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The association of lncRNA SNPs and SNPs-environment interactions based on GWAS with HBV-related HCC risk and progression

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Abstract

Background: Long non-coding RNA (lncRNA) plays an essential role in hepatitis B virus-related hepatocellular carcinoma (HBV-related HCC) occurrence and development. Single nucleotide polymorphism (SNP) may affect HBV-related HCC susceptibility by altering the function of lncRNA. However, the relationship between lncRNA SNPs and HBV-related HCC occurrence and development is still unclear.

Methods: In the present study, based on HBV-related HCC genome-wide association studies, eight potentially functional SNPs from two lncRNAs were predicted using a set of bioinformatics strategies. In 643 HBV-related HCC patients, 549 CHB carriers, and 553 HBV natural clearance subjects from Southern Chinese, we evaluated associations between SNPs and HBV-related HCC occurrence or development with odds ratio (OR) and 95% confidence interval (CI) under credible genetic models.

Results: In HBV-related HCC patients, rs9908998 was found to significantly increase the risk of lymphatic metastasis under recessive model (Adjusted OR = 1.95, 95% CI = 1.20–3.17). *Lnc-RP11-150O12.3* rs2275959, rs1008547, and rs11776545 with cancer family history may show significant multiplicative and additive interactions on HBV-related HCC susceptibility (all $p_{Adjusted} < .05$). The associations of rs2275959, rs1008547, and rs11776545 with distant metastasis of HBV-related HCC patients were observed in additive model (Adjusted OR = 1.45, 95% CI = 1.06–1.97 for rs2275959; Adjusted OR = 1.45, 95% CI = 1.06–1.98 for rs1008547; Adjusted OR = 1.40, 95% CI = 1.03–1.91 for rs11776545).

Qing Liu and Guiyan Liu have contributed equally to this work.

Conclusion: Taken together, *lnc-ACACA-1* rs9908998, *lnc-RP11-150O12.3* rs2275959, rs1008547, and rs11776545 might be predictors for HBV-related HCC risk or prognosis.

KEYWORDS

association, CHB, genetic, GWAS, HBV, HBV infection, HBV-related HCC, HCC, lncRNA, SNP

1 | INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common malignancy and the second leading cause of cancer mortality worldwide (Ferlay et al., 2015). In China, the number of new and fatal cases of HCC accounts for approximately 50% and 51% of that in the world, respectively (Ferlay et al., 2015). And the 5-year survival rate of HCC is only 14% in China (Allemani et al., 2018). Chronic hepatitis B virus (HBV) infection is the most frequently underlying cause factor of HCC. However, the outcomes of HBV infection, including virus natural clearance, persistent viral infection, or developing to HCC, are influenced by environmental and genetic factors (Wang, 2003). Only 20%–30% of adults with chronic hepatitis B virus (CHB) develop cirrhosis or HCC (WHO, 2017). Under the same environment, the tumor susceptibility and progression vary in individuals depending on genetic factors. The occurrence and development of HCC is a complex process in which gene and environment work together.

Long non-coding RNAs (lncRNAs) are a diverse class of transcripts larger than 200 nucleotides (nt), and do not serve as templates for proteins. Previous studies have confirmed that lncRNAs played a critical regulatory role in histone modification, transcriptional interference, and other biological processes, and were strongly implicated in development and progression of HCC (Li et al., 2015). At present, studies have reported the relationship between lncRNA SNPs and HCC risk and prognosis. For example, Zhang et al. found that two *HOTAIR* (OMIM# 611400) SNPs (rs12427129 and rs3816153) were associated with HCC risk in Southern China population, which may play an important role in the susceptibility of HCC (Zhang et al., 2019). Wang et al. found that *HOTTIP* (OMIM# 614060) rs17501292, rs2067087, rs17427960, and *MALAT1* (OMIM# 607924) rs4102217 increased the risk of HCC, while *HOTTIP* rs3807598 and *MALAT1* rs591291 prolonged the survival time of HBV-negative HCC patients (Wang, Xu, et al., 2018). It was found that *HULC* (OMIM# 612210) rs1041279 was a risk factor for HCC, but had no effect on the survival time of HCC patients (Wang, Lv, et al., 2018).

Recently, multiple genome-wide association studies (GWASs) have identified a series of single nucleotide polymorphisms (SNPs) significantly associated with HBV-related HCC (Chan et al., 2011; Jiang et al., 2013; Li et al., 2012; Qu et al., 2016; Zhang et al., 2010; Zhou et al., 2018). However, most of these SNPs are located in non-coding regions such as introns and intergenic regions, and only explain a small part of HBV-related HCC occurrence and progression. Therefore, lncRNAs associated with HBV-related HCC need to be further explored and functional or pathogenic SNPs in HBV-related HCC susceptible regions need to be further identified in the post-GWAS era.

Considering what were mentioned above, it was hypothesized that there may exist functional lncRNA SNPs in HBV-related HCC susceptibility regions found by existing GWAS studies, which are associated with the occurrence and development of HBV-related HCC. To further test the hypothesis, we systematically screened potentially functional lncRNA SNPs in the HBV-related HCC susceptibility regions identified by GWAS with a set of bioinformatics strategies. Next, two dependent case-control studies (HBV-related HCC vs. CHB; CHB vs. HBV nature clearance) were conducted in a Southern Chinese population to investigate the relationship of these candidate SNPs with HBV-related HCC and HBV natural clearance after HBV infection. And the effect of potential interactions between these candidate SNPs and environmental factors on HBV-related HCC and HBV natural clearance were assessed. Finally, we investigated the effects of SNPs on the development of HBV-related HCC in HBV-related HCC patients.

2 | MATERIAL AND METHODS

2.1 | Ethical compliance

This study has been acquired written informed consent of each participant, and was approved by the Institutional Review Board of Guangdong Pharmaceutical University, Guangdong, China.

2.2 | Identification of lncRNA and potentially functional SNPs

TagSNPs associated with HBV-related HCC risk were obtained from GWASs of HBV-related HCC which were retrieved in the GWAS Catalog and PubMed up to April 30,

2018 and based on Chinese population. The search terms of "Hepatocellular carcinoma" in GWAS Catalog, "HBV," "Hepatitis B," "Chronic hepatitis B," "CHB," "genome wide association study," "GWAS," "liver cancer," "Hepatocellular Carcinoma," and "HBV related HCC" in PubMed were used, respectively. Then, SNAP Online tool was used to calculate the linkage disequilibrium (LD) blocks of each tagSNP by analyzing the Chinese Han Beijing (CHB) genotype information of ± 500 kb around the tagSNPs (setting $r^2 \geq 0.8$), and these LD blocks were defined as HBV-related HCC susceptibility regions. Next, SNPs in susceptibility regions were obtained using SNPs data from dbSNP of NCBI (dbSNP v150, human GRCh37) and 1000 genome project. These SNPs were matched to the lncRNA SNPs of the lncRNASNP database which offered SNPs in lncRNAs and their potential functions in human and mouse, to obtain lncRNA SNPs in susceptibility regions. To further narrow down the potentially functional lncRNA SNPs, lncRNASNP, rVarBase, HaploReg, SNPinfo, and RegulomeDB were used to predict potential function of lncRNA SNPs, including location, conservation, presence of histone marks and DNase hypersensitive sites around SNP, effects of protein binding motifs and microRNA binding lncRNA, and function score for SNPs, etc. LncRNA SNPs with potential function in at least four functional annotation databases and minor allele frequency (MAF) > 0.05 in the southern Chinese population were retained.

Finally, eight potentially functional SNPs in two lncRNAs were selected as candidate SNPs to genotype in subsequent two case-control studies (rs9908998, rs7221955, rs9891142 in *lnc-ACACA-1*; and rs2275959, rs1008547, rs11776545, rs2298320, and rs2298321 in *lnc-RP11-150O12.3*). The screening flowchart for candidate lncRNA SNPs is shown in Figure S1. The functional prediction results of candidate lncRNA SNPs were shown in the previous study (Qing et al., 2019). GenBank reference sequences were NM_005568.5 and NM_025069.3 for *lnc-ACACA-1* and *lnc-RP11-150O12.3*, respectively. LncRNA variant nomenclature followed current guidelines of the Human Genome Variation Society (http://www.hgvs.org/rec.html).

2.3 | Participants

Two case control studies were performed in a total of 1745 subjects, including 643 HBV-related HCC patients, 549 gender- and age-matched (± 5 years) CHB and 553 HBV natural clearance subjects. All subjects were consecutively recruited between September 2010 and October 2016 from Shunde hospital of Southern medical university, Guangdong, China. In addition, all of them were residents of Shunde who lived in Shunde at least 10 years. The diagnosis of HBV-related HCC was based on pathological examination and HBV infection. Patients with two or

more malignancies or HCV infection were excluded. We only recruited CHB patients whose hepatitis B virus surface antigen (HBsAg) and hepatitis B virus core antibody (HBcAb) were both positive. HBV natural clearance subjects were diagnosed with HBsAg and hepatitis B virus e antigen (HBeAg) negative, HBcAb and hepatitis B virus surface antibody (HBsAb) positive, and without hepatitis B vaccination. For CHB and HBV natural clearance patients, we eliminated patients with other viral hepatitis infections, severe digestive problems, or other benign and malignant tumors.

Epidemiological data were collected by in-person interviews, covering information of gender, age, smoking and drinking status, and cancer family history. Additionally, clinical data of HBV-related HCC, including lymphatic metastasis, distant metastasis, TNM classification, and cancer embolus were obtained from patients' medical records. The main definitions of risk categories were as follows: (1) smoker is a person who smokes at least one cigarette per day for 6 months or more. (2) drinker is a person who consumes beer, wine, or hard liquor at least once per month for 6 months or more. (3) cancer family history is about the first-degree relatives (parents, siblings, and children). (4) lymphatic metastasis, distant metastasis, and TNM classification are confirmed by oncologists based on primary liver cancer staging criteria defined in the 2010 edition of the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC). (5) cancer embolus is diagnosed based on imaging findings of cancerous plug formation.

2.4 | SNPs genotyping

A 5 ml of peripheral blood sample was drawn from each participant. Genomic DNA was extracted from peripheral blood by means of TIANamp DNA kit (Tiangen, Beijing, China) following the manufacturer's protocol. Eight SNPs were genotyped by Sequenom MassARRAY IPLEX-GOLD system without knowledge of subjects' allocation status. About 5% samples were detected in duplicates to ensure the accuracy of this method, yielding a 100% reproducibility. The call rates of genotyping for eight SNPs were all above 98%.

2.5 | Statistical analysis

The distributions of demographic characteristics and genotype of each SNP between the case and control were compared by Pearson's χ^2 test. Hardy-Weinberg equilibrium (HWE) for genotypes was evaluated with goodness of fit χ^2 test in HBV natural clearance subjects. Prior to genetic association analysis, the most plausible genetic model was selected through the maximum of standardized version for

the three optimal tests (MAX). The MAX method requires obtaining three Cochran-Armitage Trend Test (CATT) statistics (Z_{CATT}) under the dominant, recessive, and additive genetic model, respectively, and selecting the largest absolute value of Z_{CATT} as the statistic (Z_{MAX}). Due to the linear dependence structure of three Z_{CATT} statistics, we obtained the asymptotic distribution of Z_{MAX} and the corresponding P values with either the Monte-Carlo method or the asymptotic algorithms proposed by Zang et al. (2010). This method can maximize the power and preserve the nominal type I error rate, therefore it is an efficient and robust method for genetic association analysis (Bagos, 2013; Joo et al., 2010). Next, based on the most credible genetic model, the odds ratios (ORs) and 95% confidence intervals (CIs) of multinomial logistic regression analysis were used to investigate the association between SNPs with HBV-related HCC occurrence and HBV clearance. The multiplicative and additive models, calculated by multinomial logistic regression and binomial linear regression, respectively, were used to assess the effects of SNP-environment interactions on HBV-related HCC and HBV clearance. The cumulative effects and haplotype analysis of lncRNA SNPs with HBV-related HCC and HBV clearance were performed by multinomial logistic regression as well. The relationship between SNPs and HBV-related HCC development, including lymphatic metastasis, distant metastasis, TNM classification, and cancer embolus, was investigated with ORs and 95% CIs computed by binary logistic regression analysis. For statistically significant P values after adjustment, multiple comparison corrections were performed using false discovery rate (FDR).

Test statistic Z_{MAX} and P value of the MAX test were obtained using R software's "Rassoc" package (Zang et al., 2010). The haplotype frequencies for genetic variation on lncRNA were constructed through PHASE v2.1 software. The rest of statistical analyses was carried out with SAS v9.4 software (SAS Institute, Cary, NC). All statistical tests were two-tailed with a significant level of $p < .05$.

3 | RESULTS

3.1 | Subjects characteristics

The demographic details of 643 HBV-related HCC patients, 549 CHB patients, and 553 HBV natural clearance subjects enrolled in our study are shown in Table S1. As expected, there were similar distributions of gender and age between these three groups (all $p > .05$). However, there were statistically significant differences between these three groups in smoking, drinking, and cancer family history (all $p < .001$). Comparing with HBV natural clearance subjects, HBV-related HCC patients and CHB patients had higher rates of smoking (67.19% vs. 28.23% vs. 25.14%) and drinking

(53.97% vs. 24.77% vs. 17.00%). However, fewer individuals with cancer family history were seen in CHB patients. Therefore, we took these epidemiological characteristics as potential confounders into the following genetic association analyses.

3.2 | Associations of SNPs with HBV-related HCC occurrence and HBV clearance

There were no significant differences in the genotype distributions of eight SNPs among HBV-related HCC patients, CHB patients, and HBV natural clearance subjects (all $p \geq .178$; Table 1). The genotype frequencies for these eight SNPs in HBV natural clearance subjects conformed to HWE (all $p \geq .192$; Table 1).

Table S2 showed the results of the MAX test. As a result, for *lnc-ACACA-1* rs9908998 in the HBV-related HCC vs. CHB case-control, dominant model was statistically significant and was the most plausible inheritance model ($Z_{MAX} = 2.62$, $p = .020$). However, the results of the MAX test for the remaining seven SNPs were not statistically significant (all $p > .05$) in all case control study. Considering the problem of the sample size of each genotype of SNPs, the dominant model was the most reliable model for all SNPs when studying the association of SNPs with HBV-related HCC occurrence or HBV clearance.

The results of association analysis between SNP in *lnc-ACACA-1* or *lnc-RP11-150O12.3* and HBV-related HCC occurrence or HBV clearance are shown in Table 2. Eight SNPs in *lnc-ACACA-1* or *lnc-RP11-150O12.3* had no effect on HBV-related HCC occurrence or HBV clearance (all $p > .05$).

3.3 | Associations of SNP-environment interactions with HBV-related HCC occurrence and HBV clearance

Under the credible dominant genetic model, the effects of multiplicative and additive interactions of eight SNPs with major environmental risk factors on HBV-related HCC occurrence and HBV clearance were further explored. After FDR adjustment, the multiplicative and additive interactions of *lnc-ACACA-1* rs9908998, rs7221955, and rs9891142 with major environmental factors had no effect on HBV-related HCC occurrence and HBV clearance (all $p > .05$, Table S3).

lnc-RP11-150O12.3 rs2275959, rs1008547, rs11776545, and the cancer family history had significant multiplicative and additive interactions with HBV-related HCC susceptibility ($p_{mul} = .008$, $p_{add} = .012$ for rs2275959; $p_{mul} = .012$, $p_{add} = .017$ for rs1008547; $p_{mul} = .014$, $p_{add} = .017$ for rs11776545; Table 3). No evidence was found for *lnc-RP11-150O12.3*

TABLE 1 The distribution of SNPs genotype

lncRNA	SNP	HBV-related HCC HW/HT/HV[a]	CHB HW/HT/HV[a]	HBV clearance HW/HT/HV[a]	P^b	P^c
lnc-ACACA-1	rs9908998	237/319/87	233/245/71	224/249/80	.296	.425
lnc-ACACA-1	rs7221955	480/149/7	430/108/5	429/97/8	.206	.355
lnc-ACACA-1	rs9891142	478/148/9	430/108/5	426/96/9	.178	.192
lnc-RP11-150O12.3	rs2275959	217/293/132	172/272/102	198/258/90	.236	.731
lnc-RP11-150O12.3	rs1008547	205/299/136	166/274/105	180/264/92	.384	.775
lnc-RP11-150O12.3	rs11776545	211/291/141	167/270/107	181/259/94	.293	.935
lnc-RP11-150O12.3	rs2298320	167/297/173	142/270/131	153/262/120	.381	.697
lnc-RP11-150O12.3	rs2298321	560/77/2	478/68/0	466/69/1	.765	.345

Note: GenBank reference sequences were NM_005568.5 and NM_025069.3 for *lnc-ACACA-1* and *lnc-RP11-150O12.3*, respectively.

Abbreviations: CHB, chronic hepatitis B; HBV, hepatitis B virus; HCC, hepatocellular carcinoma.

[a]HW represented wild homozygote; HT represented mutant heterozygote; HV represented mutant homozygote.

[b]*P* values for the comparison of genotype frequency among HBV-related HCC, CHB, and HBV natural clearance subjects.

[c]*P* values for Hardy-Weinberg equilibrium.

TABLE 2 Association of *lnc-ACACA-1 and lnc-RP11-150O12.3* variants with HBV-related HCC and HBV natural clearance in the dominant model

SNP	HBV-related HCC vs. CHB Crude OR(95%CI)	p_{Crude}	Adjusted OR[a](95%CI)	$p_{Adjusted}$[a]	CHB vs. HBV clearance Crude OR(95%CI)	p_{Crude}	Adjusted OR[a](95%CI)	$p_{Adjusted}$[a]
lnc-ACACA-1								
rs9908998	**1.26 (1.00–1.59)**	**.049**	1.23 (0.96–1.59)	.106	0.92 (0.73–1.17)	.515	0.92 (0.72–1.18)	.519
rs7221955	1.24 (0.94–1.63)	.130	1.13 (0.83–1.52)	.435	1.07 (0.80–1.45)	.639	1.11 (0.82–1.51)	.481
rs9891142	1.25 (0.95–1.64)	.111	1.13 (0.83–1.52)	.438	1.07 (0.79–1.44)	.673	1.11 (0.82–1.50)	.502
lnc-RP11-150O12.3								
rs2275959	0.90 (0.71–1.15)	.400	0.85 (0.65–1.10)	.219	1.24 (0.96–1.59)	.097	1.21 (0.94–1.56)	.137
rs1008547	0.93 (0.73–1.19)	.561	0.86 (0.65–1.12)	.263	1.15 (0.89–1.49)	.271	1.13 (0.87–1.46)	.365
rs11776545	0.91 (0.71–1.16)	.436	0.85 (0.65–1.11)	.243	1.16 (0.90–1.49)	.262	1.13 (0.87–1.46)	.358
rs2298320	1.00 (0.77–1.29)	.980	0.88 (0.66–1.17)	.377	1.13 (0.87–1.48)	.368	1.12 (0.85–1.47)	.422
rs2298321	0.99 (0.70–1.40)	.963	0.95 (0.65–1.39)	.801	0.95 (0.66–1.35)	.763	0.94 (0.66–1.35)	.747

Note: Bold value indicates the results with statistical differences. GenBank reference sequences were NM_005568.5 and NM_025069.3 for *lnc-ACACA-1* and *lnc-RP11-150O12.3*, respectively.

Abbreviations: CHB, chronic hepatitis B; CI, confidence interval; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; OR, odds ratio.

[a]Adjusting by gender, age, smoking, drinking, and cancer family history.

rs2298320, rs2298321-environmental multiplicative and additive interactions associated with HBV-related HCC and HBV clearance risk (all $p > .05$; Table S4).

3.4 | Combined effects of SNPs on HBV-related HCC occurrence and HBV clearance

Furthermore, we evaluated the combined risk allelic effects of three SNPs in *lnc-ACACA-1* and five SNPs in *lnc-RP11-150O12.3* on HBV-related HCC occurrence and HBV clearance, respectively. Whether it was in *lnc-ACACA-1* or

lnc-RP11-150O12, we did not detect any significant allele-dosage association between the number of adverse alleles and HBV-related HCC occurrence or HBV clearance risks (all $p > .05$; Tables 4 and S5).

3.5 | Haplotype analyses of SNPs with HBV-related HCC occurrence and HBV clearance

In addition, haplotype analyses were performed to assess the effect of the haplotype containing *lnc-ACACA-1* and *lnc-RP11-150O12.3* variant alleles on HBV-related HCC

TABLE 3 Gene-environmental interaction analyses of variants in *lnc-RP11-150O12.3* with the risk of HBV-related HCC and HBV natural clearance under the dominant model

SNP	Cancer family history	HBV-related HCC vs. CHB					CHB vs. HBV clearance				
		Adjusted OR[a](95%CI)	$p_{mul}^{a,b}$	p_{mul}^{b}	$p_{add}^{a,d}$	$p_{add}^{a,c,d}$	Adjusted OR[a](95%CI)	$p_{mul}^{a,b}$	p_{mul}^{b}	$p_{add}^{a,d}$	$p_{add}^{a,c,d}$
rs2275959	No	0.76(0.57–1.01)	**.008**	**.040**	**.012**	.060	1.29 (0.99–1.70)	.085	.425	.129	.645
	Yes	1.90(0.90–4.00)					0.58 (0.27–1.28)				
rs1008547	No	0.77(0.57–1.03)	**.012**	.060	**.017**	.085	1.18 (0.90–1.56)	.191	.515	.300	.580
	Yes	1.82(0.86–3.85)					0.62 (0.28–1.36)				
rs11776545	No	0.77(0.57–1.03)	**.014**	.070	**.017**	.085	1.18 (0.89–1.55)	.232	.422	.352	.587
	Yes	1.72(0.82–3.63)					0.65 (0.29–1.43)				
rs2298320	No	0.82(0.60–1.11)	.086	.270	.088	.440	1.17 (0.88–1.56)	.281	.770	.365	.750
	Yes	1.68(0.75–3.73)					0.64 (0.27–1.49)				
rs2298321	No	1.01(0.67–1.52)	.652	.775	.920	.920	0.95 (0.64–1.40)	.983	.983	.843	.942
	Yes	0.74(0.26–2.08)					0.94 (0.32–2.71)				

Note: Bold value indicates the results with statistical differences. GenBank reference sequences were NM_005568.5 and NM_025069.3 for *lnc-ACACA-1* and *lnc-RP11-150O12.3*, respectively.

Abbreviations: CHB, chronic hepatitis B; CI, confidence interval; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; OR, odds ratio.

[a]Adjusting by gender, age, smoking, drinking, and cancer family history.

[b]*P* values for interaction between gene and environmental were obtained based on multiplicative model.

[c]*P* values for multiple comparison correction using the FDR method.

[d]*P* values for interaction between gene and environmental were obtained based on additive model.

T A B L E 4 Combined effects of lncRNA variants on HBV-related HCC risk

Risk allele number[a]	HBV-related HCC (%) (N = 643)	CHB(%) (N = 549)	Crude OR (95%CI)	p_{Crude}	Adjusted OR[b](95%CI)	$p_{Adjusted}$[b]
lnc-ACACA-1						
0	232 (36.65)	230 (42.36)	1.00		1.00	
1–2	247 (39.02)	201 (37.02)	1.22 (0.94–1.58)	.138	1.19 (0.89–1.60)	.246
3–6	154 (24.33)	112 (20.63)	**1.36 (1.01–1.85)**	**.046**	1.22 (0.86–1.72)	.258
Trend				**.037**		.211
lnc-RP11-150O12.3						
0–5	202 (31.86)	164 (30.26)	1.00		1.00	
6–9	266 (41.96)	236 (43.54)	0.92 (0.70–1.20)	.521	0.94 (0.69–1.28)	.692
10	166 (26.18)	142 (26.20)	0.95 (0.70–1.29)	.737	1.09 (0.77–1.54)	.626
Trend				.714		.660

Note: Bold value indicates the results with statistical differences. GenBank reference sequences were NM_005568.5 and NM_025069.3 for *lnc-ACACA-1* and *lnc-RP11-150O12.3*, respectively.

Abbreviations: CI, confidence interval; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; OR, odds ratio.

[a]The risk allele were rs9908998(T), rs7221955(C), and rs9891142(C) for *lnc-ACACA-1*, rs2275959(A), rs1008547(C), rs11776545(A), rs2298320(G), and rs2298321(G) for *lnc-RP11-150O12.3*, respectively.

[b]Adjusting by gender, age, smoking, drinking, and cancer family history.

T A B L E 5 Association between *lnc-ACACA-1* variants and lymphatic metastasis in HBV-related HCC patients based on credible genetic models

SNP	Crude OR(95%CI)	p_{Crude}	Adjusted OR[a](95%CI)	$p_{Adjusted}$[a]	P_{FDR}[a,b]
rs9908998	**1.87 (1.16–3.03)**	**.011**	**1.95 (1.20–3.17)**	**.007**	**.022**
rs7221955	1.09 (0.72–1.66)	.671	1.09 (0.71–1.65)	.697	.697
rs9891142	1.14 (0.75–1.73)	.530	1.14 (0.75–1.72)	.550	.697

Note: Bold value indicates the results with statistical differences. GenBank reference sequence was NM_005568.5 for *lnc-ACACA-1*.

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

[a]Adjusting by gender, age, smoking, drinking, and cancer family history.

[b]P values for multiple comparison correction using the FDR method.

occurrence and HBV clearance, respectively (Table S6). However, regardless of whether it was in *lnc-ACACA-1* or *lnc-RP11-150O12*, all other haplotypes were neither significantly associated with HBV-related HCC occurrence nor HBV clearance, compared with the most frequent haplotype (all $p > .05$).

3.6 | Associations of SNPs with lymphatic metastasis, distant metastasis, TNM classification, and cancer embolus in HBV-related HCC patients

Finally, we analyzed the associations of eight SNPs with lymphatic metastasis, distant metastasis, TNM classification, and cancer embolus in HBV-related HCC patients. The results of the MAX test are shown in Table S7. If the MAX test showed that the selected SNP genetic model was not statistically significant, considering the sample size problem, the dominant model was considered to be the most trusted genetic model of the SNPs.

Under the recessive model, CC genotype of *lnc-ACACA-1* rs9908998 was significantly associated with lymphatic metastasis risk of HBV-related HCC patients (Adjusted OR = 1.95, 95% CI = 1.20–3.17, $p_{Adjusted}$ = .007, P_{FDR} = .022; Table 5), compared with the *lnc-ACACA-1* rs9908998TT+TC genotypes. *Lnc-ACACA-1* rs7221955 and rs9891142 had no significant association with lymphatic metastasis, distant metastasis, TNM classification, and cancer embolus in HBV-related HCC patients (all $p > .05$; Tables 5 and S8).

In additive model, *lnc-RP11-150O12.3* rs2275959, rs1008547, and rs11776545 may be the risk factors of distant metastasis for HBV-related HCC patients (Adjusted OR = 1.45, 95% CI = 1.06–1.97, $p_{Adjusted}$ = .020 for rs2275959; Adjusted OR = 1.45, 95% CI = 1.06–1.98, $p_{Adjusted}$ = .021 for rs1008547; Adjusted OR = 1.40, 95% CI = 1.03–1.91, $p_{Adjusted}$ = .030 for rs11776545; Table 6). No evidence of the association was detected between *lnc-RP11-150O12.3*

SNP	Crude OR(95%CI)	p_{Crude}	Adjusted OR[a](95%CI)	$p_{Adjusted}$[a]	P_{FDR}[a,b]
rs2275959	**1.48 (1.08–2.01)**	**.014**	**1.45 (1.06–1.97)**	**.020**	.051
rs1008547	**1.48 (1.08–2.02)**	**.014**	**1.45 (1.06–1.98)**	**.021**	.051
rs11776545	**1.43 (1.05–1.94)**	**.023**	**1.40 (1.03–1.91)**	**.030**	.051
rs2298320	**1.39 (1.01–1.89)**	**.041**	1.36 (1.00–1.87)	.054	.068
rs2298321	1.25 (0.66–2.38)	.489	1.23 (0.65–2.36)	.525	.525

Note: Bold value indicates the results with statistical differences. GenBank reference sequence was NM_025069.3 for *lnc-RP11-150O12.3*.

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

[a]Adjusting by gender, age, smoking, drinking, and cancer family history.

[b]P values for multiple comparison correction using the FDR method.

rs2298320 or rs2298321 and lymphatic metastasis, distant metastasis, TNM classification or cancer embolus in HBV-related HCC patients (all $p > .05$; Tables 6 and S8).

4 | DISCUSSION

In this study, we screened out eight potentially functional SNPs from two lncRNA located in HBV-related HCC susceptibility regions based on GWAS and conducted two dependent case-control studies in the Southern Chinese population to investigate the associations of these candidate SNPs with HBV-related HCC occurrence and progression. For *lnc-ACACA-1*, we found that rs9908998 may significantly increase the lymphatic metastasis risk of HBV-related HCC patients. For *lnc-RP11-150O12.3*, rs2275959, rs1008547, rs11776545, and cancer family history had significantly multiplicative and additive interaction on HBV-related HCC susceptibility. We also observed the potential associations of rs2275959, rs1008547, and rs11776545 with distant metastasis of HBV-related HCC patients. These results suggested that there may exist functional SNPs in *lnc-ACACA-1* and *lnc-RP11-150O12.3* promoted HBV-related HCC occurrence and progression.

Lnc-ACACA-1 is a lncRNA of 75026 nt in length (chr17:35218935-35293960). The quality score of *lnc-ACACA-1* computed by GeneCards database was 13, suggesting that it may be a functional lncRNA with expression. The lncRNASNP database predicted that *lnc-ACACA-1* may contain multiple miRNA targets by Pita, MiRanda and TargetScan tools and may be associated with HCC by TAM tool. Our previous research displayed that the expression of *lnc-ACACA-1* in tissues of HBV-related HCC was higher than that in adjacent tissues based on TCGA (Qing et al., 2019). To the best of our knowledge, research about *lnc-ACACA-1* is in the early phase of exploration, but *lnc-ACACA-1* is the LIM homeobox 1 (*LHX1*, OMIM# 601999) divergent transcript (minus strand) and is closed to the *LHX1* (chr17:35294772-35301915). *LHX1* is a protein coding gene, and its encoded

protein is a transcription factor (TF) important for the development of the kidney and genitourinary system. In addition, *LHX1* contains LIM motif which exists protein kinase transcription factor. The transcription factor expresses in brain, thymus and tonsil tissue and involves in the transcriptional regulation of neural and lymphoid cell. Multiple lines of evidence have reported that a subset of lncRNA are involved in the cis-regulation of target genes located at or near the same genomic locus. For example, lncRNA *HOTTIP* recruits MLL to the 5′ region of the *HOXA* (OMIM# 614060) gene cluster via WDR5, catalyzes the establishment of an activating chromatin-modified H3K4me3, and cis-activates the expression of the adjacent *HOXA13* (OMIM# 142959) gene, which plays an important role in the HCC development (Quagliata et al., 2014; Zhang et al., 2017). Similarly, we hypothesized that *lnc-ACACA-1* may exert its function by interacting with nearby genes such as *LHX1*. Previous study mentioned that copy number variation of *LHX1* increased the risk of gastric cancer occurrence (Asta et al., 2002). Recent GWAS founded that *LHX1* rs9893681 showed a significant association with HBV-related HCC (OR = 1.65, $p = 9.31 \times 10^{-4}$) (Qu et al., 2016), and had high levels of LD with *lnc-ACACA-1* rs7221955 and rs9891142 in this study. Perhaps population structure divergence may be one of the reasons that the association of rs7221955 and rs9891142 with HBV-related HCC was not found in this study. But all GWASs we used were based on Chinese populations. The lncRNA SNPs in our study are located in linkage disequilibrium blocks of gene in Chinese Han Beijing population. And we found *lnc-ACACA-1* rs9908998 may significantly increase the risk of lymphatic metastasis in HBV-related HCC patients. Studies have shown that lncRNA variant may be a predictor of both HCC risk and prognosis (Wang, Xu, et al., 2018; Yang et al., 2018). According to the result of rVarBase and lncRNASNP in our previous study, rs9908998 was in the chromatin interaction region and the transcription factor binding region, which is easier to affect the ability of lncRNA to adsorb miRNAs (Qing et al., 2019). Among these miRNAs, the soft agar assays showed that the overexpression of miR-675 in HCC

may alter the morphology of HCC cells, provide proliferative advantages, and inhibit invasive capacity in favor of tumor formation (Hernandez et al., 2013). Additionally, histone modification marker of liver tissue predicted using Haploreg that rs9908998 belonged to enhancer region, which could have impact on transcription (Qing et al., 2019). In addition, according to the result of RegulomeDB, rs9908998 had a high regulatory function (score = 2b indicating TF binding + any TF binding motif + DNase Footprint + DNase peak) (Qing et al., 2019). In short, rs9908998 could impair binding of transcription factors to enhancers, which may interfere with the expression of *lnc-ACACA-1* and further affect HCC prognosis.

Another lncRNA, *Lnc-RP11-150O12.3*, in this study, being 2379 nt in length, is located on chr8:37454998-37457376. The lncRNASNP database predicted that *lnc-RP11-150O12.3* may contain 125 miRNA targets by Pita, MiRanda and TargetScan tools and could be associated with HCC by TAM tool. Previous study has shown that *lnc-RP11-150O12.3* was differentially expressed in gastric cancer tissues and normal tissues, and may reduce the risk of death in gastric cancer patients (Ren et al., 2016). *Lnc-RP11-150O12.3* was also identified to maintain a significant prognostic value in colorectal adenocarcinoma. According to the study by Wang et al., *lnc-RP11-150O12.3* showed higher expression in HCC tissue compared to adjacent tissues and was associated with survival time of HCC patients, which had carcinogenic effects in HCC tumorigenesis and prognosis (Wang et al., 2017). Similar result of differential expression was also observed in HBV-related HCC tissues and adjacent tissues based on TCGA (Qing et al., 2019). The Gene ontology and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis has shown that *lnc-RP11-150O12.3* may be involved in RNA splicing (Ren et al., 2016). In addition, *lnc-RP11-150O12.3* belonged to the chromosome 8p12 (chr8:37440842-37480842) which contains high levels of chromatin activation marks observed in the hepatoma cell line HepG2, and there may exist lncRNA in 2.3-kb expressed sequence tag in this region (Chan et al., 2011). What's more, some studies demonstrated region 8p was one of the most frequently deleted regions in HCC, and the loss/inactivation of potential tumor suppressor genes located in this region may promote the development of HCC (Kok-Lung et al., 2002). Therefore, *lnc-RP11-150O12.3* may play an important role on HBV-related HCC occurrence and progression. In this study, we found that three potentially functional SNPs (rs2275959, rs1008547, and rs11776545) in *lnc-RP11-150O12.3* may interact with cancer family history on the risk of HBV-related HCC and may increase the risk of distant metastasis of HBV-related HCC. Therein, the association of rs2275959 with HBV-related HCC had been identified by previous GWAS (Chan et al., 2011), and there was an association between rs2275959 and survival time of HBV-related HCC patients (Hazard ratio = 1.22, $p = 3.51 \times 10^{-2}$), indicating rs2275959 could be considered good prognostic candidates for HCC (Li et al., 2014). In addition, *lnc-RP11-150O12.3* rs1008547 and rs11776545 kept high linkage disequilibrium with rs2275959 ($r^2 \geq 0.99$). According to the results of rVarBase and lncRNASNP in our previous study, *lnc-RP11-150O12.3* rs2275959, rs1008547, and rs11776545 located in the chromatin interaction region, could affect the binding of lncRNA to miRNAs (Qing et al., 2019). Among these miRNAs, miRNA-191 was found to be upregulated in HCC cell lines compared with normal hepatocytes (Elyakim et al., 2010). Based on multiple tissue experiments, study found that the inhibition of miR-191 could induce cellular changes, trigger proliferation inhibition, and apoptosis in HCC cell lines (Elyakim et al., 2010). What's more, the results of haploreg displayed that they impaired the motifs bound with transcription factor (Qing et al., 2019). Histone modification marker of liver tissue indicated that rs2275959 and rs11776545 were positioned on the promoter region, which could have impact on transcription (Qing et al., 2019). Rs1008547 and rs11776545 were located in the DNase hypersensitive site, suggesting that their chromosome sequences were in an open state, which facilitates the binding of regulatory factors (Qing et al., 2019). In addition, HepG2 cell line experiments found that rs1008547 may affect the binding of transcription factors *Brachyury* and *HEY1* with motif (Qing et al., 2019), while overexpression of *Brachyury* could induce the metastasis of HCC cells (Du et al., 2014), and *HEY1* could regulate the self-renewal of HCC cells (Zhu et al., 2015). Based on the function prediction of SNPinfo, the regulatory score of rs1008547 was 0.25, suggesting that rs1008547 may have regulatory function (Qing et al., 2019). Therefore, *lnc-RP11-150O12.3* rs2275959, rs1008547, and rs11776545 may be involved in the occurrence and prognosis of HBV-related HCC.

To the best of our knowledge, this was the first time to predict lncRNA SNPs using a comprehensive bioinformatics strategy based on HBV-related HCC GWAS, and found two lncRNA (*lnc-ACACA-1* and *lnc-RP11-150O12.3*) related to HBV-related HCC initiation and progression. *lnc-ACACA-1* rs9908998, *lnc-RP11-150O12.3* rs1008547 and rs11776545, which were found to be associated with HBV-related HCC in this paper, have not been studied so far. However, our study had some limitations. The sample size available for analysis was small so that there was insufficient power after considering multiple comparisons. But the interaction between *lnc-RP11-150O12.3* SNPs and cancer family history suggested that there may be gene-gene interactions. In addition, the study couldn't provide the association result of lncRNA SNPs with metabolic syndrome and non-alcoholic fatty liver disease, which are now considered among the leading causes of HCC incidence and are expected to become the leading cause of HCC incidence in western countries by the next decade.

Therefore, subsequent studies could explore the relationship among lncRNA SNP, metabolic syndrome and HCC, and conduct the downstream pathways to explore the interactions of this gene in a larger sample.

5 | CONCLUSION

Taken together, lnc-ACACA-1 rs9908998, lnc-RP11-150O12.3 rs2275959, rs1008547, and rs11776545 may be involved in the occurrence and development of HBV-related HCC. Further research would be needed to validate these resultsAQ8.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.