDH2 and CYP2E1 genes in the primary liver tumor tissue and normal liver tissue. Furthermore, interaction networks of them are shown in Figure 4C and D in gene and protein level, respectively.

At a functional significance (FS) score of 0.856, *ALDH2* rs671 was depicted in the following three functional categories: protein coding, splicing regulation, and post-translation modification. In regard to protein coding, most prediction tools brought about damaging effects, and a nonsynonymous mutation was confirmed by Ensembl-NS. In regard to splicing regulation, the results of the four prediction tools were consistently changing. In regard to post-translation modification, both OGPET and Sulfinator gave rise to nonexistent results. In a FS score of 0.398, *CYP2E1* rs2031920 was depicted in the functional category of transcriptional regulation. Ensembl-NS indicated a frameshift result in the coding sequence, while GoldenPath showed no role in regulation, and the Ensembl-TR results confirmed the result of a regulatory region. Detailed data are presented in Table 5. SNP functional prediction results showed that a mutation to rs671 was correlated with splicing, exonic splicing enhancer (ESE), or exonic splicing silencer (ESS), as well as a nonsynonymous mutation, and a mutation in rs2031920 that was associated with the transcription factor binding site (TFBS).

Discussion

In the present study, we first investigated the association of different genotypes of *ALDH2* and *CYP2E1* with HCC susceptibility in the Guangxi Zhuang Autonomous Region, China. The results of this study demonstrated that, in non-drinking individuals, the mutant genotypes of *ALDH2* (G/A and A/A) had significantly different effects on HCC susceptibility, as compared with the wild genotype (G/G) in the same C1/C1 genotype individuals. The mutant genotypes of *CYP2E1* (C1/C2 and C2/C2) resulted in significant differences in HCC susceptibility, as compared with the wild genotype (C1/C1) in the same G/G genotype individuals. Meanwhile, the joint-effects analysis of drinking status and *ALDH2* showed that alcohol consumption led to significant interactions with HCC susceptibility, as compared with non-drinking subjects with the same GG genotype. In the non-drinking individuals, mutant genotypes showed significant differences in HCC susceptibility, as compared with the wild genotype. These results indicate that mutant variants of *ALDH2* and *CYP2E1* genes may be protective factors for HCC susceptibility. Then, to verify these results, mRNA expression levels of *ALDH2* and *CYP2E1* after ethanol intervention were further studied. After intervention with different ethanol gradients, there were significant differences in *ALDH2* mRNA expression levels in all G/A + A/A genotypes, as compared with no ethanol intervention, whereas some groups with the G/G genotype showed significant differences. Meanwhile, there were

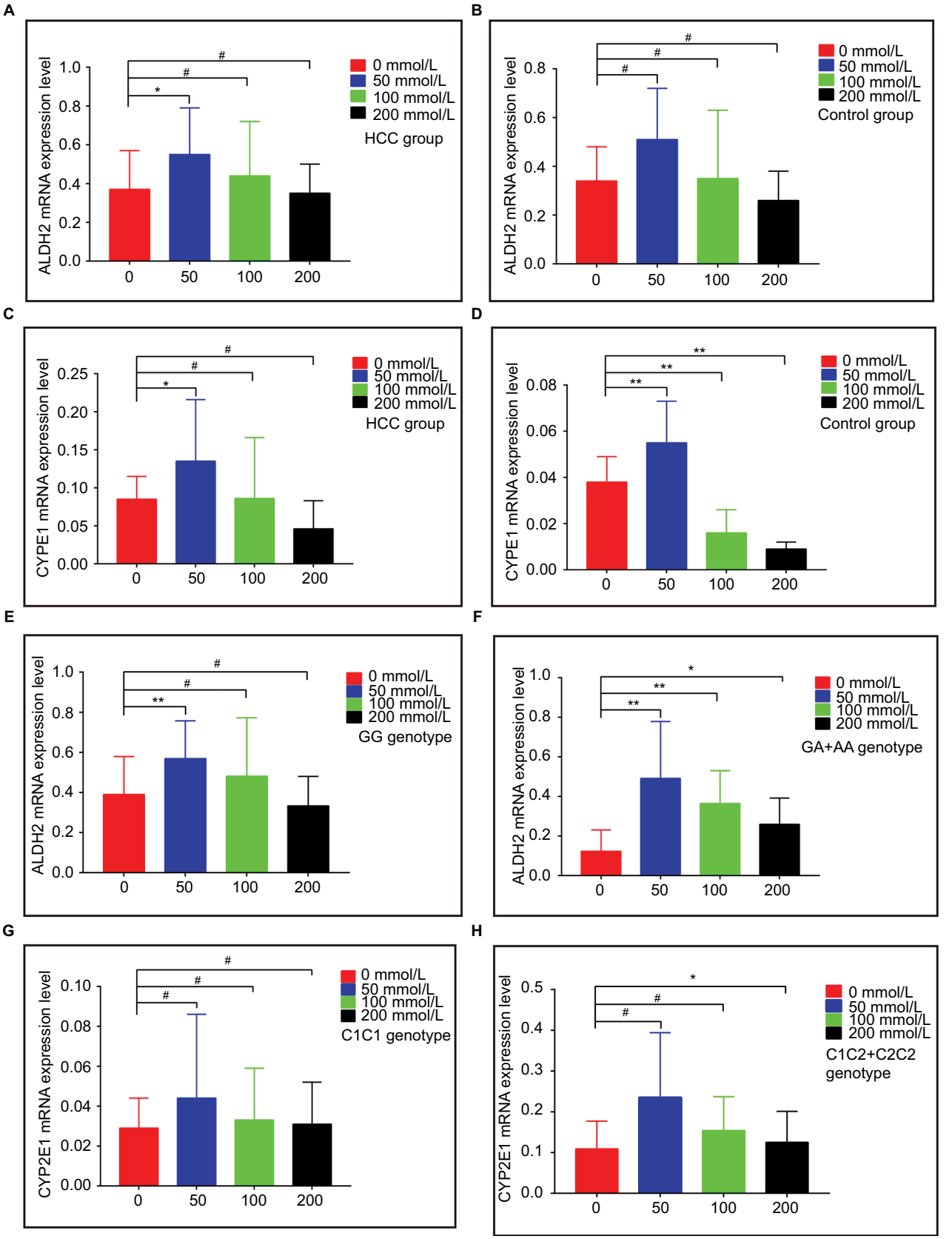

Figure 2 mRNA expression levels of *ALDH2* and *CYP2E1* in different groups. (**A** and **B**) *ALDH2* mRNA expression levels in HCC and control groups. (**C** and **D**) *CYP2E1* mRNA expression levels in HCC and control groups. (**E** and **F**) *ALDH2* mRNA expression levels of the GG and GA+AA genotypes. (**G** and **H**) *CYP2E1* mRNA expression levels of the C1/C1 and C1/C2+C2/C2 genotypes.

Note: * $P<0.05$; ** $P<0.01$; # $P>0.05$.

Abbreviation: HCC, hepatocellular carcinoma.

Table 4 Changes in *ALDH2* and *CYP2E1* mRNA expression levels after ethanol exposures

Gene	Group	Genotype	Subject (n=30)	Ethanol concentration (mmol/L)			
				0	50	100	200
ALDH2	HCC		20	0.37±0.20	0.55±0.24*	0.44±0.28	0.35±0.15
	Control		10	0.34±0.14	0.51±0.21	0.35±0.28	0.26±0.12
CYP2E1	HCC		20	0.09±0.03	0.14±0.08*	0.09±0.08	0.05±0.04
	Control		10	0.04±0.01	0.055±0.02**	0.02±0.01**	0.01±0.00**
ALDH2		G/G	18	0.39±0.19	0.57±0.19**	0.48±0.29	0.33±0.15
		G/A+A/A	12	0.12±0.11	0.49±0.29**	0.36±0.17**	0.26±0.13*
CYP2E1		C1/C1	20	0.03±0.02	0.04±0.04	0.03±0.03	0.03±0.02
		C1/C2+C2/C2	10	0.11±0.07	0.24±0.16*	0.15±0.08	0.13±0.08

Note: *P<0.05, **P<0.01; comparisons were done between before ethanol intervention (0 mmol/L) and different ethanol intervention concentrations (50, 100, 200 mmol/L); data are presented as the mean±standard deviation.
Abbreviation: HCC, hepatocellular carcinoma.

significant differences in *CYP2E1* mRNA expression levels associated with the C1/C2 + C2/C2 genotype, as compared with no ethanol intervention, whereas there was no significant difference associated with the C1/C1 genotype, as compared with no ethanol intervention.

Many investigations of the *ALDH2* rs671 polymorphism have reported its crucial role in disease, especially cancers, by alteration of *ALDH2* expression levels. Mutation reduces *ALDH2* expression owing to a dominant-negative effect and, importantly, leads to significant lowering of ALDH2 protein levels.[19] By elevating the *ALDH2* transcriptional level, *ALDH2* activity increases with the restoration of arachidonic acid levels in hepatoma cell lines.[20] Inhibition of *ALDH2* expression may provide new insights into the associations between cancers and *ALDH2*.[21] Low *ALDH2* expression is associated with *CCNE1* and *SMAD3* expression, and possesses a potential prognostic value in upper tract urothelial carcinoma.[22] It is well established that the activity of the ALDH2 enzyme, which is encoded by a mutant allele, was decreased by 30–50% in heterozygous individuals and to almost zero in individuals with the homozygous mutant.[23] Decreased ALDH2 enzymatic activity, thus, causes AA accumulation as ALDH2 serves as the principal enzyme in AA metabolism. AA is highly toxic, mutagenic, and carcinogenic,[24] and can induce ethanol-metabolizing cancers by its excessive accumulation, including esophageal squamous cell carcinoma,[25,26] oropharyngolaryngeal cancer,[27] gastric cancer,[28–30] colorectal cancer,[31–33] pancreatic cancer,[34–36] and lung cancer.[37–39]

Nevertheless, the results of studies of HCC susceptibility remain controversial. In fact, the studies by Yokoyama et al,[27] Yu et al,[40] Zhou et al,[41] and Koide et al[42] indicated that the ALDH2*2 polymorphism had limited (not statistically significant) risk for HCC onset. Meanwhile, Tomoda et al[43] revealed that ALDH2 rs671 alone was correlated with an increased risk for HCC development, and Liu et al[44] and Munaka et al[45] indicated that the *ALDH2* gene had indirect significance on HCC, in combination with the alcohol dehydrogenase gene. Furthermore, Kato et al[46] demonstrated that the combination of ALDH2 and CYP2E1 could modulate HCC development. The results of the present investigation indicated that mutant *ALDH2* genotypes displayed significant difference in individuals with the C1/C1 genotype, as well as in the non-drinking populations. These findings are consistent with those of previous studies[43,44,46,47] that genotypes, including mutant alleles, have more or less a direct or indirect impact on HCC development due to the *ALDH2* gene alone or combined with other factors.

With respect to the *CYP2E1* rs2031920 polymorphism, the role of its mutant allele (C2) on HCC susceptibility also remains controversial. Several studies[42,48] have indicated that the mutant genotypes C1/C2 and C2/C2 had no impact on HCC susceptibility. Other studies,[46,49–52] however, reported the unneglectable function on HCC vulnerability in either a direct or indirect manner. The results of the present study indicated that mutant genotypes of *CYP2E1* had significant, but different, impacts on HCC susceptibility when combining *ALDH2* and *CYP2E1* for analysis. This finding is consistent with the above reports.

After various exposure times to different ethanol concentrations, *ALDH2* mRNA expression levels in all groups with mutant genotypes increased significantly, while some groups with the wild genotype showed an increase. As a possible reason for this difference, the mutant genotypes of ALDH2 influence HCC susceptibility. In addition, there were significant differences among the mutant genotypes, while there was no significant difference in *CYP2E1* gene expression associated with the wild genotype. Thus, the mutant genotypes of CYP2E1 may play a role in HCC susceptibility.

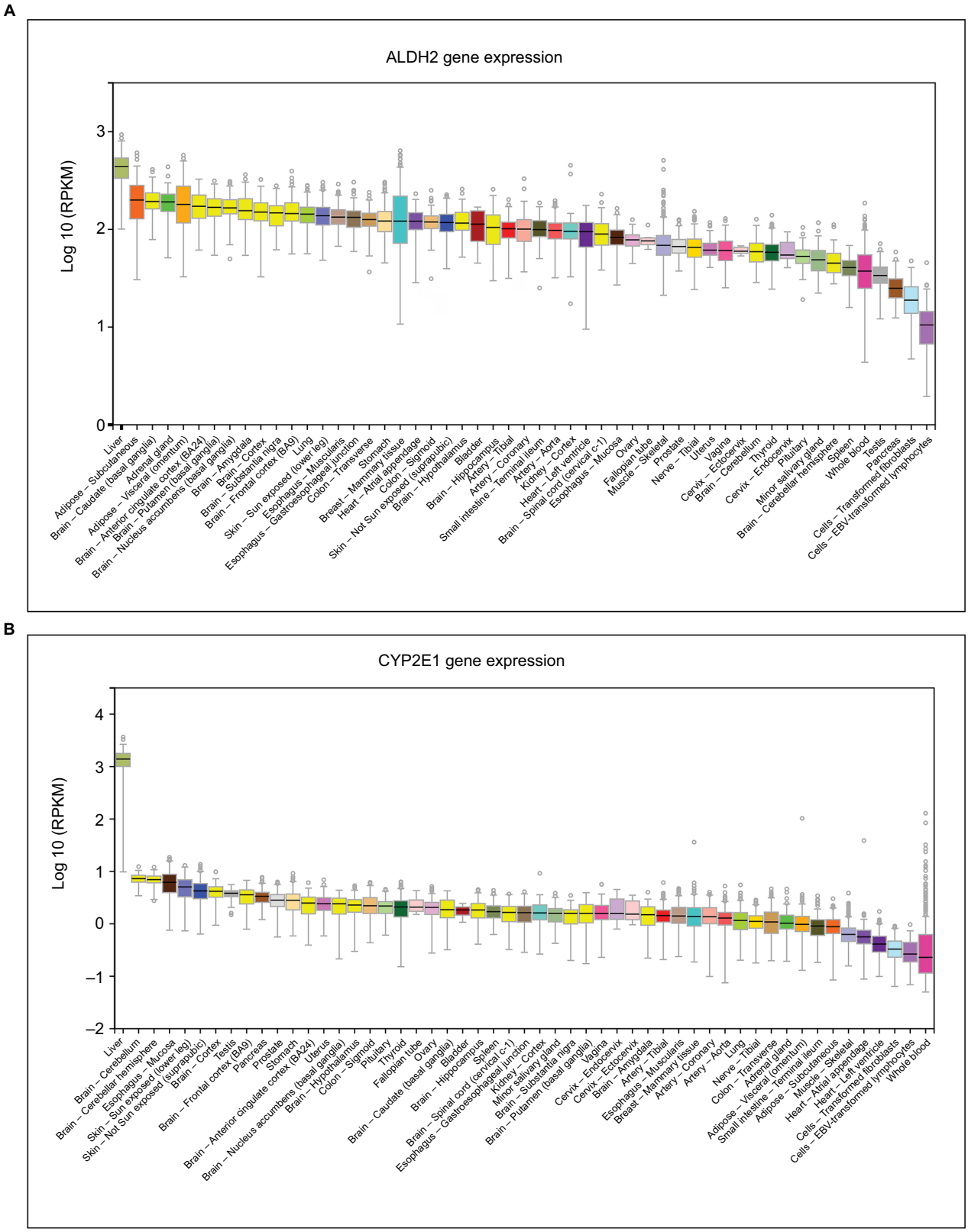

Figure 3 Expression levels of *ALDH2* and *CYP2E1* in different tissues. (**A**) *ALDH2* expression levels in different tissues. (**B**) *CYP2E1* expression levels in different tissues.

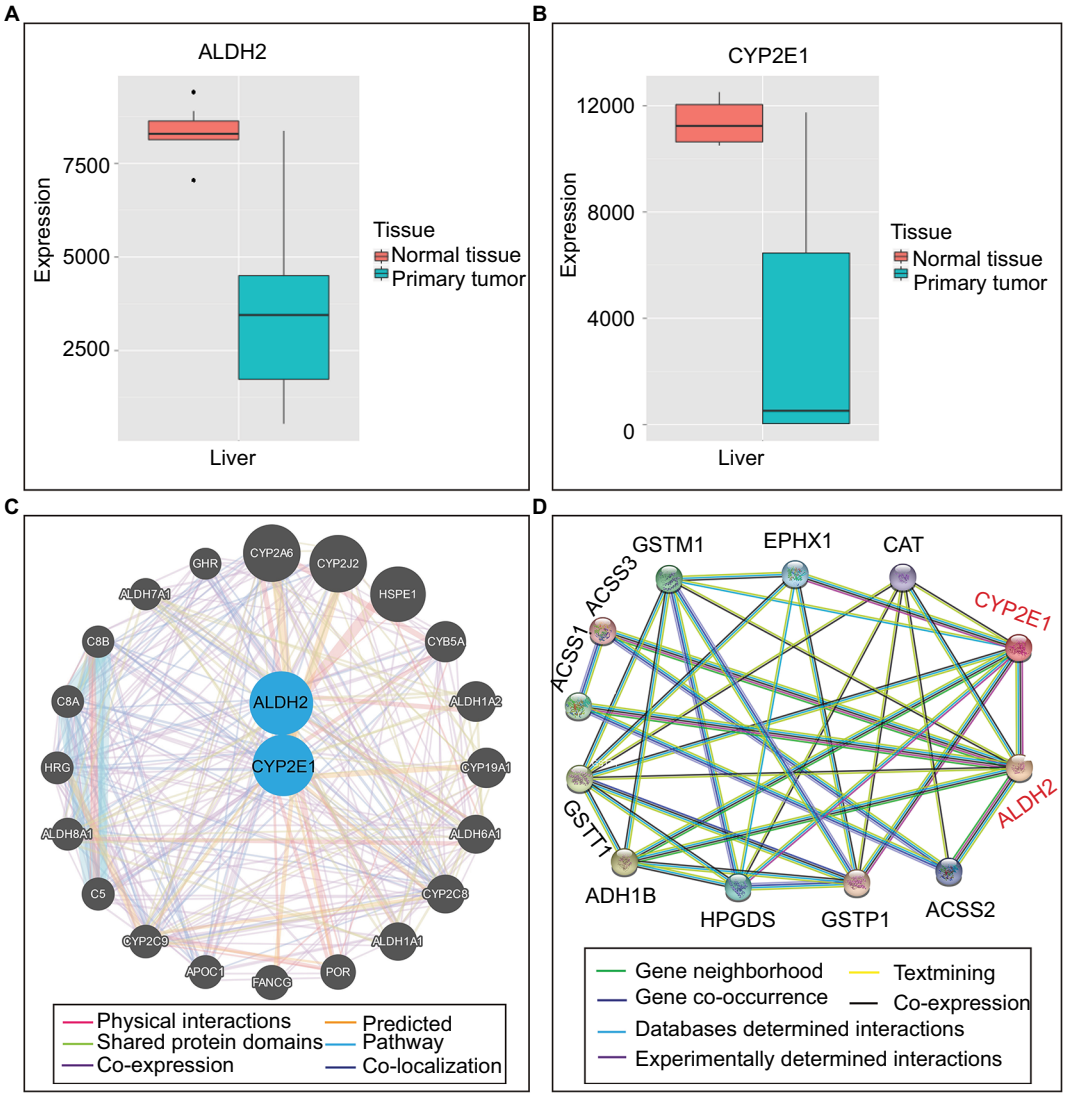

Figure 4 Gene expression levels and protein interactions. (**A** and **B**) *ALDH2* and *CYP2E1* gene expression levels in normal and primary tumor tissues. (**C**) Interactions of *ALDH2* and *CYP2E1* with other genes. (**D**) Interactions of ALDH2 and CYP2E1 with other proteins.

Table 5 Functional information of *ALDH2* rs671 and *CYP2E1* rs2031920 genetic polymorphisms

Gene site	FS score	Functional category	Prediction tool	Prediction result
ALDH2 rs671	0.856	Protein coding	PolyPhen	Probably damaging
			SIFT	Damaging
			SNP effect	Benign
			LS-SNP	Benign
			SNPs3D	Deleterious
			Ensembl-NS	Nonsynonymous
		Splicing regulation	ESEfinder	Changed
			ESRSearchi	Changed
			PESX	Changed
			RESCUE_ESE	Changed
		Post translation	OGPET	Not exist
			Sulfinator	Not exist
CYP2E1	0.398	Transcriptional regulation	Ensembl-NS	Frameshift coding
			GoldenPath	Not exist
			Ensembl-TR	Regulatory region

Abbreviation: FS, functional significance.

As shown by the results of bioinformatic analysis, the *ALDH2* rs671 mutation was correlated with splicing events (ESE or ESS). An ESE consisting of six bases within an exon is a DNA sequence motif that directs or enhances accurate splicing of pre-mRNA into mRNA. An ESS is a short region, usually 4–18 nucleotides, of an exon, and serves as silencer or inhibitor of splicing of the pre-mRNA and, thus, results in alternate and constitutive splicing.[53] To elicit its silencing or inhibiting effects, ESS recruits many proteins that negatively affect the splicing mechanism.[53]

Prediction results of changing splicing regulation were also determined with four tools: ESEfinder, ESRSearchi, PESX, and RESCUE_ESE. Abnormal splicing changes are thought to contribute to cancer onset and progression.[54–57] These splicing changes induce a nonsynonymous mutation at the rs671 site. The protein coding tools PolyPhen, SIFT, and SNPs3D predicted probable damaging effects to the rs671 site. Therefore, we hypothesized that mutation to the rs671 site would induce changes in splicing and protein coding that may serve as potential influencing factors in HCC susceptibility.

The *CYP2E1* rs2031920 polymorphism is associated with TFBS, according to the SNP function prediction tool. TFBS is a type of non-DNA molecular binding site that is bound by transcriptional factors and participates in the regulation of transcription of DNA to RNA. With varying degrees of affinity for different transcriptional factors, some binding sites have been reported to undergo rapid evolutionary change.[58] However, the results of GoldenPath, a SNP function prediction tool, indicated no transcriptional regulation of the rs2031920 polymorphism. Therefore, we speculated that the low affinity of some transcriptional factors to the rs2031920 genetic polymorphism site could explain these discrepancies. So, further studies should explore the mechanisms of both the *ALDH2* rs671 and *CYP2E1* polymorphisms. Moreover, further studies concentrating on ALDH2 and CYP2E1 expression levels with HCC prognosis are also warranted.

The findings of this study could serve as a reference to investigate the causes of the high prevalence of HCC in certain areas in China. These findings can also be utilized to distinguish individuals with *ALDH2* gene deficiency from the normal *ALDH2* genotype population in order to diminish exposure to factors associated with HCC susceptibility. Furthermore, once the roles of these two genes in HCC susceptibility are validated, therapeutic targets and serum biomarkers can be explored to improve early diagnosis and prognosis of HCC.

There were several limitations to this study that should be addressed. First, the samples size was relatively small, thus additional studies with larger sample sizes should be conducted to further validate the roles of these two genes in HCC susceptibility. Second, to eliminate potential confounding factors, more patient data should be collected for analysis of HCC susceptibility and prognosis. Third, multi-center, multi-racial, clinical studies are required to generalize these findings and clarify the underlying mechanisms.

In conclusion, this study was the first to investigate whether the *ALDH2* rs671 and *CYP2E1* rs2031920 polymorphisms are associated with HCC susceptibility in Guangxi Zhuang Autonomous Region, a region in China with a high prevalence of HCC. In addition, we found that mutant variants of *ALDH2*-rs671 and *CYP2E1*-rs2031920 genes may serve as protective factors for HCC susceptibility. These findings provide reference to identify new biomarkers for early diagnosis and treatment of HCC.

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Author contributions

Xinping Ye, Xiangkun Wang, Liming Shang, and Tao Peng designed this manuscript; Xinping Ye, Xiangkun Wang, Liming Shang, Guangzhi Zhu, Hao Su, Chuangye Han, Guanghui Li, and Tao Peng conducted the study and collected HCC

tissues and blood samples as well as corresponding baseline data of HCC and control groups; Xinping Ye and Xiangkun Wang analyzed the data and wrote this manuscript; Tao Peng guided the writing. All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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