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RESEARCH ARTICLE

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An SNP reducing SNORD105 and PPAN expression decreases the risk of hepatocellular carcinoma in a Chinese population

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Abstract

Background: With hepatocellular carcinoma (HCC) becoming a heavy disease burden in China, it is particular to reveal its pathological mechanism. Recent researches have indicated that small nucleolar RNAs (snoRNAs) may be involved in various cancers including HCC. Polymorphisms within snoRNAs may affect its function or expression level, and even its host gene, then produce series of effects related to itself or its host gene.

Methods: The association of the single nucleotide polymorphism (SNP) rs2305789 in SNORD105 with HCC susceptibility was evaluated in two independent case-control sets (712 HCC and 801 controls). The contribution of rs2305789 to HCC risk was investigated using case-control, genotype-phenotype correlation analysis, and functional assays.

Results: The SNP rs2305789 was significantly associated with a decreased risk of HCC in both case-control sets (OR = 0.80, 95% CI: 0.69–0.93, $p = 0.003$). Compared with the AA genotype, the GG genotype was significantly correlated with lower expression of both SNORD105 and PPAN ($p < 0.01$). Furthermore, the overexpressed SNORD105 up-regulated PPAN expression level ($p < 0.05$). Finally, the in vivo experiment showed that the overexpressed SNORD105 increased cell viability and motility in both HepG2 and Huh7 cell lines ($p < 0.05$).

Conclusions: To sum up, our results suggested that rs2305789 decreased the risk of HCC by reducing the expression of both SNORD105 and PPAN, which reduced HCC cell viability and motility.

KEYWORDS
hepatocellular carcinoma, PPAN, rs2305789, SNORD105

Xuekun Chen and Qing Zhang authors contributed equally to this work.

1 | INTRODUCTION

Liver cancer comes fourth in cancer incidence and second in cancer-related mortality in China, and hepatocellular carcinoma (HCC) comprises 83.9%–92.3% of the primary hepatic carcinoma cases in China.[1] Because of no typical symptoms in the early stage, HCC is often found in the advanced stage, and both the lack of early-stage screen markers and effective therapies for advanced stage contribute to its high mortality, thus producing a heavy disease burden in China.[2] HCC is a typical multistage and multifactorial carcinoma, except for environmental factors, such as chronic hepatitis B or C virus infections and aflatoxin B1 exposure, genetic factors were also indicated to play a critical role in HCC carcinogenesis, including small nucleolar RNAs (snoRNA).[3–5]

As a class of non-coding RNAs, snoRNAs are 60–300 nucleotides in length and involved in post-transcriptional modification of other non-protein-coding RNAs.[6] SnoRNAs are divided into two families based on their structure and main function: Box C/D snoRNAs (SNORDs) and box H/ACA snoRNAs (SNORAs), the former is responsible for 2'-O-ribose methylation, and the latter guides pseudouridylation of nucleotides.[7] Both groups of snoRNAs work through interacting with their targets in a sequence-specific manner, so single nucleotide polymorphism (SNP) in snoRNA loci or the target RNA might have effect on their interaction.[8] Because of involved in the modifications of rRNAs at post-transcriptional level, and overactivation of ribosomes is a common feature of cancer, based on these, dysregulation of snoRNAs may also be involved in cancer.[9] Recent studies do suggest that snoRNAs play a key role in the development and progression of various cancers,[10–12] including HCC.[13–15] What's more, Dong et al.[16,17] found that U50 with a 2bp (TT) deletion was associated with prostate cancer and breast cancer.

SNORD105, firstly found by Vitali et al.[18] in 2003, is a kind of Box C/D snoRNAs and lies within the third intron of Peter Pan protein (PPAN), which is essential for cell growth and proliferation. So far, there is no research on polymorphism in SNORD105 and the function of SNORD105 in HCC; in the current case-control study, we evaluated the association between the SNP (rs2305789) within SNORD105 and HCC susceptibility in a Chinese population.

2 | METHODS

2.1 | Study populations

We analyzed 712 newly diagnosed HCC cases and 801 matched controls from two independent case-control groups. The 373 HCC patients from Jiangsu were diagnosed, hospitalized, and treated in the affiliated hospitals of Soochow University from 2010 to 2013. The 339 HCC patients of Shanghai group were from Qidong Liver Cancer Research Institute of Jiangsu Province during the same time. 801 gender and age-matched controls (418 from Suzhou and 383 from Shanghai) were cancer-free individuals. Tissue samples were obtained from 72 patients with HCC and immediately frozen in liquid nitrogen. All the recruited specimens were unrelated Han Chinese. The HCC cases diagnosis, the exclusion and inclusion criteria for both the control and case, and the drinkers and smokers definition were the same as described before.[19,20] This study was approved by the Ethical Committee of Soochow University. All subjects signed informed consent.

2.2 | DNA extraction and genotyping

Peripheral blood genomic DNA and HCC tissue DNA were extracted using genomic DNA purification kit (Qiagen, Germany). DNA fragments containing the rs2305789 polymorphism were amplified with genotyping primers (forward primer: 5-ATCCTGAGCAAAACAGAGACCAATG-3, reverse primer: 5-ACTTTGAAGCCAGAAATCACAGCAG-3). The PCR cycle parameters were as follows: 5 min, 94°C; 34 cycles of 30 s, 94 °C, 30 s, 60 °C and 30 s, 72°C; and with a final elongation for 5 min at 72°C. After the amplification, the genotype of rs2305789 was analyzed using sequencing technique.

2.3 | RNA extraction and quantitative real-time PCR

Total RNA was isolated from different genotype tissue samples using RNA isolation kit (Qiagen). cDNA was generated from target RNA using the Reverse Aid First Strand cDNA Synthesis Kit (Cat#K1622, Thermo Scientific). Reverse transcription primers for SNORD105 and U6 were designed and provided by Gene Pharma (Shanghai, China). Then, SYBR Green real-time PCR was performed on Roche Light Cycler 480 to quantify the relative SNORD105 and PPAN expression levels in these samples. U6 was chosen as the internal control for SNORD105 and GAPDH for PPAN. Primer sequences used for PPAN and GAPDH are shown in Table 1, available at genes Online, and SNORD105 and U6 were designed and synthesized by Gene Pharma (Shanghai, China). The PCR conditions were 3 min, 95℃, followed by 40 cycles of 12 s, 95℃ and 40 s, 62℃. In addition, the melting curve analysis was performed to verify primer specificity. The $2^{-\triangle\triangle CT}$ algorithm was applied to calculate the differences in target gene expression levels.

TABLE 1 Primers sequences used in the study

Primer name	Sequences (5′-3′)
PPAN -F	CGCCCAGGGAAGAGAGTG
PPAN -R	TGAAAGGAAACCGAGGGC
GAPDH -F	CTCTCTGCTCCTCCTGTTCGAC
GAPDH -R	TGAGCGATGTGGCTCGGCT

2.4 | Vector construction

Approximately 100-bp fragments containing the sequence of SNORD105 with A or G allele were directly synthesized and amplified by Genewiz Company (Suzhou, China) and cloned into EcoRI and BamHI sites of pEGFP-N1. The sequence and direction of the obtained constructions were validated by DNA sequencing. Then, the SNORD105 plasmids (SNORD105pDNA) were extracted with SanPrep Column Plasmid Mini-Preps Kit (cat # B518191-0100, sangon Biotech) according to the instructions.

2.5 | Cell culture and transfection

The HepG2 and Huh7 hepatoma cell lines used in this study were obtained from Shanghai Cell Bank of Chinese Academy of Sciences and cultured at 37°C in a humidified 5% CO2 incubator using DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin.

Two cell lines of HepG2 and Huh7 were respectively seeded in 96-well plate (2×10^3 cells/well) or 6-well plate (2.0×10^5 cells/well). 24 h later, cells were transfected with SNORD105pDNA using JetPRIME® transfection reagent (cat # PT114-15, Polyplus Transfection) following the manufacturer's protocol. The cells were divided into experimental group (transfected with plasmid SNORD105ApDNA/ SNORD105GpDNA) and control group (non-transfected group) for the following analyses.

2.6 | Cell viability

According to the manufacturer's instructions, the effect of SNORD105 on HCC cell proliferation was determined using an Enhanced Cell Counting Kit-8 (cat#C0042, Beyotime Biotechnology). Briefly, HepG2 and Huh7 cells were plated in 96-well plates (cat # 3799, Corning) at 2000 cells/well. 24 h later, cells were transfected with SNORD105pDNA. Then, 0, 6, 12, and 24 h later, 10 ul cck-8 solution was added to each well, and the cells were incubated for 45 min under the same incubator conditions. The relative viability of cells was measured at 450 nm in FilterMax F5.

2.7 | The wound-healing migration assays

One day before the wound-healing assay performed, two cell lines of HepG2 and Huh7 were transfected with SNORD105pDNA in 6-well plates (cat # 3516, Corning), respectively. Scratch wound was generated using a 10 ul pipette tip when cellular density reached nearly 100%. Cells were then washed three times with a sufficient volume

TABLE 2 Demographic characteristics of HCC cases and controls recruited from Suzhou and Shanghai

	Suzhou		Shanghai		Overall	
Characteristic	Case ($n = 373$)	Control ($n = 418$)	Case ($n = 339$)	Control ($n = 383$)	Case ($n = 712$)	Control ($n = 801$)
Age (mean ± S.D.)	53.5 ± 9.1	53.6 ± 10.1	52.4 ± 9.8	53.1 ± 9.5	53.2 ± 9.9	53.8 ± 10.0
Gender						
Male	230 (0.62)	271 (0.65)	247 (0.73)	255 (0.67)	477 (0.67)	526 (0.66)
Female	143 (0.38)	147 (0.35)	92 (0.27)	128 (0.33)	235 (0.33)	275 (0.34)
Smoking status						
Non-smokers	265 (0.71)	281 (0.67)	243 (0.72)	277 (0.72)	508 (0.71)	558 (0.70)
Former smokers	44 (0.12)	47 (0.11)	36 (0.11)	42 (0.11)	80 (0.11)	89 (0.11)
Current smokers	64 (0.17)	90 (0.22)	60 (0.17)	64 (0.17)	124 (0.17)	154 (0.19)
Drinking status						
Non-drinkers	225 (0.60)	280 (0.67)	190 (0.56)	247 (0.64)	415 (0.58)	527 (0.66)
Light drinkers	104 (0.28)	107 (0.26)	88 (0.26)	77 (0.20)	192 (0.27)	184 (0.23)
Heavy drinkers	44 (0.12)	31 (0.07)	61 (0.18)	59 (0.15)	105 (0.15)	90 (0.11)
Tumor stages						
Ia + Ib	261 (0.70)		235 (0.69)		496 (0.70)	
IIa + IIb	80 (0.21)		61 (0.18)		141 (0.20)	
IIIa + IIIb	32 (0.09)		43 (0.13)		75 (0.10)	
HBsAg						
Positive	254 (0.68)	35 (0.08)	220 (0.65)	27 (0.07)	474 (0.67)	62 (0.08)
Negative	119 (0.32)	383 (0.92)	119 (0.35)	356 (0.93)	238 (0.33)	739 (0.92)

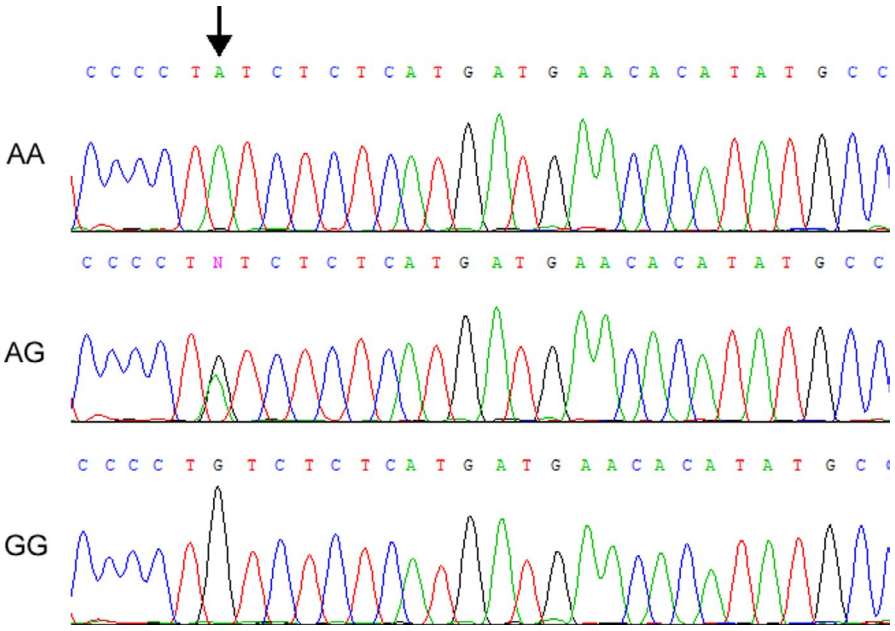

FIGURE 1 Example sequencing output for rs2305789 polymorphism. The upper, middle, and lower panels showed the sequence of AA, AG, and GG genotype, respectively. The arrow indicates the position of the polymorphism

TABLE 3 Associations between rs2305789 and HCC susceptibility in Suzhou and Shanghai case-control sets

	Genetic model	Genotype	Cases	(%)	Control	(%)	OR (95% CI)[a]	*p* Value
Suzhou	Codominant model	AA	144	38.6	142	34.0	1.00 (Reference)	
		AG	177	47.5	193	46.2	0.90 (0.66–1.23)	0.523
		GG	52	13.9	83	19.8	0.62 (0.41–0.94)	0.023
	Dominant model	AA	144	38.6	142	34.0	1.00 (Reference)	
		AG+GG	229	61.4	276	66.0	0.82 (0.61–1.09)	0.176
	Recessive model	AA+AG	321	86.1	335	80.2	1.00 (Reference)	
		GG	52	13.9	83	19.8	0.65 (0.45–0.96)	0.027
	Additive model	A allele	465	62.3	477	57.1	1.00 (Reference)	
		G allele	281	37.7	359	42.9	0.80 (0.66–0.98)	0.033
Shanghai	Codominant model	AA	129	38.0	130	33.9	1.00 (Reference)	
		AG	164	48.4	176	46.0	0.94 (0.68–1.30)	0.703
		GG	46	13.6	77	20.1	0.60 (0.39–0.93)	0.023
	Dominant model	AA	129	38.1	130	33.9	1.00 (Reference)	
		AG+GG	210	61.9	253	66.1	0.84 (0.62–1.13)	0.250
	Recessive model	AA+AG	293	86.4	306	79.9	1.00 (Reference)	
		GG	46	13.6	77	20.1	0.62 (0.42–0.93)	0.020
	Additive model	A allele	422	62.2	436	56.9	1.00 (Reference)	
		G allele	256	37.8	330	43.1	0.80 (0.65–0.99)	0.040
Overall	Codominant model	AA	273	38.3	272	34.0	1.00 (Reference)	
		AG	341	47.9	369	46.0	0.92 (0.74–1.15)	0.469
		GG	98	13.8	160	20.0	0.61 (0.45–0.83)	0.001
	Dominant model	AA	273	38.3	272	34.0	1.00 (Reference)	
		AG+GG	439	61.7	529	66.0	0.83 (0.67–1.02)	0.076
	Recessive model	AA+AG	614	86.2	641	80.0	1.00 (Reference)	
		GG	98	13.8	160	20.0	0.64 (0.49–0.84)	0.001
	Additive model	A allele	887	62.3	913	57.0	1.00 (Reference)	
		G allele	537	37.7	689	43.0	0.80 (0.69–0.93)	0.003

Abbreviations: CI, confidence interval; OR, odds ratio.

[a]Adjusted for age, sex, smoking status, drinking status, and HBV infection

of PBS to remove the floating cells and cultured for 0 and 48 h before being photographed with an inverted microscope (OLYMPUS CKX53, Japan).

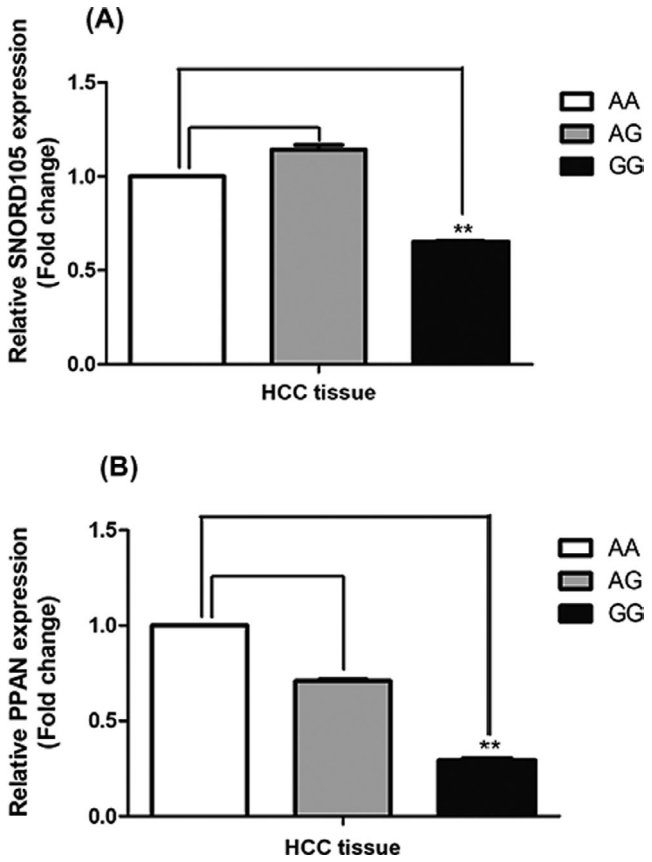

FIGURE 2 Expression of SNORD105 and PPAN in HCC tissues with different genotypes. (A) SNORD105 expression (mean ± SEM) in HCC tissue samples with AA, AG, and GG genotype of rs2305789; (B) PPAN mRNA expression (mean ± SEM) in HCC tissue samples with AA, AG, and GG genotype of rs2305789. (GG, $n = 24$, AG, $n = 30$, and AA, $n = 18$)

2.8 | Statistical analysis

Chi-square test was used to analyze the genotype distributions in controls for Hardy-Weinberg equilibrium. Logistic regression was used to analyze the association between rs2305789 and HCC risk, adjusted by age, gender, HBV infection, drinking, and smoking. Non-parametric Mann–Whitney U test was used to compare the relative SNORD105 and PPAN expression levels in tissue samples among different genotypic groups. Statistical analysis was implemented with Statistic Analysis System software (version 8.0, SAS Institute, Cary, NC). All statistical tests were two-sided, with $p < 0.05$ set as the significance level. The G*Power 3.1 software was used to calculate the statistical power of the current sample size.

3 | RESULTS

3.1 | The association of rs2305789 with HCC susceptibility

The demographic characteristics of the subjects were listed in Table 2, available at Genes online, in the study. Age and gender were adequately matched between two case-control sets. Smoking status and alcohol consumption were similar between the case and control. Approximately 67% of the cases and 8% of the controls were HBsAg-positive. Sequencing result examples of rs2305789 were shown in Figure 1. Genotype distributions in control group had no deviation from Hardy-Weinberg equilibrium ($p > 0.05$). The rs2305789 genotype distributions between the controls and cases were shown in Table 3. In each single case-control set and pooled analysis, subjects with the homozygous GG genotype had a significantly decreased risk of HCC under co-dominant model, compared with the AA genotype (adjusted OR = 0.61; $p = 0.001$). Similar trends were observed in both Recessive and Additive models but not Dominant model (Table 3). Collectively, these results suggest the association between

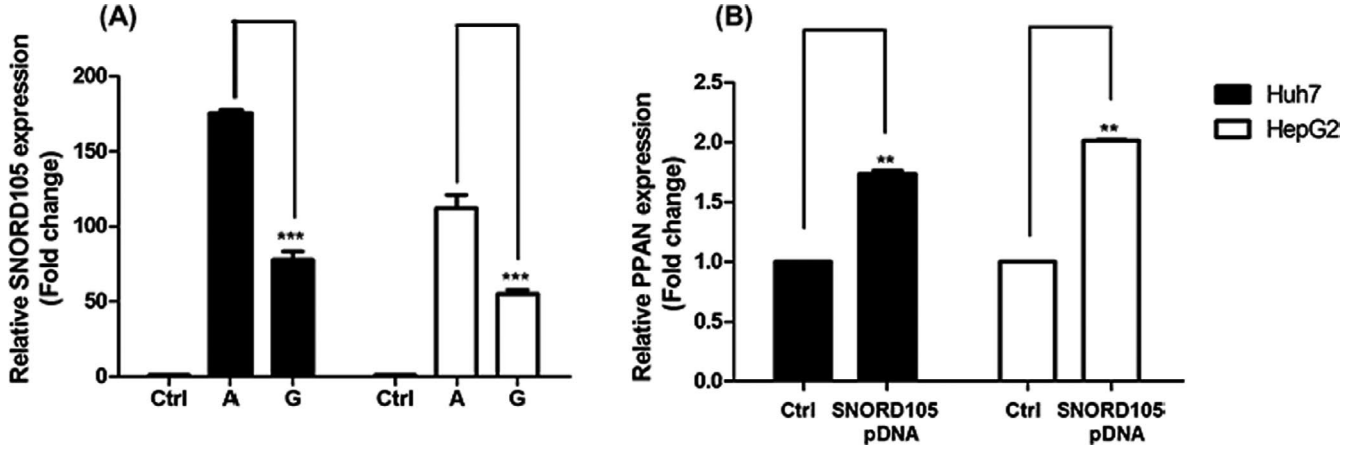

FIGURE 3 The effect of SNORD105 on gene transcriptional activity. (A) qRT-PCR analysis of SNORD105 expression in Huh7 and HepG2 cells transfected with rs2305789-AA or GG in pEGFP-N1 (***$p < 0.001$ vs. rs2305789-GG); (B) qRT-PCR analysis of PPAN mRNA expression in Huh7 and HepG2 cells transfected with SNORD105 pDNA (**$p < 0.01$ vs. empty vector)

rs2305789 and HCC susceptibility. A statistical power of 1.000 was examined with α set at 0.05 under the dominant model using the G*Power 3.1 software.

3.2 | The correlation between rs2305789 and SNORD105/PPAN expression in HCC tissues

Next, we examined the SNORD105 and PPAN expression levels in HCC tissue samples with different genotypes. As shown in Figure 2A, the SNORD105 expression level in HCC tissues with GG genotype was significantly lower than that in samples with AA genotype, while the difference between tissues with AG and AA genotype is not significant. As expected, the expression level of PPAN showed a similar trend (Figure 2B).

3.3 | The effect of rs2305789 on SNORD105 expression

To validate whether rs2305789 polymorphism has effect on SNORD105 expression, we constructed two SNORD105 vectors containing A or G allele, respectively, and transfected them into Huh7 and HepG2 cell lines. The results shown in Figure 3A suggested that the SNORD105 expression level in the SNORD105 vector-transfected groups was significantly higher than that in the empty vector-transfected group in Huh7 cell line, and that the SNORD105 expression level in the SNORD105 vector containing G allele transfected group was significantly lower than that in the SNORD105 vector containing A allele-transfected group. The results in HepG2 cell line showed a similar trend.

3.4 | The overexpressed SNORD105 increased PPAN expression

To further explore the possible effect of SNORD105 on its host gene PPAN expression, we transfected vectors into Huh7 and HepG2 cell lines. As shown in Figure 3B, the PPAN expression level in the SNORD105ApDNA-transfected group was significantly higher than that in the empty vector-transfected group in both Huh7 and HepG2 cell lines.

3.5 | Overexpressed SNORD105 increased cell viability

To further explore the effect of SNORD105 on cell viability, SNORD105ApDNA was transfected into HepG2 and Huh7 cell lines, and the results showed that the cell viability of HepG2 in the SNORD105ApDNA-transfected group increased significantly (Figure 4A), and in the Huh7 cell line, it showed a similar trend, and more significant than that in HepG2 cell line (Figure 4B).

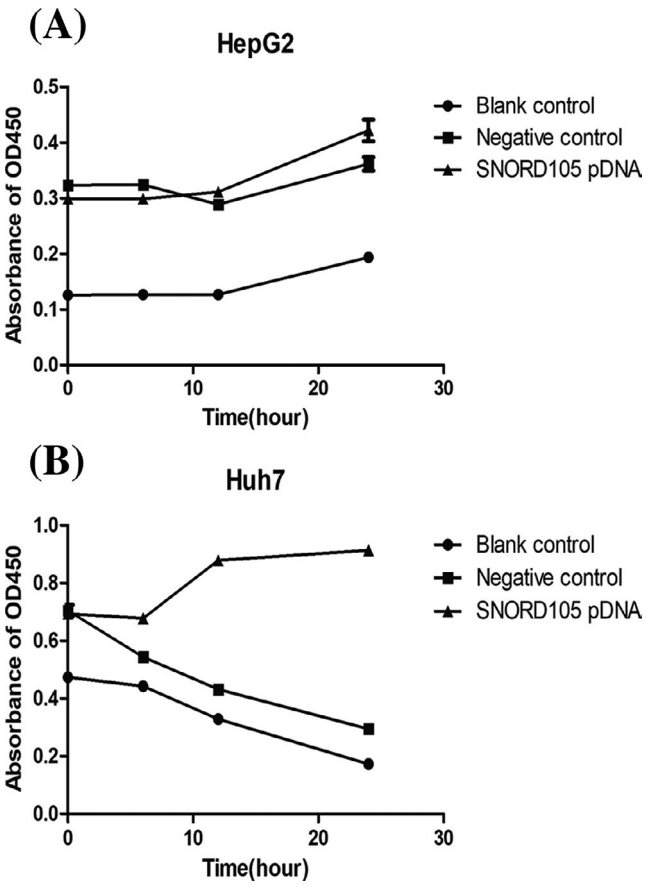

FIGURE 4 The effect of overexpressed SNORD105 on cell viability. (A) The cell viability reflected by the absorbance in OD 450 nm of HepG2 cells (2×10^4/well) in SNORD105 pDNA, empty vector, and blank control transfected group after 0, 6, 12, and 24 h analyzed using the CCK-8 assay; (B) The cell viability reflected by the absorbance in OD 450 nm of Huh7 cells (2×10^4/well) in SNORD105 pDNA, empty vector, and blank control transfected group after 0, 6, 12, and 24 h analyzed using the CCK-8 assay

3.6 | Overexpressed SNORD105 increased cell motility

Finally, we analyzed the effect of SNORD105 on the motility of HCC cell lines. The scratch assay results showed that the overexpressed SNORD105 increased the motility of both HepG2 and Huh7 cell lines (Figure 5).

4 | DISCUSSION

In the present study, we found that the polymorphism rs2305789 within SNORD105 and PPAN gene was associated with HCC susceptibility, and the later, phenotype-genotype and functional assay demonstrated that the protective GG genotype was related to the lower expression of SNORD105 and PPAN in the HCC tissue samples, which can inhibit cell viability and motility, thus be associated with HCC susceptibility.

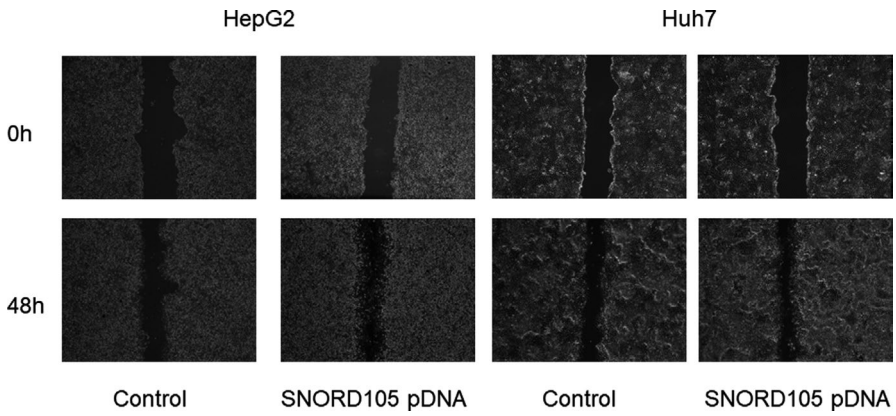

FIGURE 5 The effect of overexpressed SNORD105 on cell motility. The effect of overexpressed SNORD105 on cell motility in both HepG2 and Huh7 cell lines (40×)

SNORD105 was firstly cloned from a rat cDNA library, whose human orthologue was identified in 2003.[18] SNORD105 is predicted to guide the 18S rRNA U799 2'O-ribose methylation, while its function in HCC is still unclear. In this case-control study, the results suggested that the GG genotype of rs2305789 polymorphism within SNORD105 was associated with HCC susceptibility, and the expression level of SNORD105 in the HCC tissues with GG genotype was significantly lower than that in the HCC tissues with AA genotype, and the overexpressed SNORD105 increased the cell viability and motility of both HepG2 and Huh7. That is, the rs2305789 polymorphism played its role in HCC through regulating SNORD105 expression. Till now, there are no relative researches on the roles of SNORD105 in HCC, and further studies in other ethnic groups should be taken to verify our results.

Peter Pan protein, an evolutionarily conserved protein, was initially identified in a screen for growth-defective mutants in Drosophila,[21] and localized in both nucleoli and mitochondria,[22,23] and also a ribosome biogenesis factor mediating the pre-rRNA precursor processing.[24] In 2015, Pfister advocated it for the first time that PPAN knockdown induced cell apoptosis,[23] and Pfister's later research found that PPAN knockdown induced cell cycle arrest and impaired cell proliferation in cancer cells, and also showed the precise localization and its regulation function in cell cycle.[24] In Pfister's latest research, they further demonstrated that the loss of PPAN affected mitochondrial homeostasis and autophagy.[25] In our study, we found that the PPAN expression level in HCC tissues with the protective GG genotype was significantly lower than that in the HCC tissues with AA genotype, and the overexpressed PPAN induced by SNORD105 increased the cell viability and motility of both HepG2 and Huh7. That is, the rs2305789 polymorphism also played its role in HCC through regulating PPAN expression. Moreover, what was found in Pfister's researches that the loss of PPAN induced cell cycle arrest and apoptosis and impaired cell proliferation supported our results to some extent.

At last, the limitations in this study should be emphasized. Although we observed an association between rs2305789 and HCC susceptibility, the evidence of the underlying mechanism of how rs2305789 affects the expression of SNORD105 and PPAN needs further functional investigations.

In summary, we found that rs2305789 was involved in the carcinogenesis of HCC through affecting the expression of SNORD105 and PPAN. Our findings also indicated that SNORD105 might act as a proto-oncogene in HCC. Therefore, SNORD105 may be a promising marker for HCC personalized diagnosis. Our study still needed replications in other populations, and the molecular mechanisms underlying SNORD105 regulation also needed further systematic investigations.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Xuekun Chen, Qing Zhang, and Lijuan Li conceived and designed the experiments, analyzed the data, and prepared the manuscript. Xuekun Chen, Zhenzhen Yang, Huan Yu, Yiling Qu, and Rui Tan conducted the experiments. Yuzhen Gao and Yan He contributed to data collection and analysis. Lijuan Li supervised the study. All authors read and approved the final version.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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