Original Article

Genome-wide association study of the TP53 R249S mutation in hepatocellular carcinoma with aflatoxin B1 exposure and infection with hepatitis B virus

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Background: Exposure to dietary aflatoxin B1 (AFB1) induces DNA damage and mutation in the TP53 gene at codon 249, known as the TP53 R249S mutation, and is a major risk factor for hepatocellular carcinoma (HCC). AFB1 and the hepatitis B virus (HBV) together exert synergistic effects that promote carcinogenesis and TP53 R249S mutation in HCC.

Methods: A genome-wide association study (GWAS) of whole genome exons was conducted using 485 HCC patients with chronic HBV infection. This was followed by an independent replication study conducted using 270 patients with chronic HBV infection. Immunohistochemistry was used to evaluate TP53 expression in all samples. This showed a correlation between codon 249 mutations and TP53 expression. Susceptibility variants for the TP53 R249S mutation in HCC were identified based on both the GWAS and replication study. The associations between identified variants and the expression levels of their located genes were analyzed in 20 paired independent samples.

Results: The likelihood of positive TP53 expression was found to be higher in HCC patients with the R249S mutation both in the GWAS ($P<0.001$) and the replication study ($P=0.006$). The combined analyses showed that the TP53 R249S mutation was significantly associated with three single nucleotide polymorphisms (SNPs): ADAMTS18 rs9930984 (adjusted $P=4.84\times10^{-6}$), WDR49 rs75218075 (adjusted $P=7.36\times10^{-5}$), and SLC8A3 rs8022091 (adjusted $P=0.042$). The TP53 R249S mutation was found to be highly associated with the TT genotypes of rs9930984 (additive model, $P=0.01$; dominant model, $P=6.43\times10^{-5}$) and rs75218075 (additive model, $P=0.002$; dominant model, $P=2.16\times10^{-4}$). Additionally, ADAMTS18 mRNA expression was significantly higher in HCC tissue compared with its expression in paired non-tumor tissue ($P=0.041$), and patients carrying the TT genotype at rs9930984 showed lower ADAMTS18 expression in non-tumor tissue compared with patients carrying the GT genotype ($P=0.0028$). WDR49 expression was markedly lower in HCC tissue compared with paired non-tumor tissue ($P=0.0011$).

Conclusions: TP53 expression is significantly associated with the R249S mutation in HCC. Our collective results suggest that rs9930984, rs75218075, and rs8022091 are associated with R249S mutation susceptibility in HCC patients exposed to AFB1 and HBV infection.

Keywords: Hepatocellular carcinoma (HCC); hepatitis B virus (HBV); genome-wide association study (GWAS); TP53 mutation; aflatoxin B1 (AFB1)

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Introduction

Primary liver cancer is one of the most prevalent types of cancer with over 841,000 new cases recorded and causing approximately 782,000 deaths worldwide in 2018. Hepatocellular carcinoma (HCC) represents 75–85% of total liver cancer cases (1). The main risk factors for HCC are chronic infection with hepatitis B virus (HBV) or hepatitis C virus, consuming food contaminated with aflatoxin, heavy alcohol intake, obesity, smoking, and type 2 diabetes (2,3). Chronic HBV infection is associated with 53% of HCC cases worldwide (4) and an even higher proportion of cases (75%) in Asia (5). Aflatoxin B1 (AFB1) is the major carcinogenic form of aflatoxin and has been classified as a group I carcinogen in humans by the International Agency for Research on Cancer (IARC) (6). It is also a major risk factor for HCC. In 2013, 65.9% of male liver cancer-related deaths in China and 58.4% of female liver cancer-related deaths in China could be attributed to HBV infection, and an estimated 25.0% of all liver cancer-related deaths in China could be attributed to aflatoxin exposure (7).

TP53 is a tumor suppressor gene that responds to diverse cellular stress signals and regulates the expression of target genes that induce cell cycle arrest, apoptosis, senescence, DNA repair, and changes in metabolism. Mutations in the TP53 gene are the most common to occur in human malignancies (8). TP53 mutations are reportedly associated with the prognosis of multiple cancers and are biomarkers of environmental carcinogens (9). In particular, the R249S mutation in exon 7 of TP53 is a recognized molecular fingerprint of aflatoxin exposure (10-13) and is associated with poor prognosis in patients with HCC (14). Previous reports suggest that HBV and AFB1 exposure have synergistic carcinogenic effects and increase the likelihood of the TP53 R249S mutation occurring in HCC (10,11,15). Studies have shown that complex trans-activation exists between HBx and TP53, in which HBx directly inhibits TP53 activity by binding its C-terminal (16). Kew *et al.* (17) investigated the effects of wild-type and mutant HBx on TP53 gene, and found that HBx mutants could inhibit TP53 expression and downstream signal transduction. Recent studies have shown that overexpression of HBx C-terminal mutants in HHT4 cells (normal liver cell lines) significantly increased colony formation efficiency, while the corresponding wild-type allele significantly decreased colony formation efficiency in HHT4 cells. At the same time, TP53 R249S mutation interact with HBx mutants to regulate cell proliferation and mitochondrial stability (18). Due to high levels of both dietary exposure to AFB1 and HBV infection rates in Guangxi, the incidence of HCC is higher in this region than the collective incidence in China; the frequency of TP53 R249S mutations in Guangxi is up to 64.9% (10,19).

A genome-wide association study (GWAS) is a study that examines characteristic genetic alterations and epigenetic profiles associated with various diseases and detects the contribution of individual genetic factors to complex diseases, enabling potential targets associated with the occurrence, development, and treatment of multiple diseases to be identified (20). We conducted a GWAS using Illumina HumanExome BeadChip-12-1_A chip, to analyze genetic susceptibility to the TP53 R249S mutation and identify genetic factors that influence this mutation in the TP53 gene in HCC patients exposed to AFB1 and HBV in the Guangxi region.

We present the following article in accordance with the MDAR reporting checklist (available at http://dx.doi.org/10.21037/jgo-20-510).

Methods

Study participants

A total of 485 HCC patients that received hepatectomy

surgery at the Department of Hepatobiliary Surgery of the First Affiliated Hospital of Guangxi Medical University between January 2001 and November 2013 were enrolled in the GWAS. An additional 270 HCC patients were recruited between December 2013 and August 2016 for the replication study. All patients were positive for hepatitis B surface antigen and HCC after receiving hepatectomies; this was confirmed by histopathology. The clinicopathological variables of the enrolled participants were obtained from their clinical records and pathological reports. These variables included age, sex, smoking status, drinking status, pathological grade, the biobehavior of their cancer, serum alpha fetoprotein (AFP) levels, hepatic cirrhosis, radical resection status, and the use of transcatheter hepatic arterial chemoembolization (TACE). Tumor status was classified according to the Barcelona Clinic Liver Cancer (BCLC) staging system (21). Child-Pugh classification was carried out as described in previous literature (22). The presence of portal vein tumor thrombus (PVTT) was determined using previously described criteria (23). Smoking status, drinking status, and radical resection status were defined according to previous literature (24).

These studies were approved by the Ethical Review Committee of the First Affiliated Hospital of Guangxi Medical University {Approval Number: 2015 [KY-E-072]}. All participants signed written informed consent. This study conformed to the provisions of the Declaration of Helsinki (as revised in 2013).

Specimens and TP53 R249S mutation detection

HCC tissue samples were collected during surgery and immediately stored at −80 ℃. Total DNA was extracted using TIANamp Genomic DNA Kits [TIANGEN Biotech (Beijing) Co, Ltd., China]. DNA concentration and purity were measured using the NanoDrop2000 system (Thermo Fisher Scientific, Waltham, MA, USA). TP53 R249S mutations were detected using Sanger sequencing after amplification by polymerase chain reaction (PCR). The sequencing primers are listed in Table S1.

Immunohistochemistry

The expression of TP53 in HCC tissue samples was evaluated using immunohistochemical staining (Figure S1). Antigen retrieval was conducted using Tris-EDTA at a high temperature for 2.5 min. Tissue slices were treated with 3% hydrogen peroxide to inactivate endogenous peroxidases. After washing with PBS, the slices were incubated with mouse antibody against human TP53 (1:150) (ZSGB-BIO ORIGENE, Beijing, China) at 4 ℃, followed by incubation with a secondary antibody (Dako Cytomation, Glostrup, Denmark) for 30 min at 37 ℃. Finally, the tissue slices were visualized using *diaminobenzidine* (DAB) (Dako Cytomation) and counterstained with hematoxylin. Positive and negative controls were included for each sample. All tissue sections were reviewed and scored blindly by two experienced pathologists. In cases of disagreement, where possible, joint review consensuses were obtained. The proportion of TP53 positive cells was calculated using the following formula: number of positive cells/total number of the cells ×100%. A TP53 positive stain was defined as ≥10% of cancer cells positive for TP53 (25,26).

Genotyping and quality control in the GWAS

All samples in the GWAS were genotyped using Illumina HumanExome BeadChip-12-1_A, which includes 242,901 markers of protein-altering genetic variants. The study flow chart and the genotyping procedure are depicted in Figures S2,S3, respectively. Genotype calling was carried out using Genotyping Module v1.0 in GenomeStudio v2011.1 with an average call rate of 99.84%. A total of 50 samples (>10%) were randomly selected, and sequence analysis of candidate loci was performed using ABI PRISM 3100 (Applied Biosystems, Shanghai Sangon Biological Engineering Technology & Services Co, Ltd, Shanghai, China). The sequencing results were 100% consistent with the BeadChip-12-1_A genotyping results.

A quality control (QC) procedure was used prior to association analysis. Firstly, samples were excluded if they met any of the following criteria: (I) a genotyping rate of <95%; (II) ambiguous sex; (III) a genome-wide identity-by-descent (IBD) of >0.1875; and (IV) being an outlier in a principal components analysis (PCA) for ancestry and population stratification. Secondly, single nucleotide polymorphisms (SNPs) were removed that had (I) a genotype call rate of <95%, (II) a P value in Hardy-Weinberg equilibrium (HWE) of $<1\times10^{-6}$, and (III) a minor allele frequency (MAF) of <0.05. Thirdly, an analysis of population stratification by PCA was performed using the EIGENSOFT package to eliminate multi-ethnic interference. Genomic inflation factors (GIF) were used to investigate residual population stratification using MATLAB 7.0. The QC procedure was performed using Plink v1.07, R 3.0.1, and the EIGENSOFT package. After QC, the

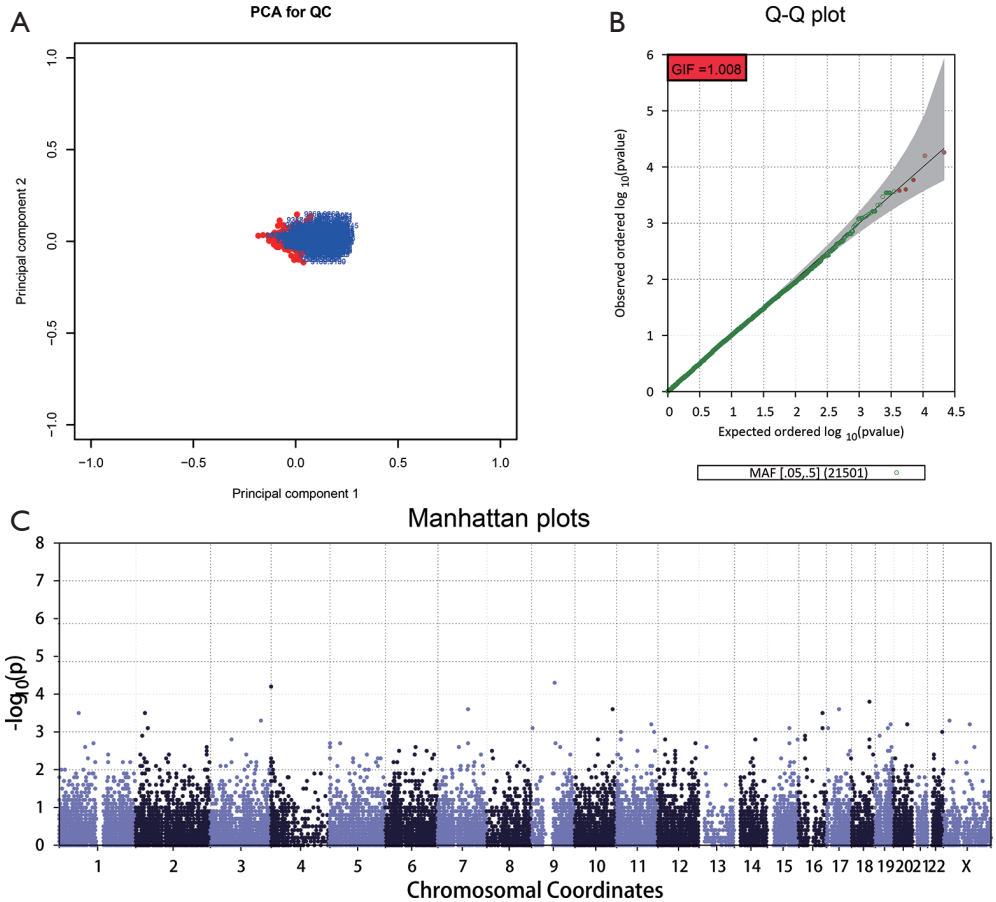

Figure 1 GWAS results. (A) Results of principal component analysis across all individuals. (B) Q-Q plots of single variant logistic score test P values. (C) Manhattan plots for association analysis. The results of the single variant test (–log10 P values) were plotted against genomic position (GRCh37/hg19). GWAS, genome-wide association study.

total genotyping rate in the remaining individuals was determined. This is shown in Figure S4A. The genotype failure rate and heterozygosity across all individuals are shown in Figure S4B. The PCA plot showed either no stratification or mild stratification in our study population (*Figure 1A*). The genomic control inflation factor was determined using a Quantile-Quantile (Q-Q) plot (*Figure 1B*). Ultimately, 21,501 SNPs from 459 cases were used in the GWAS following QC.

SNP selection for the replication study

Based on the presence or absence of the TP53 R249S mutation, subjects were placed into either the mutation group or the non-mutation group. Genetic differences between the two groups were explored in the GWAS. The EPACTS package was used to perform association analyses. According to the Manhattan plot produced (*Figure 1C*), 35 SNPs with top-ranking P values were selected. However, only 29 primer pairs could be designed for these SNPs in the replication study. The 29 SNPs used for the validation analysis are shown in Table S2, and their corresponding primers are shown in Table S3.

Genotyping and quality control in the replication study

A total of 270 patients were enrolled in the replication study. Genotyping analyses were carried out using iPLEX Gold SNP genotyping technology based on the MassARRAY array platform. The QC procedure was carried out as follows. Firstly, samples with (I) a genotyping rate of <95% and (II) a genome-wide IBD of >0.1875 were excluded. Secondly, SNPs with (I) a genotype call rate of <95%, (II) a HWE P value of $<1\times10^{-6}$, and (III) a MAF of <0.05 were

removed. Following QC, 258 samples (80 in the mutation group and 178 in the non-mutation group) were included for analysis. The results of the replication study using the 29 SNPs are shown in Table S4, and their corresponding primers are shown in Table S5.

Quantitative real time PCR

Tumor and paired non-tumor liver tissue samples were collected for gene expression analysis from 20 HCC patients randomly selected from the independent replication study. All samples used to extract RNA were treated with RNA preservation solution and then stored in a –80° refrigerator. Candidate SNPs were selected from the GWAS, and replication analyses were then carried out to analyze potential associations between these SNPs and expression levels of their located gene. The tissue samples stored in the RNA protection solution were taken out and 10 mg of tissue was put into the centrifuge tube with Trizol reagent, and then homogenized fully with a grinder. Then the RNA extraction was carried out in accordance with Trizol's instructions. Briefly, tissue sample Trizol lysis buffer was placed at room temperature (25 ℃) for 10 minutes to further fully decompose; Then 200 μL chloroform was added and shaken vigorously for 15 seconds. Placed at room temperature (25 ℃) for 5 minutes. Centrifuged at 12,000 g at 4 ℃ for 15 minutes. At this time, the liquid in the centrifuge tube was divided into three layers, and the RNA was mainly in the upper colorless aqueous phase. The upper colorless aqueous phase (about 500 μL) was placed into a new sterile centrifuge tube with RNase removed. Five hundred μL isopropyl alcohol was added and placed at room temperature 25 ℃) for 10 minutes. The liquid was centrifuged at 12,000 g at 4 ℃ for 10 minutes and the supernatant was discarded. Then add 1 mL 75% ethanol for washing and discard the superfine. Let the precipitated RNA dry and then dissolve the RNA precipitate in 50 μL RNase-free water. The integrity of total RNA was determined by gel electrophoresis. cDNA reverse transcription was performed using PrimeScript™ RT Reagent Kit with gDNA Eraser (TAKARA BIO INC). Then the real-time PCR reaction was performed according to Roche FastStart Universal SYBR Green Master (ROX) kit and ABI 7500 fluorescence PCR system operating guidelines. The primers used in amplification are shown in Table S6. Quantitative real time (RT)-PCRs were conducted in triplicate on an ABI7500 RT-PCR System using FastStart Universal SYBR Green Master (Rox). The expression of genes in all samples was normalized to that of GAPDH using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Results are expressed as mean ± standard deviation (SD) for normally distributed variables and as a median for non-normal distributions. The logistic regression model was applied to analyze differences in clinicopathological factors between the TP53 R249S mutation group and the non-mutation group. Odds ratio (OR) and 95% confidence interval (CI) values were calculated using univariate analyses. Analyses of the associations between the TP53 R249S mutation and SNPs were implemented using single variant logistic score test in EPACTS package v3.2.6 (27). Q-Q and Manhattan plots were also generated. Chi-squared tests and the logistic regression model were applied to identify any associations between genetic models and gene types. Local linkage disequilibrium (LD) and recombination patterns were analyzed using LocusZoom (28). Differences in expression between tumor tissue samples and paired adjacent non-tumor tissue samples were assessed using the Student's *t* test, and differences between SNP genotypes and gene expression were assessed using a non-parametric trend test. Statistical analysis was performed using SPSS v18.0 (SPSS, Chicago, IL, USA). Data were considered statistically significant when $P<0.05$.

Results

Baseline characteristics

A total of 165 HCC patients with the TP53 R249S mutation were analyzed in this GWAS, as well as 320 patients without this mutation. Our replication study included 80 patients with this mutation and 178 without. When subgroups were stratified by the TP53 R249S mutation, no difference in clinicopathological parameters was found between these subgroups in either the GWAS or the replication study. These parameters included age, sex, race, BMI, smoking status, drinking status, Child-Pugh score, BCLC stage, TACE status, cirrhosis, serum AFP levels, pathological grade, antiviral therapy, tumor size, capsule status, regional invasion, intrahepatic metastasis, vascular invasion, and PVTT. There was, however, a significant difference in tumor number found in the replication study between these subgroups (HR =2.72, 95% CI: 1.00–7.38, P<0.049) (*Table 1* and *Table 2*). TP53 expression status was found to be significantly different between the mutation group and

Table 1 Clinicopathological characteristics of subjects analyzed in the GWAS

Variable	Codon 249 mutation *TP53* gene			
	Non-mutation group (n=320)	Mutation group (n=165)	OR* (95% CI)	P value*
Age (years)				
≤46	178	82	1	
>46	142	83	1.17 (0.64–2.15)	0.605
Sex				
Male	282	148	1	
Female	38	17	0.79 (0.54–1.15)	0.215
Race				
Han Chinese	211	109	1	
Minority	109	69	0.72 (0.49–1.06)	0.940
BMI				
≤25	265	136	1	
>25	55	29	1.03 (0.63–1.69)	0.915
Smoking status				
Never smoked	211	107	1	
Has smoked	109	58	1.05 (0.71–1.56)	0.811
Drinking status				
Never drunk	198	97	1	
Has drunk	122	68	1.14 (0.78–1.67)	0.509
Child-Pugh class				
A	267	136	1	
B	53	29	1.07 (0.65–1.77)	0.778
BCLC stage				
A	189	95	1	
B	47	33	1.40 (0.84–2.32)	
C	84	37	0.88 (0.55–1.39)	0.238
TP53 expression[a]				
Negative	133	26	1	
Positive	132	115	4.46 (2.73–7.27)	< 0.001
TACE status				
Before hepatectomy				
No	244	138	1	
Yes	76	27	0.63 (0.39–1.02)	0.061
After hepatectomy				
No	137	75	1	
Yes	183	90	0.90 (0.62–1.31)	0.578
Cirrhosis				
No	37	22	1	
Yes	283	143	0.83 (0.47–1.46)	0.511
Serum AFP[b]				
≤400 (ng/mL)	165	82	1	
>400 (ng/mL)	134	68	1.02 (0.69–1.51)	0.917

Table 1 *(continued)*

Table 1 *(continued)*

Variable	Codon 249 mutation *TP53* gene			
	Non-mutation group (n=320)	Mutation group (n=165)	OR* (95% CI)	P value*
Radical resection[c]				
Yes	166	102	1	
No	146	59	1.52 (1.03–2.25)	0.035
Pathological grade[d]				
Good	22	5	2.28 (0.84–6.17)	
Moderate	245	127	1.96 (0.43–9.00)	0.262
Poor	9	4		
Antiviral therapy				
No	210	105	1	
Yes	110	60	1.09 (0.74–1.62)	0.664
Oncological behavior				
Tumor size				
≤5 cm	103	52	1	
>5 cm	217	113	1.03 (0.69–1.54)	0.880
No. of tumors				
Single (n=1)	238	118	1	
Multiple (n>1)	82	47	1.16 (0.76–1.76)	0.500
Capsule				
Complete	134	64	1	
Incomplete	129	76	0.92 (0.53–1.60)	
Absent	57	25	1.23 (0.82–1.86)	0.459
Regional invasion				
Absent	272	140	1	
Present	48	25	1.01 (0.60–1.71)	0.965
Intrahepatic metastasis				
Absent	146	75	1	
Present	174	90	1.01 (0.69–1.47)	0.972
Vascular invasion				
Absent	260	139	1	
Present	60	26	0.81 (0.49–1.34)	0.414
PVTT				
No	269	140	1	
vp1	5	6	2.31 (0.69–7.69)	
vp2	14	3	0.41 (0.12–1.46)	
vp3	27	13	0.93 (0.46–1.85)	
vp4	5	3	1.15 (0.27–4.89)	0.414

[a], TP53 expression information was unavailable for 79 patients; [b], AFP information was unavailable for 36 patients; [c], radical resection information was unavailable for 12 patients; [d], pathological grade information was unavailable for 73 patients; *, OR and P values for the logistic regression model univariate analysis. GWAS, genome-wide association study; AFP, alpha-fetoprotein; TACE, transarterial chemoembolization; BMI, body mass index; PVTT, portal vein tumor thrombus; vp1, PVTT in the distal to second order portal branches; vp2, PVTT in the second order portal branches; vp3, PVTT in the first order branches; vp4, PVTT in the main trunk; MST, median survival time; MRT, median recurrence time; OR, odds ratio; 95% CI, 95% confidence interval.

Table 2 Clinicopathological characteristics of subjects analyzed in the replication study

Variable	Codon 249 mutation in *TP53* gene			
	Non-mutation group (n=178)	Mutation group (n=80)	OR* (95% CI)	P value*
Age (years)				0.718
≤47	92	44	1	
>47	86	36	0.91(0.53-1.56)	
Sex				0.151
Male	162	68	1	
Female	16	12	1.80 (0.81–4.00)	
Race				0.063
Han Chinese	92	33	1	
Minority	72	43	1.68 (0.97–2.91)	
NA	14	4		
BMI				0.550
≤25	138	66	1	
>25	38	14	0.82 (0.42–1.59)	
NA	2			
Smoking status				0.080
Never smoked	123	46	1	
Has smoked	53	33	1.63 (0.94–2.83)	
NA	2	1		
Drinking status				0.153
Never drunk	133	53	1	
Has drunk	43	26	1.53 (0.86–2.74)	
NA	2	1		
Child-Pugh class				0.172
A	173	74	1	
B	2	3	3.53 (0.58–21.55)	
NA	3	3		
BCLC stage				
A	139	53	1	0.104
B	5	4	2.11 (0.55–8.17)	
C	14	12	2.26 (0.98–5.21)	
NA	20	11		
TP53 expression				0.006
Negative	45	8	1	
Positive	116	65	3.15 (1.40–7.09)	
NA	17	7		

Table 2 *(continued)*

Table 2 *(continued)*

Variable	Codon 249 mutation in *TP53* gene			
	Non-mutation group (n=178)	Mutation group (n=80)	OR* (95% CI)	P value*
TACE status				
Before hepatectomy				0.458
No	151	71	1	
Yes	25	9	0.74 (0.33–1.65)	
NA	2			
Serum AFP				0.672
≤400 (ng/mL)	88	37	1	
>400 (ng/mL)	75	36	1.13 (0.65–1.96)	
NA	15	7		
Pathological grade				
Good	3	3	1	0.424
Moderate	143	60	0.42 (0.82–2.12)	
Poor	11	7	0.63 (0.10–4.09)	
NA	21	10		
Oncological behavior				
Tumor size				0.268
≤5 cm	82	31	1	
>5 cm	76	40	1.37 (0.78–2.41)	
NA	20	9		
No. of tumors				0.049
Single (n=1)	149	62	1	
Multiple (n>1)	8	9	2.72 (1.00–7.38)	
NA	21	9		
Regional invasion				0.425
Absent	159	69	1	
Present	9	6	1.55 (0.53–4.51)	
NA	20	5		
PVTT				0.226
No	154	65	1	
Yes	14	10	1.70 (0.72–4.03)	
NA	10	5		

*, OR and P value for univariate analysis of logistic regression model. NA, not available; AFP, alpha-fetoprotein; TACE, transarterial chemoembolization; BMI, body mass index; PVTT, portal vein tumor thrombus; vp1, PVTT in the distal to second order portal branches; vp2, PVTT in the second order portal branches; vp3, PVTT in the first order branches; vp4, PVTT in the main trunk; MST, median survival time; MRT, median recurrence time; OR, odds ratio; 95% CI, 95% confidence interval.

Table 3 Correlation analysis of TP53 R249S mutation and TP53 expression in the GWAS and replication study

Subjects	Number	TP53 R249S mutation			
		Non-mutation group (n)	Mutation group (n)	OR* (95% CI)	P value*
GWAS					
Negative	159	133	26	1	
Positive	247	132	115	4.46 (2.73–7.27)	<0.001
NA	79	55	24		
Replication					
Negative	53	45	8	1	
Positive	181	116	65	3.15 (1.40–7.09)	0.006
NA	24	17	7		

*, OR and P value for univariate analysis of the logistic regression model. GWAS, genome-wide association study; OR, odds ratio; 95% CI, 95% confidence interval.

Table 4 Association analysis of candidate SNPs in GWAS, replication study, and combined studies

SNP	Chr	Gene type	GWAS			Replication			Combined		
			Number	MAF	P*	Number	MAF	P*	Number	MAF	P*
rs9930984	16	ADAMTS18_G/T	437	0.126	3.34×10^{-4}	252	0.091	0.0255	689	0.113	4.84×10^{-6}
rs75218075	3	WDR49_T/C	459	0.179	4.7×10^{-4}	252	0.147	0.0295	711	0.167	7.36×10^{-5}
rs8022091	14	SLC8A3_A/C	437	0.360	1.55×10^{-3}	248	0.347	0.0232	685	0.356	0.042

*, adjusted for age, sex, race, smoking status, and drinking status. SNP, single nucleotide polymorphism; GWAS, genome-wide association study; Chr, chromosome; MAF, minor allele frequency.

the non-mutation group in the GWAS (OR =4.46, 95% CI: 2.73–7.27, P<0.001) (*Table 3*) and in the replication study (OR =3.15, 95% CI: 1.40–7.09, P=0.006) (*Table 3*).

Quality control

Following QC, 459 samples and 21,501 SNPs were identified. The total genotyping rate in the remaining samples was 98.35% (Figure S4A). The PCA plot showed either no stratification or mild stratification of our study population (*Figure 1A*). The Q-Q plot showed a genomic control inflation factor (λ) of 1.008 (*Figure 1B*).

Association analysis

A total of 258 samples met the experimental requirements for the replication study. As shown in *Table 4*, rs8022091, rs9930984, and rs75218075 were three SNPs found to be significantly associated with the TP53 R249S mutation in the GWAS (MAF =0.36, P=0.00155; MAF =0.126, P=0.000334; MAF =0.179, P=0.00047), in the replication study (MAF =0.347, P=0.0232; MAF =0.091, P=0.0255; MAF =0.147, P=0.0295), and in the combined analysis after adjustment for age, sex, race, smoking status, and drinking status (MAF =0.356, P=0.042; MAF =0.113, $P=4.84\times10^{-6}$; MAF =0.167, $P=7.36\times10^{-5}$). Ultimately, rs8022091, rs9930984, and rs75218075 were identified as SNPs associated with the TP53 R249S mutation and were subjected to further analysis.

Genetic model analysis

We constructed additive and dominant genetic models to establish the potential associations between genotype variants of the candidate SNPs and the TP53 R249S mutation. These results are shown in *Table 5*. In the additive genetic model, the TP53 R249S mutation was significantly associated with the TT genotypes of rs9930984 (OR

Table 5 Combined analysis of associated SNP genotypes and TP53 R249S mutation

SNPs	TP53 R249S mutation		OR (95% CI)	P	Test power
	Non-mutation	Mutation			
rs9930984					
Additive					0.85
GG	26	3	1	3.06×10^{-4}	
GT	79	19	2.08 (0.57–7.62)	0.267	
TT	358	204	4.94 (1.48–16.52)	0.010	
Dominant					0.84
GG + GT	105	22	1		
TT	358	204	2.27 (1.67–4.44)	6.43×10^{-5}	
rs75218075					
Additive					NA
CC	19	2	1	0.001	
CT	147	49	3.17 (0.71–14.89)	0.130	
TT	307	187	5.79 (1.33–25.13)	0.002	
Dominant					0.65
CC + CT	166	51	1		
TT	307	187	1.98 (1.38–2.85)	2.16×10^{-4}	
rs8022091					
Additive					0.82
AA	65	40	1	0.413	
AC	185	92	0.81 (0.51–1.29)	0.371	
CC	209	94	0.73 (0.46–1.16)	0.184	
Dominant					0.79
AA	65	40	1		
AC + CC	394	186	0.77 (0.50–1.18)	0.228	

P values, OR, and 95% CI were calculated under the additive model using logistic regression adjusting for age, sex, race, smoking status, and drinking status. SNP, single nucleotide polymorphism; OR, odds ratio; 95% CI, 95% confidence interval.

=4.94, 95% CI: 1.48–16.52, P=0.010) and rs75218075 (OR =5.79, 95% CI: 1.33–25.13, P=0.002). In the dominant genetic model, HCC patients carrying the TT genotype of rs9930984 were shown to have a greater risk of TP53 R249S mutation then those carrying GG and GT genotypes (OR =2.27, 95% CI: 1.67–4.44, $P=6.43\times10^{-5}$). A similar result was found in HCC patients carrying the TT genotype of rs75218075, which was associated with a higher risk of TP53 R249S mutation than those carrying CC and CT genotypes (OR =1.98, 95% CI: 1.38–2.85, $P=2.16\times10^{-4}$). However, no significant difference in rs8022091 genotypes was found between the mutation group and non-mutation group in both the additive and dominant genetic models.

Linkage disequilibrium and haplotype analysis

LD and haplotype analysis of SNPs (rs8022091, rs9930984, and rs75218075) was performed within 2 Mb. rs9930984 in the a disintegrin and metallopeptidase with thrombospondin type 1 motif 18 (ADAMTS18) on chromosome 16, within

2 Mb of rs75218075 in WD repeat domain 49 (WDR49) on chromosome 3, and within 2 Mb of rs8022091 in solute carrier family 8 member A3 (SLC8A3) on chromosome 14 (*Figure 2A*,*B*,*C*). Our data shows that rs9930984 is in LD with rs11640912. However, there was no significant association betweenrs11640912 and the TP53 R249S mutation in the replication study (Table S4), and there was no LD found between rs8022091 and rs75218075.

Quantitative RT-PCR

We assessed the mRNA expression of ADAMTS18, WDR49, and SLC8A5 to establish the effects of different SNP genotypes on their gene expression. ADAMTS18 expression was significantly higher in HCC tissue compared with paired non-tumor tissue (P=0.041) (*Figure 3A*), and populations with the TT genotype of rs9930984 showed significantly less ADAMTS18 expression in non-tumor tissue than individuals with the GT genotype (P=0.0028) (*Figure 3A*). However, there was no significant difference in ADAMTS18 expression found between GT and TT genotypes of rs9930984 in HCC tissue, or between the TP53 R249S mutation group and the non-mutation group in either HCC tissue or non-tumor tissue (*Figure 3A*). WDR49 expression was markedly lower in HCC tissue compared with paired non-tumor tissue (P=0.0011). Similarly, we found no significant difference in WDR49 expression between different rs75218075 genotypes, or between the TP53 R249S mutation group and the non-mutation group in either HCC tissue or non-tumor tissue (*Figure 3B*). No significant difference in SLC8A5 expression was found between HCC tissue or paired non-tumor tissue, between different rs75218075 genotypes, or between the TP53 R249S mutation group and the non-mutation group in non-tumor tissue (*Figure 3C*).

Discussion

To identify novel loci susceptible to the TP53 R249S mutation in HCC patients both exposed to AFB1 and infected with HBV, we performed a GWAS. A trend of positive TP53 expression was evident in HCC patients with the TP53 R249S mutation compared with HCC patients without this mutation in both the GWAS and the replication study. This was consistent with previous findings (12). Following a combined analysis of all subjects from the GWAS and the subsequent replication study, three SNPs, rs8022091, rs9930984, and rs75218075, remained significantly associated with the TP53 R249S mutation after adjusting for age, sex, race, smoking status, and drinking status.

We further conducted a combined analysis of the genotypes of these three SNPs and the TP53 R249S mutation. Our results suggest that the TT genotype of both rs9930984 and rs75218075 are risk factors associated with the incidence of TP53 R249S mutation in HCC combined with AFB1 exposure and HBV infection. Consistent with these findings, the TT genotypes of rs9930984 and rs75218075 were significantly associated with the TP53 R249S mutation in HCC in the additive genetic model. In contrast, the different genotypes of rs8022091 showed no significant associations with the TP53 R249S mutation in either the additive or dominant model. We performed further analysis for LD within 2 Mb of rs8022091, rs9930984, and rs75218075, and this showed that rs9930984 was in strong LD with rs11640912. However, rs11640912 was not significantly associated with the TP53 R249S mutation in our replication study.

The rs9930984 SNP is located in the ADAMTS18 gene, which encodes a member of the ADAMTS protein family. ADAMTS18 has been identified as a novel functional tumor suppressor (29). More and more studies (29-33) have found that mutations in this gene, as well as hypermethylation of promoters, is closely related to the growth of multiple types of tumors. This suggests that ADAMTS18 plays a role in tumor suppression. Compared with paired adjacent non-tumor tissue or normal tissue, ADAMTS18 CpG methylation is significantly upregulated in gastric, colorectal, and pancreatic cancer tissue (31). Pharmacological and genetic demethylation analyses indicate that CpG methylation directly inhibits the expression of ADAMTS18. *In vitro* studies have found that the restoration of ADAMTS18 gene expression previously downregulated or silenced in tumor cell lines inhibits tumor cell clonality (29). However, a study on malignant melanoma showed that mutated ADAMTS18 promoted cell growth, migration, and metastasis (32). In our study, ADAMTS18 rs9930984 was significantly correlated with the TP53 R249S mutation, and participants carrying the TT genotype of rs9930984 showed a higher risk of TP53 R249S mutation compared with those carrying GG and GT genotypes. The results of our separate experiments both showed significantly higher ADAMTS18 mRNA expression in HCC tissue compared with non-tumor tissue. However, no differences in ADAMTS18 expression were found between the TP53 R249S mutation group and the non-mutation group for both tumor tissue and non-tumor

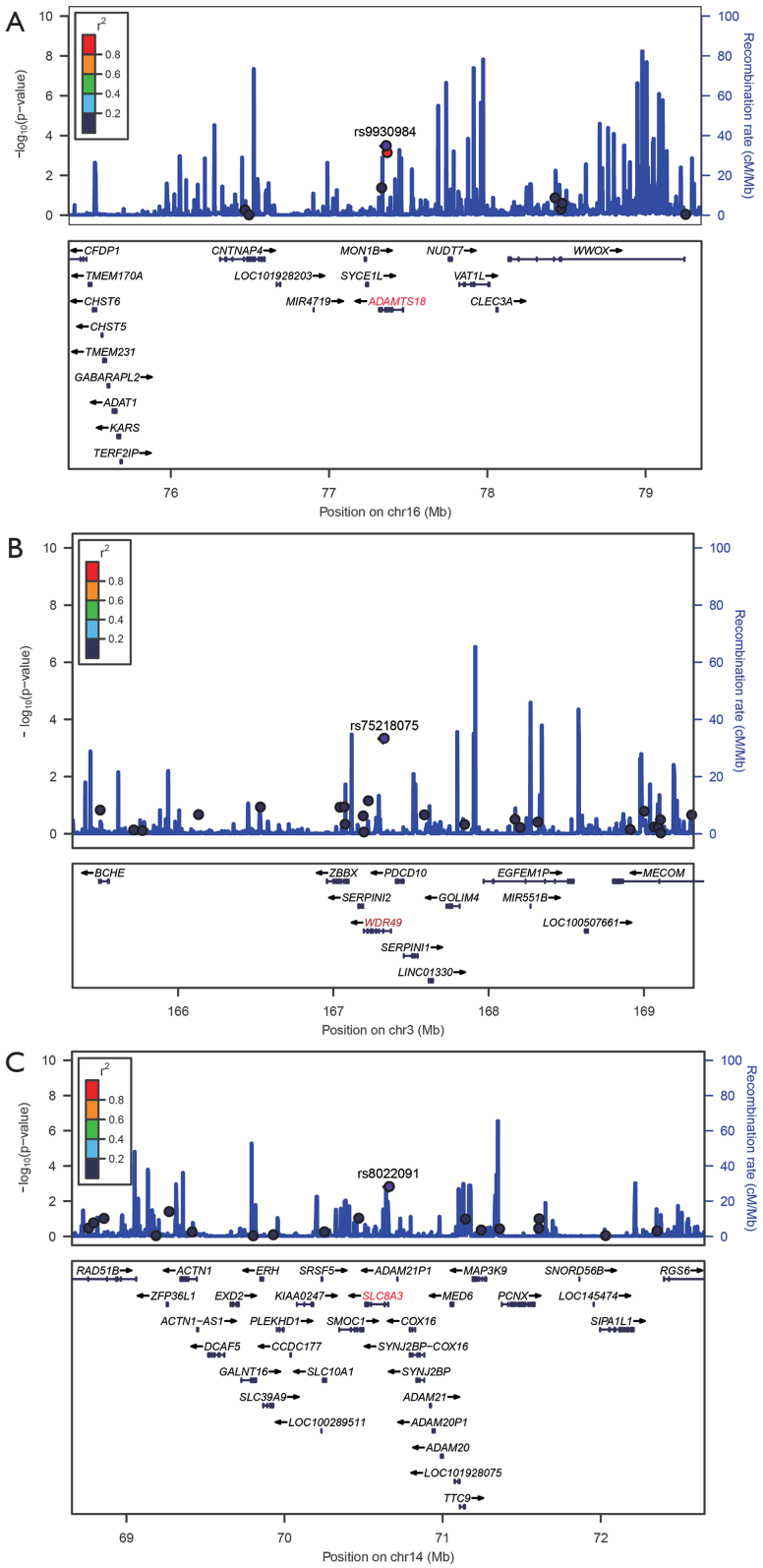

Figure 2 LocusZoom plot of local LD for candidate SNPs. (A) LocusZoom plot for rs8022091. (B) LocusZoom plot for rs9930984. (C) LocusZoom plot for rs752180. SNP, single nucleotide polymorphism.

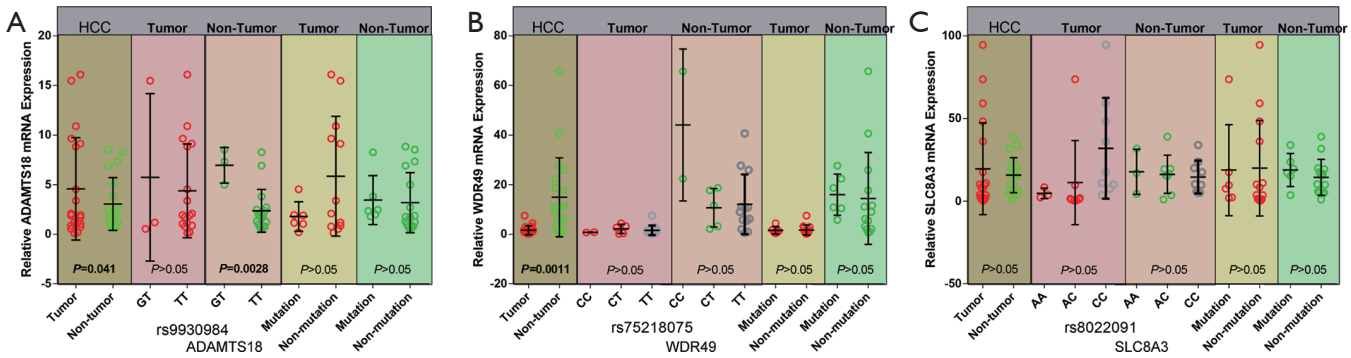

Figure 3 Relative expression of candidate genes in HCC. (A) Relative expression of ADAMTS18 in HCC tumor tissue and non-tumor tissue, in tumor tissue and non-tumor tissue with and without the TP53 R249S mutation, and according to rs9930984 genotype. (B) Relative expression of WDR49 in HCC tumor tissue and non-tumor tissue, in tumor tissue and non-tumor tissue with and without the TP53 R249S mutation, and according to rs75218075 genotype. (C) Relative expression of SLC8A3 in HCC tumor tissue and non-tumor tissue, in tumor tissue and non-tumor tissue with and without the TP53 R249S mutation, and according to rs8022091 genotype. HCC, hepatocellular carcinoma.

tissue. ADAMTS18 expression in non-tumor tissue with the rs9930984 TT genotype was lower than that expressed in tissue with the GT genotype. To our knowledge, no studies to date have demonstrated an association between ADAMTS18 and HCC pathogenesis or the TP53 R249S mutation. Moreover, no relevant research on rs9930984 and its potential role in HCC is presently documented.

The rs75218075 SNP is located in the WDR49 gene on chromosome 3, which encodes a member of the WDR protein family with nine WD repeats. These proteins are involved in multiple cellular processes, including cell cycle progression, signal transduction, apoptosis, and gene regulation (34,35). WDR79 is reportedly involved in the development and progression of non-small cell lung cancer (36). A previous *in vitro* study on bladder cancer by Chen *et al.* (37) showed that WDR5 is upregulated in tumor tissue and promotes the proliferation of bladder cancer cells and the autologous renewal of cells, as well as increasing chemoresistance. However, no studies on the biological function of WDR49 have been reported to date, and its role in tumor development remains unknown. Our results suggest that WDR49 rs75218075 is significantly associated with the TP53 R249S mutation (*Table 4*, Tables S2,S4), and patients carrying the rs75218075 TT genotype might be at greater risk of expressing the TP53 R249S mutation compared with those carrying CC and CT genotypes. WDR49 mRNA expression was significantly lower in HCC tissue compared with non-tumor tissue, but was not correlated with any genotypes of rs75218075. We also found no correlation between WDR49 mRNA expression and either the TP53 R249S mutation group or non-mutation group in either HCC or non-tumor tissue.

The rs8022091 SNP is located in the SLC8A gene on chromosome 14 and encodes a member of the sodium/calcium exchanger integral membrane protein family. Recent studies have shown that SLC8A3 is an essential component of PKCα activation by Ca^{2+} in endothelial cell membranes, a necessary step of VEGF-induced ERK1/2 phosphorylation and angiogenesis (38,39). Reverse transport of SLC8A3 has been reported in cardiac fibroblasts (39) and neuroblastoma cells (40); reverse transport is required for ERK1/2 activation. A recent study showed a significant negative correlation between promoter CpG island methylation and SLC8A3 gene expression in clear-cell renal cell cancer (41), indicating that the SLC8A3 gene may play a functional epigenetic control role in tumors. In both our GWAS and replication study, SLC8A3 rs8022091 was significantly associated with the TP53 R249S mutation, though there was no correlation between any rs8022091 genotypes and the TP53 R249S mutation.

In addition, TP53 mutations are reportedly associated with the prognosis of multiple cancers and are biomarkers of environmental carcinogens. Our previous study has shown that patients with TP53 R249S mutation have a higher risk of recurrence within 2 years after surgery in HBV-relative HCC (42). Nogueira *et al.* showed that TP53 R249S mutation was associated with tumor diameter and tumor differentiation in Brazilian HCC patients (43). However, in our previous study, we did not find such those association, possibly because our study population

was all HBV-associated HCC, and their study population included people with HBV, HCV and non-hepatitis virus backgrounds. Moreover, their study sample was only 53, and the reliability of their results was questionable. Several studies on Japanese HCC patients have shown (35,36) that *TP53* gene mutation is significantly correlated with short-term tumor recurrence and tumor differentiation, which was similar to our previous results (44,45). Other studies in China have also shown that TP53 R249S mutation is an indicator of poor prognosis in HCC (46,47). Hence, TP53 R249S mutations may be an independent prognostic biomarker for HCC.

In our study, no significant differences were detected between SLC8A3 mRNA expression and either HCC tissue or non-tumor tissue. We also found no associations between SLC8A3 mRNA expression and any rs8022091 genotypes, or SLC8A3 mRNA expression and either the TP53 R249S mutation group or non-mutation group in either HCC tissue or non-tumor tissue.

Conclusions

We successfully conducted a comprehensive GWAS and replication study on HCC patients exposed to AFB1 and HBV in the Guangxi region. The SNPs ADAMTS18 rs9930984 and WDR49 rs75218075 were found to be associated with susceptibility to the TP53 R249S mutation, and the TT genotypes of rs9930984 and rs75218075 were further identified as risk factors for this mutation. Further large-scale, multi-center studies are necessary to verify our findings and establish the specific associations between these regions and this codon 249 TP53 gene mutation in HCC patients exposed to both AFB1 and HBV.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. These studies were approved by the Ethical Review Committee of the First Affiliated Hospital of Guangxi Medical University {Approval Number: 2015 [KY-E-072]}. All participants signed written informed consent. This study conformed to the provisions of the Declaration of Helsinki (as revised in 2013).

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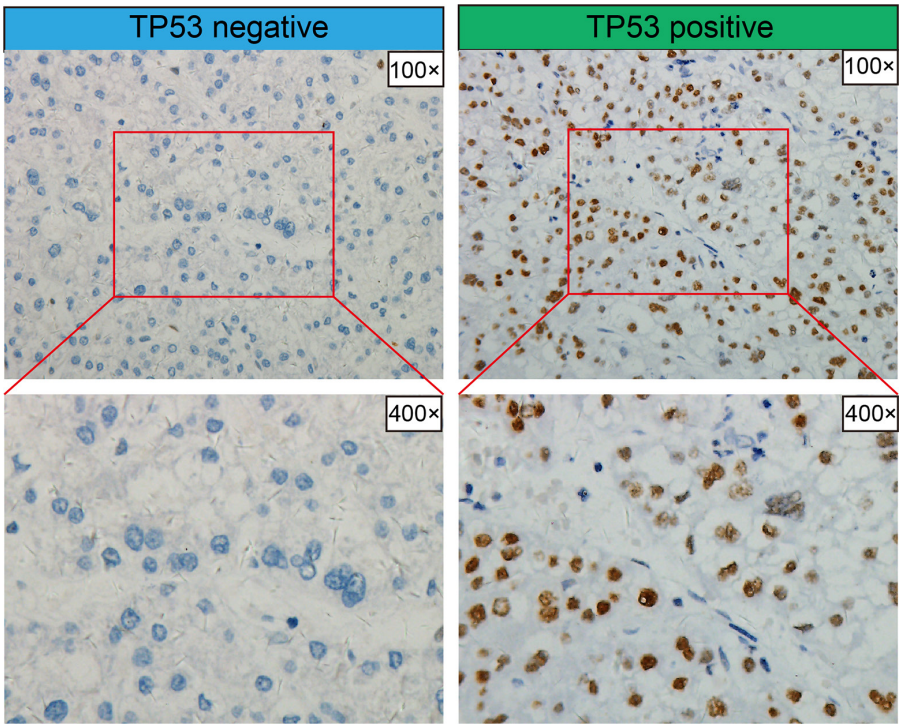

Figure S1 Expression of TP53 in hepatocellular carcinoma tissues by immunohistochemical staining.

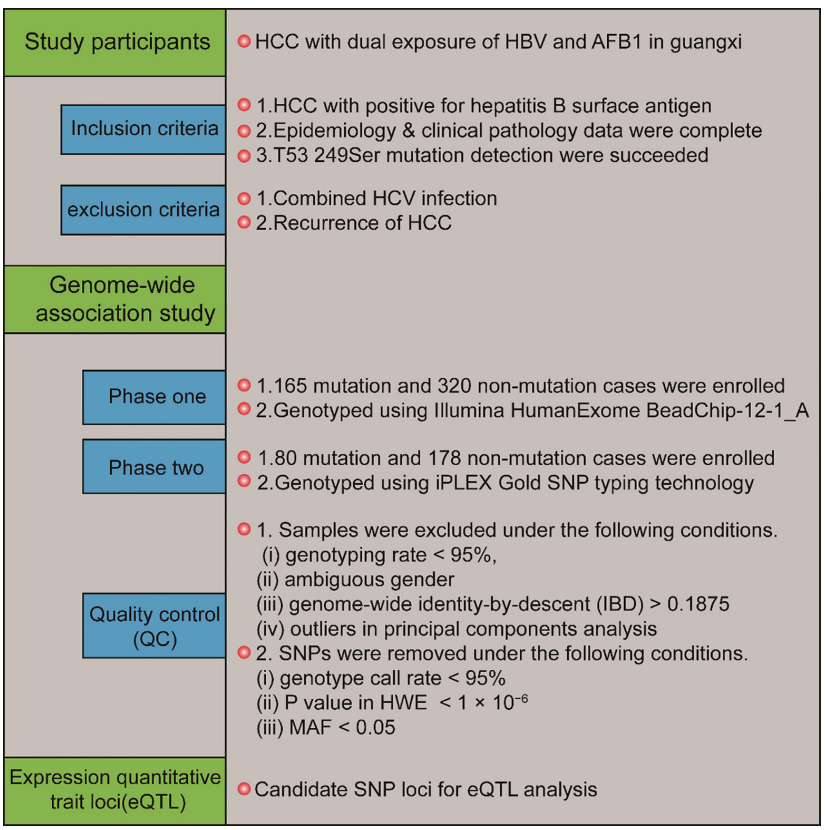

Figure S2 Study flowchart.

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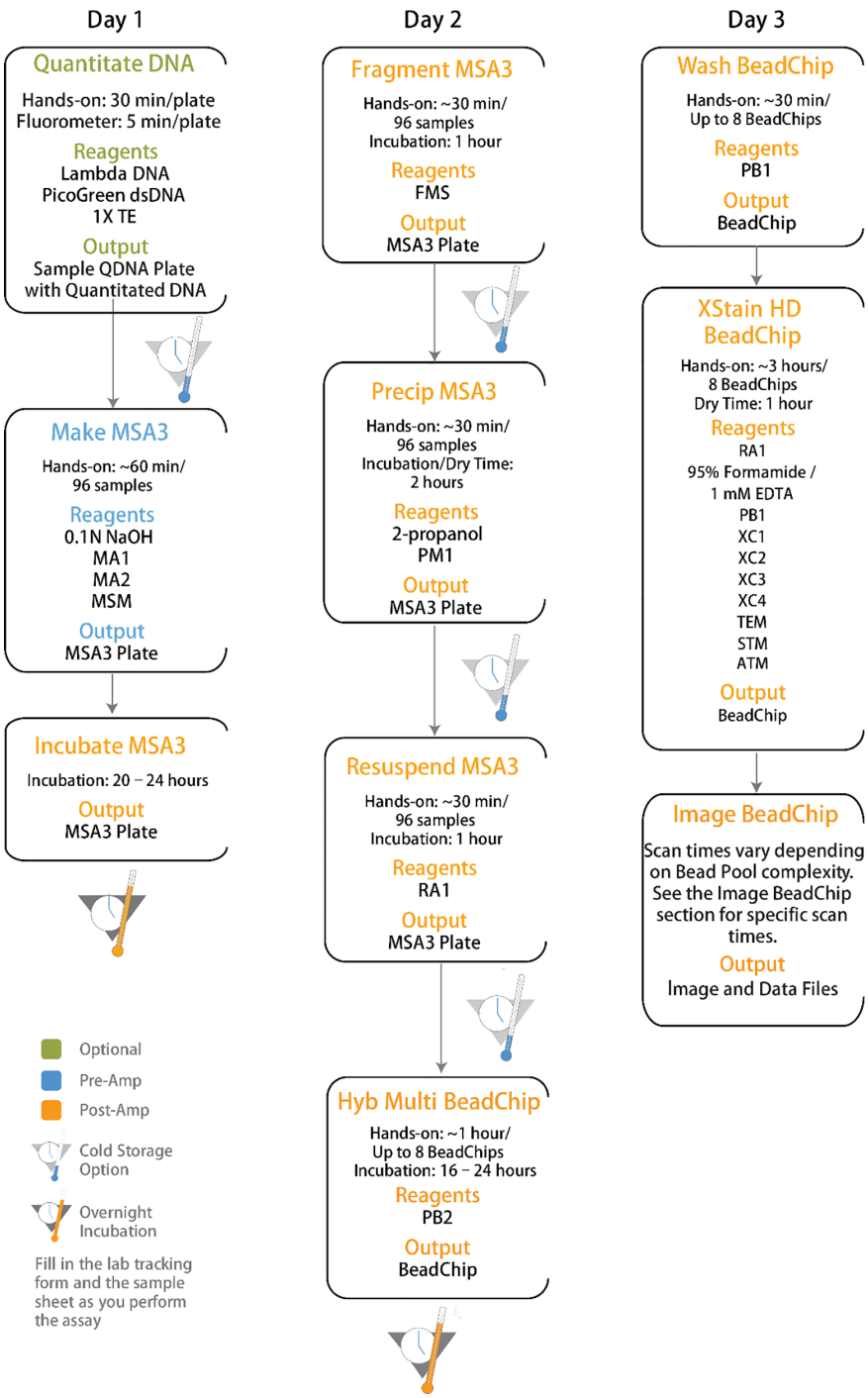

Figure S3 Genotyping procedure of BeadChip-12-1_A in the GWAS. GWAS, genome-wide association study.

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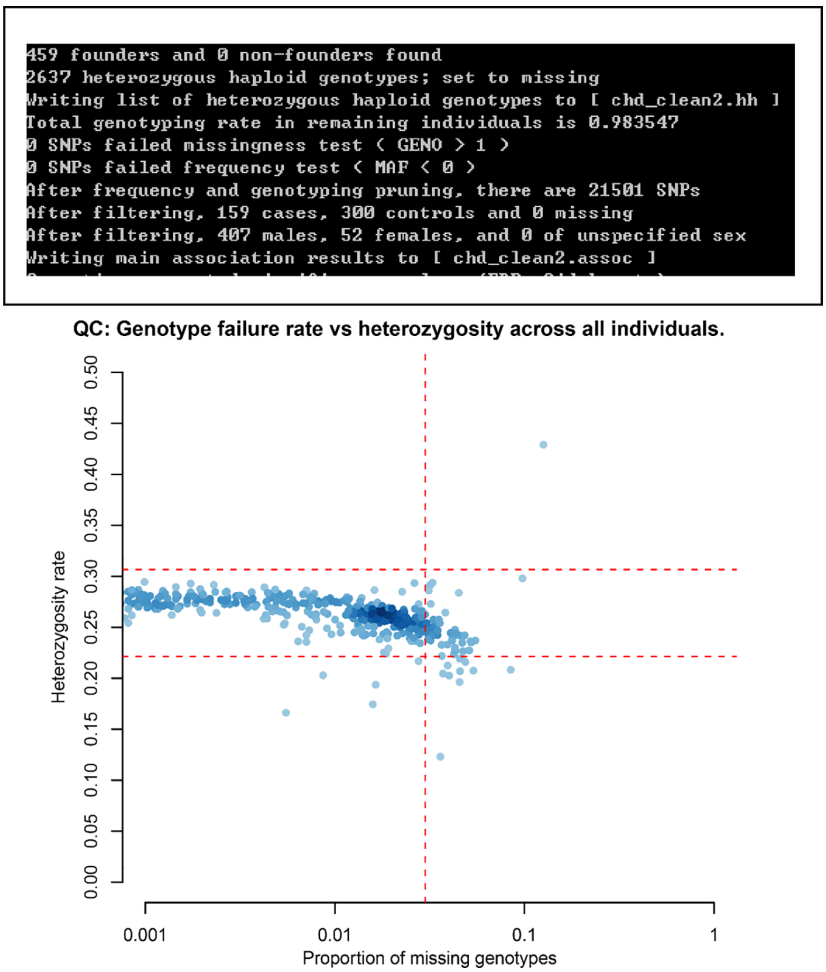

Figure S4 Quality control overview chart. (A) Overall overview of BeadChip-12-1_A after QC. (B) Genotype failure rate and heterozygosity across all individuals.

Table S1 Primers for TP53 R249S mutation detection

Gene	Primers	Sequences
TP53	Forward	*5'-CTTGCCACAGGTCTCCCCAA-3'*
	Reverse	*5'-AGGGGTCAGAGGCAAGCAGA-3'*

The following conditions were applied: 35 cycles of denaturation for 30 sec at 94 ℃; 30 sec of annealing at 62 ℃; and primer extension for 1 min at 72 ℃.

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Table S2 Associations between TP53 R249S mutation and whole-exon SNPs (TOP-29)

SNP	Chr	Position and gene	Number	HWE	MAF	P*	Mutation group	Non-mutation group	AF of mutation group	AF of non-mutation group
rs3758354	9	9:75764565_A/C_Intergenic	447	0.974	0.109	0.0000543	155	292	0.048	0.140
rs10001249	4	4:1535004_G/A_Intergenic	447	0.974	0.272	0.0000627	154	293	0.351	0.230
rs6104	18	18:61570529_C/G_ Nonsynonymous:SERPINB2	448	0.976	0.462	0.000168	155	293	0.626	0.491
rs4646450	7	7:99266318_G/A_ Intron:CYP3A5	453	0.987	0.328	0.000249	156	297	0.407	0.286
rs9895154	17	17:42267949_G/A_ Nonsynonymous:TMUB2	459	1.000	0.063	0.000261	159	300	0.104	0.042
rs6585827	10	10:124165615_G/A_ Intron:PLEKHA1	444	0.967	0.368	0.000270	151	293	0.715	0.589
rs7414489	1	1:64165243_C/A_Intergenic	444	0.967	0.452	0.000285	152	292	0.536	0.408
rs2288101	2	2:31135184_G/T_ Nonsynonymous:GALNT14	458	0.998	0.216	0.000285	159	299	0.283	0.181
rs9930984	16	16:77353973_G/T_ Nonsynonymous:ADAMTS18	437	0.952	0.126	0.000334	148	289	0.936	0.843
rs5951426	X	X:22291606_C/A_ Nonsynonymous:ZNF645	437	0.952	0.489	0.000460	154	283	0.620	0.452
rs75218075	3	3:167320010_T/C_ Nonsynonymous:WDR49	459	1.000	0.179	0.00047	159	300	0.120	0.210
rs1619714	11	11:115552918_A/G_ Intron:AP000797.3	443	0.965	0.409	0.000609	152	291	0.671	0.550
rs12012519	X	X:87190349_T/C_Intergenic	459	1.000	0.107	0.000648	159	300	0.170	0.073
rs11901030	2	2:40998751_A/G_Intergenic	451	0.983	0.269	0.000728	156	295	0.340	0.232
rs11640912	16	16:77359919_A/T_ Nonsynonymous:ADAMTS18	443	0.965	0.138	0.000749	151	292	0.921	0.832
rs714106	19	19:42083849_C/A_ Nonsynonymous:CEACAM21	453	0.987	0.373	0.000783	156	297	0.551	0.667
rs3745936	19	19:42092815_T/A_Essential_ Splice_Site:CEACAM21	437	0.952	0.365	0.000798	149	288	0.557	0.675
rs893817	15	15:74229065_G/A_ Intron:LOXL1	457	0.996	0.479	0.000832	158	299	0.405	0.518
rs11023787	11	11:15952294_C/T_Intergenic	447	0.974	0.497	0.000999	155	292	0.571	0.457
rs55752830	11	11:124947396_A/G_ Nonsynonymous:SLC37A2	452	0.985	0.108	0.00109	156	296	0.154	0.084
rs2056822	19	19:15739597_A/C_ Exon:CYP4F8	444	0.967	0.274	0.00139	149	295	0.792	0.693
rs3812629	10	10:75407290_G/A_ Nonsynonymous:SYNPO2L	453	0.987	0.205	0.00154	156	297	0.141	0.239
rs34051490	16	16:20966362_T/C_ Nonsynonymous:DNAH3	442	0.963	0.181	0.00154	155	287	0.126	0.211
rs8022091	14	14:70655751_A/C_ Utr5:SLC8A3	437	0.952	0.360	0.00155	148	289	0.561	0.680
rs1442801	15	15:98768182_C/T_Intron:CTD-2544M6.1	449	0.978	0.296	0.00161	155	294	0.639	0.738
rs922948	3	3:69442637_G/A_ Intron:FRMD4B	451	0.983	0.369	0.00174	155	296	0.700	0.595
rs10842496	12	12:25311489_G/T_ Nonsynonymous:CASC1	453	0.987	0.171	0.00177	155	298	0.226	0.143
rs117866676	12	12:123342706_C/T_ Nonsynonymous:HIP1R	457	0.996	0.053	0.00183	157	300	0.083	0.037
rs12192544	6	6:46620252_C/G_ Nonsynonymous:CYP39A1	459	1.000	0.149	0.00294	159	300	0.195	0.125

*, adjusted for age, sex, race, smoking status, and drinking status. SNP, single nucleotide polymorphism; Chr, chromosome; MAF, minor allele frequency; AF, allele frequency; HWE, Hardy-Weinberg equilibrium.

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Table S3 Primers of candidate SNPs in the GWAS (TOP-29)

SNP	5' to 3'		Product (bp)	Tm (℃)
	Forward primer	Reverse primer		
rs10001249	GCCTGCCAGGAACATCAGTT	GTGGCGATTCGGCGTTA	477	62
rs10842496	ATTACTGGTAGCTATGGGCTGAGA	TCTTGAAGAAAGGAGAACTGACACT	358	60
rs11023787	ATGGAAGCACATTCATTTGGG	AGGGCATTTGGACCGTGAG	332	60
rs11640912	TTTGATACAAGCAGCAATTTCTTCTG	TGAGTGAACAGTGGTAATGAAAAGAA	507	62
rs117866676	GTGGCCCATGGGTCATTCCAG	TCTGTAAACCTCCCAAAAGGCAAGA	508	62
rs11901030	TTGTAAATGGCAGAGTTGAGTGTTG	CTTGTGCAGGGGAATGACG	556	63
rs12012519	AAGGCGTCAAAGGTCAGGAA	GCATCGGATATGCCATAGAGTG	315	60
rs12192544	TGTTCTAGCCCTGCACTTAAGAGT	CGCGCTGGCTGAGATTCAAAGA	495	62
rs1442801	CGTTATGGCTGTAGGCTGTTTG	GGCTCCAATTTCTTTGTTTTCC	291	60
rs1619714	GCCAGGGTGGTAGGAATGAG	GGGAGGCTGAGGTAGGACAA	398	60
rs2056822	ATCCCCAAAGGTGCCCTCCAT	AAGTCAGGCATCTGACAAGGCA	493	62
rs2288101	TTTCTCTCTTTTCCTGCTCATCAGA	TTGTTGTTCAGACTCCTGCCCAAA	505	62
rs34051490	ACCGTATCTTACTGGCAACCCTATC	GCTCTAAAACGTTGGGTCCATTGT	394	60
rs3745936	GCAGTGAAAATAACAGGAGGTGGTGA	GCCATAGGCCCATTCACTTTCC	502	62
rs3758354	ATGAGGAAGCTGTGGGGAGA	TGTTGCTGCTATTACCAGTGCC	793	62
rs3812629	ACCACATACCTGTCCGCACG	CTCGCGAGCAGCGCATCT	496	60
rs4646450	ACAGGACCTCTATGCTGAAAACTAT	GTGTCAATTCAACTGTCCGTGAT	635	60
rs55752830	GGGACATAGAATCTGGTAGGAGGCA	GGATGGCTGTGGGTGGATTGTA	536	60
rs5951426	TGAACAAGATGCCTGCTGGTGA	GCATATGTTGCACAGACGGTAGTGA	588	60
rs6104	TCTGAGAAGCATGGGCATGGA	GGGTAGCAGAAGTTGTTCAGAAGAGCA	411	62
rs6585827	TCCTCTTTCCTTCTCCCCTCT	ACCTTGCTAAGTTCAACACCGT	592	60
rs714106	GTGAAGAGACCTGCACCCAGGA	TGACAATCCTGCGTATGTGAAGCA	509	60
rs7414489	TGATTCCCTTCGCCTCAAG	ACCACAATAAACACCACTCCAAC	658	60
rs75218075	TCAGAGAAATTCAGATCCCAGAGTT	GGGACTATATGTTTTCTTTCACCTCAT	502	60
rs8022091	CAGCTGTCAGGGAGCTGCG	TCCCAGCTGATAGGGTGACGTT	493	60
rs893817	CCCGCTCAGCACATATTTCA	TCTCACAACCCCTACAGACGAT	377	62
rs922948	TGGAGGTGGGAGGTCAGTCA	CCTATTCAGCGTCTATTCTTTGG	420	62
rs9895154	GCCTGTTTGCCTTCTCATTCC	CTGCCCACATTGACACCAAG	274	62
rs9930984	CCTTTGAACTAGAAGGACTGGAGTT	GCCACATACATTTTCTCTAGTGTTCT	545	60

SNP, single nucleotide polymorphism; GWAS, genome-wide association study.

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Table S4 Association results of SNPs in the replication study

SNP	Chr	Position and gene	Number	HWE	MAF	P	Mutation group	Non-mutation group	AF of mutation group	AF of Non-mutation group
rs8022091	14	14:70655751_A/C_Utr5:SLC8A3	248	0.961	0.347	0.0232	78	170	0.731	0.618
rs9930984	16	16:77353973_G/T_ Nonsynonymous:ADAMTS18	252	0.977	0.091	0.0255	78	174	0.962	0.885
rs75218075	3	3:167320010_T/C_ Nonsynonymous:WDR49	252	0.977	0.147	0.0295	79	173	0.095	0.171
rs11023787	11	11:15952294_C/T_Intergenic	251	0.973	0.478	0.0592	78	173	0.545	0.448
rs117866676	12	12:123342706_C/T_ Nonsynonymous:HIP1R	256	0.992	0.064	0.0858	79	177	0.095	0.051
rs12012519	X	X:87190349_T/C_Intergenic	258	1.000	0.118	0.130	80	178	0.163	0.098
rs6104	18	18:61570529_C/G_ Nonsynonymous:SERPINB2	255	0.988	0.410	0.154	78	177	0.468	0.384
rs3758354	9	9:75764565_A/C_Intergenic	250	0.969	0.142	0.167	78	172	0.109	0.157
rs5951426	X	X:22291606_C/A_ Nonsynonymous:ZNF645	258	1.000	0.426	0.223	80	178	0.519	0.598
rs11640912	16	16:77359919_A/T_ Nonsynonymous:ADAMTS18	249	0.965	0.094	0.259	79	170	0.930	0.894
rs893817	15	15:74229065_G/A_Intron:LOXL1	253	0.981	0.476	0.338	77	176	0.442	0.491
rs9895154	17	17:42267949_G/A_ Nonsynonymous:TMUB2	250	0.969	0.062	0.377	76	174	0.046	0.069
rs55752830	11	11:124947396_A/G_ Nonsynonymous:SLC37A2	252	0.977	0.099	0.414	79	173	0.082	0.107
rs10842496	12	12:25311489_G/T_ Nonsynonymous:CASC1	243	0.942	0.200	0.425	77	166	0.221	0.190
rs2056822	19	19:15739597_A/C_Exon:CYP4F8	247	0.957	0.292	0.487	76	171	0.730	0.699
rs3745936	19	19:42092815_T/A_Essential_ Splice_Site:CEACAM21	247	0.957	0.370	0.501	75	172	0.607	0.640
rs2288101	2	2:31135184_G/T_ Nonsynonymous:GALNT14	254	0.985	0.209	0.639	79	175	0.222	0.203
rs3812629	10	10:75407290_G/A_ Nonsynonymous:SYNPO2L	252	0.977	0.177	0.653	77	175	0.188	0.171
rs34051490	16	16:20966362_T/C_ Nonsynonymous:DNAH3	247	0.957	0.200	0.677	77	170	0.188	0.206
rs7414489	1	1:64165243_C/A_Intergenic	248	0.961	0.413	0.726	80	168	0.425	0.408
rs1442801	15	15:98768182_C/T_Intron:CTD-2544M6.1	251	0.973	0.299	0.730	78	173	0.712	0.697
rs4646450	7	7:99266318_G/A_Intron:CYP3A5	257	0.996	0.296	0.785	79	178	0.304	0.292
rs714106	19	19:42083849_C/A_ Nonsynonymous:CEACAM21	257	0.996	0.181	0.860	80	177	0.825	0.816
rs922948	3	3:69442637_G/A_Intron:FRMD4B	250	0.969	0.418	0.862	79	171	0.576	0.585
rs11901030	2	2:40998751_A/G_Intergenic	252	0.977	0.266	0.917	78	174	0.263	0.267
rs10001249	4	4:1535004_G/A_Intergenic	255	0.988	0.235	0.938	80	175	0.238	0.234
rs1619714	11	11:115552918_A/G_ Intron:AP000797.3	249	0.965	0.392	0.987	78	171	0.609	0.608
rs12192544	6	6:46620252_C/G_ Nonsynonymous:CYP39A1	254	0.985	0.152	0.992	76	178	0.151	0.152
rs6585827	10	10:124165615_G/A_ Intron:PLEKHA1	245	0.950	0.400	1.000	75	170	0.600	0.600

SNP, single nucleotide polymorphism.

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Table S5 Primers for MassARRAY genotyping

SNP	2ND-PCRP	1ST-PCRP	Product (bp)	UEP_SEQ	EXT1_SEQ	EXT2_SEQ
rs8022091	ACGTTGGATGCATTAAGCTGCCGGAAGAAG	ACGTTGGATGTGTCTCCCAGCTGATAGGGT	102	ATCTCCAAGCCTCCA	ATCTCCAAGCCTCCAC	ATCTCCAAGCCTCCAA
rs75218075	ACGTTGGATGTTGGACGGCTATTACACTGG	ACGTTGGATGTTGCCTTTGGAATCCCTATG	99	GGACACCCACTGGTT	GGACACCCACTGGTTC	GGACACCCACTGGTTT
rs7414489	ACGTTGGATGTTCCCAGCCTTGATCCATAC	ACGTTGGATGAAGACTCATGCCCAGCATAC	105	CCTTGCCTGCCTGCAC	CCTTGCCTGCCTGCACG	CCTTGCCTGCCTGCACT
rs55752830	ACGTTGGATGACCTGATGAACATGCCGATG	ACGTTGGATGCCATCTGCTTTCAGACAAGG	115	ACGATGGCATAGGCGA	ACGATGGCATAGGCGAC	ACGATGGCATAGGCGAT
rs4646450	ACGTTGGATGGCCTTGTCCAGAATACACAC	ACGTTGGATGTAACAAAGAGCGAGAGGACG	103	ATTCACTTCACGTGGCA	ATTCACTTCACGTGGCAC	ATTCACTTCACGTGGCAT
rs11023787	ACGTTGGATGATGGCATGTCCTACCTTCTG	ACGTTGGATGAACATTGAGGGAAGGGTCAG	99	TCCTACCTTCTGCTCCTT	TCCTACCTTCTGCTCCTTC	TCCTACCTTCTGCTCCTTT
rs3812629	ACGTTGGATGTTACAAGACCCTGCCTCACG	ACGTTGGATGGAGTCTTAGGAGTCATAGGG	103	CGACACCTAAGACCCCCC	CGACACCTAAGACCCCCCC	CGACACCTAAGACCCCCCT
rs34051490	ACGTTGGATGCCATTCTGGAGAATGCTGAC	ACGTTGGATGCGTTGGGTCCATTGTTCTTC	100	GGAAACTTCTCTGATGGA	GGAAACTTCTCTGATGGAC	GGAAACTTCTCTGATGGAT
rs2288101	ACGTTGGATGTCTCTTTCTCCCCAGGTATG	ACGTTGGATGACAAGGTGATGACTGACAGG	102	GGCCTTCACATACACCCAG	GGCCTTCACATACACCCAGC	GGCCTTCACATACACCCAGA
rs12012519	ACGTTGGATGCTGTATTCTTGCAGACAGGG	ACGTTGGATGAAGAAGGACCGCTTCTAGAC	90	ACAGGGTGCCTTTAATTTA	ACAGGGTGCCTTTAATTTAC	ACAGGGTGCCTTTAATTTAT
rs117866676	ACGTTGGATGAGCTTGCTCACGGCATCCTG	ACGTTGGATGAGGCTGCTGGACGAGCAGTT	103	GGAGCGGCAGCAGCGCCCC	GGAGCGGCAGCAGCGCCCCA	GGAGCGGCAGCAGCGCCCCG
rs10001249	ACGTTGGATGATGTGAGATGCGCCCGAGA	ACGTTGGATGAAATCCACCTGGACGCGAAC	120	CACACCCAGGCCCAGAAAAC	CACACCCAGGCCCAGAAAACC	CACACCCAGGCCCAGAAAACT
rs1619714	ACGTTGGATGAAGGATGACTGTCTCTCTGG	ACGTTGGATGCAGTATCCTGCTTCCCTAAG	99	GGGTCGTTATGAGGATTCAA	GGGTCGTTATGAGGATTCAAC	GGGTCGTTATGAGGATTCAAT
rs922948	ACGTTGGATGTCTCCCTTAAAGAAGGTGGC	ACGTTGGATGAAAAGCATCCAAATGGGAGG	101	ACCATGATCCACTGGACTTCC	ACCATGATCCACTGGACTTCCC	ACCATGATCCACTGGACTTCCT
rs3745936	ACGTTGGATGATCTGCTTTGTGGTCGATCC	ACGTTGGATGGGGTCAAGACCTCTTCTCTG	103	TCGTCAGAGTGTAGCAATTCC	TCGTCAGAGTGTAGCAATTCCA	TCGTCAGAGTGTAGCAATTCCT
rs3758354	ACGTTGGATGCTCCAGAGAAATGACTCACC	ACGTTGGATGGTTTGTAAAGTAAGTGGAGC	94	GCTGTTTCTAGAAGATGATGA	GCTGTTTCTAGAAGATGATGAG	GCTGTTTCTAGAAGATGATGAT
rs714106	ACGTTGGATGTGCATTTCCAGAACGTCACC	ACGTTGGATGGGTGAGATGCCTGTTCAATC	110	GGGAGGGACACGGGATACTACA	GGGAGGGACACGGGATACTACAC	GGGAGGGACACGGGATACTACAA
rs6585827	ACGTTGGATGCCATTCACATTCCTACCAAC	ACGTTGGATGCAGGTGCTAACAACCAGTTC	97	ACCACATACACATTTTATTTCAT	ACCACATACACATTTTATTTCATC	ACCACATACACATTTTATTTCATT
rs1442801	ACGTTGGATGCTGATTTCTGAGCCAAGTCC	ACGTTGGATGCCCCTGTAAAATCTAAATGC	111	ATTTATGAGCCAAGTCCACTGTT	ATTTATGAGCCAAGTCCACTGTTA	ATTTATGAGCCAAGTCCACTGTTG
rs11640912	ACGTTGGATGAATACGGCTAGAACCTGGAC	ACGTTGGATGTGAGCCCTTTGATTACTAAC	110	ACGTCTAGAACCTGGACAGAATA	ACGTCTAGAACCTGGACAGAATAA	ACGTCTAGAACCTGGACAGAATAT
rs893817	ACGTTGGATGAAACAGGTGAGGTGTGGACG	ACGTTGGATGAAATCTGTTCCCTCCTGCCC	120	AAGTTGTGGACGAGCAATGGGAA	AAGTTGTGGACGAGCAATGGGAAA	AAGTTGTGGACGAGCAATGGGAAG
rs9930984	ACGTTGGATGGGTGGAATTCAGACATGTTC	ACGTTGGATGCTCCTGGATTTCGATGCTTC	112	GGTTGAGAATATTATCCGGTGGTC	GGTTGAGAATATTATCCGGTGGTCC	GGTTGAGAATATTATCCGGTGGTCA
rs6104	ACGTTGGATGGTGGCAGATCATCCTTTTCT	ACGTTGGATGAGCAGCACGCTTAGTTTTAG	120	TGCCTTTTATTTTTCGGCAGATTTT	TGCCTTTTATTTTTCGGCAGATTTTC	TGCCTTTTATTTTTCGGCAGATTTTG
rs5951426	ACGTTGGATGTAGCTTAGATGGTCCCTGTC	ACGTTGGATGGTTCGTCCTCATATTGCTCC	100	CCATGTCTTGCAGTCTTTTAGGGAT	CCATGTCTTGCAGTCTTTTAGGGATG	CCATGTCTTGCAGTCTTTTAGGGATT
rs2056822	ACGTTGGATGATCAGCAGCCTTAACTTGCC	ACGTTGGATGAAGCCATAGGTGACCTCTTC	108	CTCGATGACCCCTTCCGCTTCGACCC	CTCGATGACCCCTTCCGCTTCGACCCC	CTCGATGACCCCTTCCGCTTCGACCCA
rs10842496	ACGTTGGATGGCTGTATTTCAAGCCTTTCC	ACGTTGGATGACAGTGATCTATTCACTGAG	119	CCGTTCTTCTTTCTCATATTTCAAAC	CCGTTCTTCTTTCTCATATTTCAAACG	CCGTTCTTCTTTCTCATATTTCAAACT
rs12192544	ACGTTGGATGCCTTGCTCTGTTCTTACTCC	ACGTTGGATGCTCCAATCCAAGGAATCCAG	100	GACTGGCTCTGTTCTTACTCCTTCAGC	GACTGGCTCTGTTCTTACTCCTTCAGCC	GACTGGCTCTGTTCTTACTCCTTCAGCG
rs11901030	ACGTTGGATGTGGTTTCAGACACTAAAAGC	ACGTTGGATGGGACTGCAGTTTCTCAGAAC	106	AAAATCAACTAATGTTGACACTTAAAT	AAAATCAACTAATGTTGACACTTAAATA	AAAATCAACTAATGTTGACACTTAAATG
rs9895154	ACGTTGGATGTGAAACTGATCTACCAGGGC	ACGTTGGATGGTGAATCACACAGTTGTCGG	106	ATGCGACAAGACCCAGCCCGCACACTGC	ATGCGACAAGACCCAGCCCGCACACTGCA	ATGCGACAAGACCCAGCCCGCACACTGCG

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Table S6 Primers used in quantitative RT-PCR assays

Genes	Primers	Sequences
GAPDH	Forward	*GTCAGCCGCATCTTCTTT*
	Reverse	*CGCCCAATACGACCAAAT*
ADAMTS18	Forward	*GAGGTGCAGCAATGCTTCTAT*
	Reverse	*CAGCACACGTAGACACAGC*
WDR49	Forward	*CCATTATGAGTTGCCAGAAAGC*
	Reverse	*CCCACGCTGAGTTGGTTTT*
SLC8A3	Forward	*GACGACAAAGGGCTACTGGAA*
	Reverse	*AGCATTAGGAAGCAAGAGCGT*

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