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Research Paper

A genetic variant in pseudogene *E2F3P1* contributes to prognosis of hepatocellular carcinoma

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ABSTRACT

Certain pseudogenes may regulate their protein-coding cousins by competing for miRNAs and play an active biological role in cancer. However, few studies have focused on the association of genetic variations in pseudogenes with cancer prognosis. We selected six potentially functional single nucleotide polymorphisms (SNPs) in cancer-related pseudogenes, and performed a case-only study to assess the association between those SNPs and the prognosis of hepatocellular carcinoma (HCC) in 331 HBV-positive HCC patients without surgical treatment. Log-rank test and Cox proportional hazard models were used for survival analysis. We found that the *A* allele of rs9909601 in *E2F3P1* was significantly associated with a better prognosis compared with the *G* allele [adjusted hazard ratio (HR) = 0.69, 95% confidence interval (CI) = 0.56–0.86, $P = 0.001$]. Additionally, this protective effect was more predominant for patients without chemotherapy and transcatheter hepatic arterial chemoembolization (TACE) treatment. Interestingly, we also detected a statistically significant multiplicative interaction between genotypes of rs9909601 and chemotherapy or TACE status on HCC survival (P for multiplicative interaction < 0.001). These findings indicate that rs9909601 in the pseudogene *E2F3P1* may be a genetic marker for HCC prognosis in Chinese.

Keywords: pseudogene, *E2F3P1*, SNP, hepatocellular carcinoma (HCC), prognosis

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INTRODUCTION

Liver cancer is the fifth most common cancer worldwide and the second most frequent cause of cancer mortality with over 748,300 new cases every year, half of which are in China[1,2]. Hepatocellular carcinoma (HCC) is the most common type of liver cancer[3]. Although surgical resection and liver transplantation are regarded as the best treatment for a curative prognosis of early-stage HCC[4], about 85% patients are not suitable for surgery due to locally advanced tumor or distant metastasis[5]. Discovery and application of biomarkers that incorporate with traditional cancer staging improve patient care. Thus, substantial efforts have been made to identify biomarkers as prognostic factors for improving therapeutic effect and prognosis prediction.

Pseudogenes are structurally similar to genes that encode functional proteins, but unable to encode fully functional proteins in most cases. Thus, pseudogenes have long been considered as nonfunctional sequences of genomic DNA. However, emerging evidence suggests that pseudogenes may harbor the potential to regulate the expression of their ancestral protein-coding genes by serving as a source of small interfering RNAs (siRNAs), antisense transcripts, microRNA (miRNA) binding sites, or competing mRNAs[6–8]. Furthermore, pseudogenes regulate tumor suppressors and oncogenes by acting as microRNA decoys[9–14]. To date, several studies have reported the association between pseudogene expression and multiple cancer risk. Ishiguro et al. reported that two pseudogenes (*NANOG1* and *NANOGP8*) were differentially expressed in colon cancer cells, and their expression might contribute to the proliferation of colon cancer cells[15]. Similar results were found for the association of *POU5F1P1* expression with prostatic carcinoma[16], *PTENP1* expression with melanoma[17], and *NANOGP8* expression with gastric cancer[18]. However, little is known about pseudogenes and cancer prognosis.

Functional polymorphisms in pseudogenes, such as single nucleotide polymorphisms (SNPs) influencing miRNAs binding, may affect the expression or function of the proteins[19,20]. Thus, we speculate that potentially functional polymorphisms in pseudogenes may affect the expression of pseudogenes or its original protein-coding genes by influencing miRNA binding affinity, and thus play a role in the development and progression of human cancer. In this study, we examined the associations between six genetic variants of pseudogenes and prognosis of 331 patients with intermediate or advanced HCC in Chinese.

SUBJECTS AND METHODS

Subjects

The protocol was approved by the local institutional review board at the authors′ affiliated institution. Written informed consent was obtained from every subject. The enrollments of subjects were described previously[21,22]. For constructing a relatively homogenous population, our current study was restricted to HCC patients without surgery in intermediate stage (B) or advanced stage (C) according to the Barcelona Clinic Liver Cancer (BCLC) staging system[23,24]. We recruited 414 intermediate or advanced HCC patients from Nantong Tumor Hospital and the First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu, China. All patients were followed up prospectively every three months from the time of enrollment by personal or family contacts until death or last time of follow-up. As a result, a total of 331 intermediate or advanced HCC patients who had completed follow-ups and clinical information were enrolled in our study with a response rate of 80.0%. The maximum follow-up time (MFT) for the 331 patients involved in the present study was 60.7 months (last follow-up in January 2013) and the median survival time (MST) was 14.5 months.

Serological tests

HBsAg, anti-HBs, anti-HBc and anti-HCV were detected by the enzyme-linked immunosorbent assay (Kehua Bio-engineering Co., Ltd., Shanghai, China) following the manufacturer′s instructions as described previously[21].

Selection and genotyping of SNPs

We included eight key cancer-related pseudogenes from a review conducted by Poliseno et al.[8] (***Supplemental Table 1*** available online). By blasting the identical sequence between pseudogenes and their parental genes, we found 31 common SNPs located in pseudogenes, with at least 50 bps flanking regions identical to their parental genes. Then, we searched miRBase (http://www.mirbase.org/) and Patrocles (http://www.patrocles.org/) to find whether the allelic change of 31 SNPs may influence miRNAs binding. Eventually, we selected six potentially functional SNPs that might affect miRNA binding (rs11682718 in *DNMT3AP1*, rs1838149 and rs9909601 in *E2F3P1*, rs2004079 in *NANOGP8*, rs9889937 in *FOXO3B*, and rs6913881 in *KRASP1*).

Genomic DNA was extracted from a leukocyte pellet by traditional proteinase K digestion, phenol-chloroform extraction and ethanol precipitation. All SNPs were genotyped using the TaqMan allelic discri–

Table 1 **Genotyping results of survival of HCC patients**

SNP	Base change[a]	Pseudogene	Location	Genotyping rate	MAF[b]	Log-rank *P*		
						Additive model	Dominant model	Recessive model
rs11682718	G>A	*DNMT3AP1*	2p14	99.1%	0.110	0.986	0.948	0.869
rs1838149	C>T	*E2F3P1*	17q12	99.7%	0.094	0.916	0.923	0.675
rs9909601	G>A	*E2F3P1*	17q12	97.3%	0.362	0.026	0.007	0.541
rs2004079	G>T	*NANOGP8*	15q14	98.2%	0.338	0.552	0.858	0.333
rs9889937	T>C	*FOXO3B*	17p11.2	99.1%	0.372	0.184	0.860	0.089
rs6913881	G>A	*KRASP1*	6p12.1	98.8%	0.130	0.204	0.082	0.484

HCC: hepatocellular carcinoma; SNP: single nucleotide polymorphism; MAF: minor allele frequency. [a]major > minor allele, [b]MAF in patients.

mination assay on a 7900 system (Applied Biosystems, Carlsbad, CA, USA). The primers and probes for the six SNPs are shown in ***Supplemental Table 2*** (available online). Two blank (water) controls in each 384-well plate were performed for quality control, and more than 5% samples were randomly selected and repeated, yielding a 100% concordance. The success rates of genotyping for the six SNPs were all above 95%.

Statistical analysis

Mean survival time was presented when the MST could not be calculated. Kaplan-Meier method and log-rank test were performed to compare the survival time in different subgroups categorized by patient characteristics, clinical features and genotypes. Univariate and multivariable Cox proportional hazard regression analyses were performed to estimate the crude or adjusted hazard ratio (HR) and their 95% confidence intervals (CI), with adjustment of age, gender, smoking status, drinking status, BCLC stage, and chemotherapy or transcatheter hepatic arterial chemoembolization (TACE) status. Cox stepwise regression model was also conducted to determine predictive factors of HCC prognosis, with a significance level of 0.050 for entering and 0.051 for removing the respective explanatory variables. The Chi-square-based Q test was applied to test the heterogeneity of associations between subgroups. Analyses were carried out using Statistical Analysis System software (version 9.1.3; SAS Institute, Cary, NC, USA). All tests were two-sided and the criterion of statistical significance was set at $P < 0.05$.

RESULTS

Demographic and baseline characteristics of the study subjects

The demographic characteristics and clinical information of the 331 HCC patients in stage B or C included in the study were described previously[22]. Totally, 258 of them died from HCC, and two died from other causes during up to 60.7 months of follow-up. For disease-specific survival analysis, the latter were considered as censored data in the analyses. Drinking and chemotherapy or TACE status was significantly associated with survival time (log-rank $P = 0.006$ and < 0.001 for drinking status and chemotherapy or TACE status, respectively). Notably, compared to those who received neither chemotherapy nor TACE therapy (MST = 3.4 months), patients with chemotherapy or TACE therapy (MST = 16.8 months) had a 61% significantly decreased risk of death (HR = 0.39; 95% CI = 0.29–0.51).

Table 2 **Genotypes of *E2F3P1* rs9909601 and survival of HCC patients**

Genotype	Patients	Deaths	MST (mo)	Crude HR (95% CI)	Adjusted HR (95% CI)[a]	*P*[a]
rs9909601						
GG	122	104	12.6	1	1	
AG	167	121	16.0	0.71(0.55–0.92)	0.54(0.41–0.71)	<0.001
AA	33	26	15.4	0.72(0.47–1.11)	0.67(0.43–1.05)	0.080
AG/AA	200	147	15.8	0.71(0.55–0.91)	0.56(0.43–0.73)	<0.001
Additive model				0.79(0.65–0.97)	0.69(0.56–0.86)	0.001

HCC: hepatocellular carcinoma; MST: median survival time; HR: hazard ratio; CI: confidence intervals. [a]Adjusted for age, gender, smoking and drinking status, BCLC stage, and chemotherapy or TACE status.

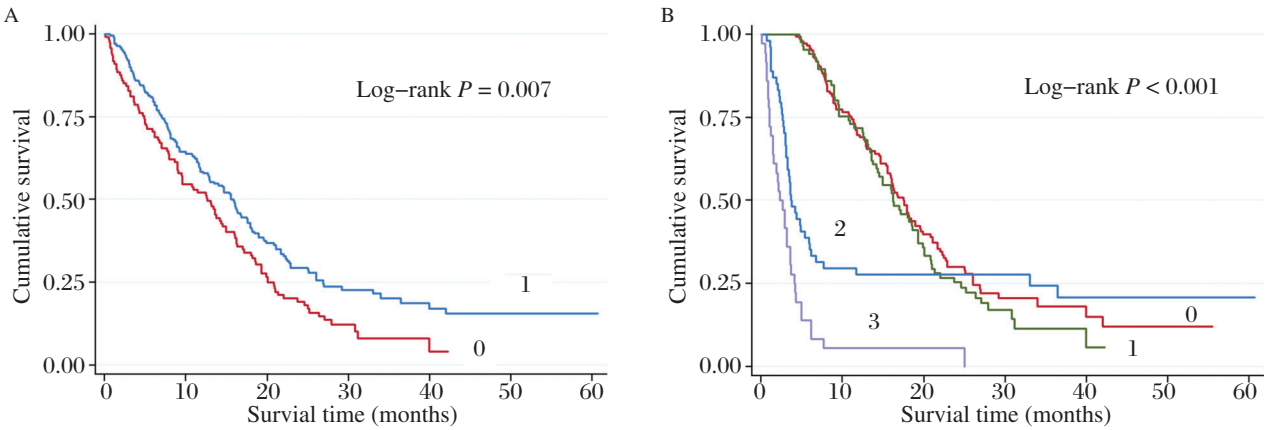

Fig. 1 **Kaplan-Meier plots of survival by *E2F3P1* rs9909601 genotypes in the survival of hepatocellular carcinoma (HCC) patients.** A: *E2F3P1* rs9909601 genotypes and HCC survival (log-rank $P = 0.007$ for AG/AA vs. GG) in a dominant model. 0, patients with common genotype GG; 1, those with variant genotypes (AG/AA). B: Kaplan-Meier plots of survival by the combination of rs9909601 genotypes and chemotherapy or transcatheter hepatic arterial chemoembolization (TACE) therapy status in HCC-specific survival (log-rank $P < 0.001$). 0, patients with variant genotypes (AG/AA) and receiving chemotherapy or TACE therapy; 1, those with common genotype (GG) and receiving chemotherapy or TACE therapy; 2, those with variant genotypes (AG/AA) and without chemotherapy and TACE therapy; 3, those with GG genotype and without chemotherapy and TACE therapy.

Effects of polymorphisms in pseudogenes on HCC survival

Kaplan-Meier method and log-rank test were performed to examine the associations of the six SNPs with HCC survival in different genetic models (additive model, dominant model and recessive model). As shown in ***Table 1***, the difference between the survival time of HCC patients and variant genotypes of rs9909601 located in *E2F3P1* was statistically significant (log-rank test: $P = 0.007$ in the dominant model and $P = 0.026$ in the additive model, respectively). Patients carrying rs9909601 AG/AA genotypes survived significantly longer time (MST = 15.8 months) than those carrying rs9909601GG genotypes (MST = 12.6 months; ***Fig. 1A***). Furthermore, multivariable Cox regression analysis showed that rs9909601 remained as a significant prognostic marker for HCC (***Table 2***). After adjusting for age, gender, smoking status, drinking status, BCLC stage, and chemotherapy or TACE status, variant genotypes of rs9909601were significantly associated with favorable prognosis of HCC (additive model: adjusted HR = 0.69, 95% CI = 0.56–0.86, $P = 0.001$; dominant model: adjusted HR = 0.56, 95% CI = 0.43–0.73, $P < 0.001$).

Stepwise Cox regression analysis on HCC survival

Then, we performed stepwise Cox proportional hazard analysis to estimate the effects of demographic characteristics, clinical features, and *E2F3P1* rs9909601 on HCC survival. As shown in ***Table 3***, four variables (age, drinking status, chemotherapy or TACE status,

Table 3 **Multivariable Cox regression analysis on survival of HCC patients**

Variables	β[a]	SE[b]	HR	95% CI	*P*
Stepwise regression analysis					
Chemotherapy or TACE (yes vs. no)	-1.2569	0.1564	0.29	0.21–0.39	< 0.001
rs9909601 (AG/AA vs. GG)	-0.5653	0.1337	0.57	0.44–0.74	< 0.001
Age (> 53 vs. ≤ 53)	-0.4582	0.1366	0.63	0.49–0.83	0.001
Drinking status (yes *vs.* no)	0.4124	0.1334	1.51	1.16–1.96	0.002
Final regression model					
Age (> 53 vs. ≤53)	-0.4714	0.1380	0.62	0.48–0.82	0.001
Drinking status (yes vs. no)	0.5495	0.1740	1.73	1.23–2.44	0.002
Chemotherapy or TACE (yes vs. no)	-1.2711	0.1586	0.28	0.21–0.38	< 0.001
rs9909601 (AG/AA vs. GG)	-0.5880	0.1348	0.56	0.43–0.72	< 0.001

HCC: hepatocellular carcinoma; HR: hazard ratio; CI: confidence intervals; TACE: transcatheter hepatic arterial chemoembolization. [a]β is the estimated parameter of the regression model. [b]SE is the standard error of the regression model.

Table 4 **Stratification analysis of rs9909601 genotypes associated with survival of HCC patients**

Variables	rs9909601 (patients/deaths)		Adjusted HR (95% CI)[a]	*P* for heterogeneity
	GG	AG/AA		
Age, years				
≤53	61/51	107/83	0.65(0.45–0.93)	0.419
>53	61/53	93/64	0.52(0.35–0.78)	
Gender				
Male	113/97	165/120	0.54(0.41–0.71)	0.162
Female	9/7	35/27	1.06(0.43–2.62)	
Smoking status				
No	39/32	76/53	0.56(0.36–0.88)	0.718
Yes	83/72	124/94	0.62(0.45–0.86)	
Drinking status				
No	46/37	75/52	0.60(0.38–0.94)	0.905
Yes	76/67	125/95	0.58(0.42–0.81)	
BCLC stage				
Stage B	113/96	182/133	0.54(0.41–0.71)	0.263
Stage C	9/8	18/14	0.99(0.36–2.80)	
Chemotherapy or TACE				
None	36/35	54/41	0.45(0.28–0.73)	0.029
Yes	86/69	146/106	0.85(0.62–1.15)	

HCC: hepatocellular carcinoma; BCLC: Barcelona Clinic Liver Cancer stage; HR: hazard ratio; CI: confidence intervals; TACE: transcatheter hepatic arterial chemoembolization. [a]Adjusted for age, gender, smoking and drinking status, BCLC stage, and chemotherapy or TACE status except for the stratification factor.

and *E2F3P1* rs9909601) were selected into the final regression model. Furthermore, when gender, smoking status and BCLC stage were included in the final model, the *E2F3P1* rs9909601 still remained as an independent protective factor for HCC survival (HR = 0.56, 95% CI = 0.43–0.72, $P < 0.001$).

Stratification and interaction analysis

The associations between *E2F3P1* rs9909601 and HCC survival were further investigated by stratification of age, gender, smoking status, drinking status, BCLC stage, and chemotherapy or TACE status. As shown in ***Table 4***, we found that the protective effect of rs9909601 variant genotypes was more prominent in patients without chemotherapy and TACE (adjusted HR = 0.45, 95% CI = 0.28–0.73) than those with chemotherapy or TACE therapy (adjusted HR = 0.85, 95% CI = 0.62–1.15, $P = 0.029$ for heterogeneity test). Therefore, a gene-chemotherapy or TACE status interaction analysis was carried out, and a statistically significant multiplicative interaction was observed (P for multiplicative interaction < 0.001, ***Fig. 1B***). Compared to subjects with AG/AA genotypes and with chemotherapy or TACE therapy, patients with GG genotype but without chemotherapy or TACE therapy had a significantly increased mortality risk (adjusted HR = 14.98, 95% CI = 9.20–24.37, $P < 0.001$) (***Table 5***).

DISCUSSION

In the present study, we investigated the effects of six common SNPs in cancer-related pseudogenes on the survival of advanced HCC patients and demon–

Table 5 **Interaction between rs9909601 genotypes and chemotherapy or TACE status**

Combined effects	rs9909601	Chemotherapy or TACE	Patients	Deaths	MST(mo)	Crude HR (95% CI)	Adjusted HR (95% CI)[a]	*P*[a]
0	AG/AA	Yes	146	106	17.5	1	1	
1	GG	Yes	86	69	16.2	1.15(0.85–1.56)	1.18(0.87–1.61)	0.290
2	AG/AA	None	54	41	3.7	1.78(1.23–2.58)	2.28(1.52–3.43)	< 0.001
3	GG	None	36	35	2.4	9.95(6.50–15.24)	14.98(9.20–24.37)	< 0.001
P for multiplicative interaction						< 0.001	< 0.001	

MST: median survival time; HR: hazard ratio; CI: confidence intervals; TACE: transcatheter hepatic arterial chemoembolization. [a]Adjusted for age, gender, smoking and drinking status, BCLC stage, and chemotherapy or TACE status.

strated that *E2F3P1* rs9909601 may be an independent biomarker to predict the survival of advanced HCC patients. To the best of our knowledge, this is the first report to evaluate the role of genetic variations of pseudogene in HCC survival.

E2F was reported to regulate the expression of multiple genes that are important in cell proliferation as a transcription factor[25]. Specifically, it plays a critical role in the control of cell cycle[26,27]. Recent findings suggested that the expression of *E2F3* was regulated by several miRNAs[28] and played a major role in modifying cellular proliferation rate which directly or indirectly affected clinical outcome of many types of tumors, including bladder cancer[29], prostate cancer[30], ovarian cancer[31] and breast cancer[32]. *E2F3P1* located in chromosome 17 is a pseudogene with sequence similar to *E2F3* in chromosome 6. Although they are in different chromosomes, *E2F3P1* may regulate *E2F3* expression by competing for miRNAs and their expressions are positively correlated[33]. Lees et al. have reported that genetic variations in *E2F3P1* may influence the miRNAs binding and thus may interrupt subsequent cellular activity, including proliferation and apoptosis[34]. Thus, it is biologically plausible that genetic variations in pseudogenes contribute to cancer risk or prognosis, given that miRNAs may provide linkage between pseudogenes and their parent genes.

By using two web-based prediction tools (miRBase and Patrocles), we found that the wild G allele of *E2F3P1* rs9909601 was more inclined to bind miR-24, miR-149, and miR-892b than the variant A allele. Both of the miR-24 and miR-149 have been investigated substantially in cancers. For instance, Han et al. identified that the overexpression of miR-24 was associated with the non-recurrence of hepatocellular carcinoma following liver transplantation which contributed to a better prognosis of HCC[35]. Besides, several studies have indicated that the down-regulation of miR-149 has been found in a variety of carcinomas and finally led to a worse patient survival, such as head and neck squamous cell carcinoma[36], colorectal cancer[37] and astrocytoma[38]. Thus, *E2F3P1* rs9909601 identified in our study may affect the impact of miRNAs regulation on gene expression by influencing the binding affinity of several special miRNAs, and hence play a role in the progression of HCC. Moreover, our analyses indicated a significant interaction between variant genotypes of rs9909601 and therapy status, which provided evidence that the effect of genetic variants on HCC prognosis could be modified by clinical factors.

In conclusion, rs9909601 at *E2F3P1* may be a useful biomarker for the prognosis of HCC survival. However, other studies with larger sample size and functional analysis are warranted to verify our finding.

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Research Paper

A genetic variant in pseudogene *E2F3P1* contributes to prognosis of hepatocellular carcinoma

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Supplementary Table 1 **Information of cancer-related pseudogenes**

Genes	Corresponding pseudogenes	Location
KRAS	*KRASP1*	Chr:6p12-p11
PTEN	*PTENP1*	Chr:9p21
E2F3	*E2F3P1*	Chr:17q11-q12
DNMT3A	*DNMT3AP1*	Chr:2p14
FOXO3	*FOXO3B*	Chr:17p11.2
NANOG	*NANOGP8*	Chr:15q14
CCND3	*CCND3P*	Chr:10p11.23
CDK4	*CDK4PS*	Chr:1p21.1

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Supplementary Table 2 **Information of primers and probes for TaqMan allelic discrimination**

Polymorphism		Sequence(5′-3′)
rs11682718	Primer	F: TCAAAAAGAGACAGCACCTGGAT
		R: GGAATTTGACCCTCTGGAAGTCTA
	Probe	G: FAM-CTTCCTTTTCTCAGCTGGGACAG -TAMRA
		A: HEX-TTCCTTTTCTCAACTGGGACAGG -TAMRA
rs1838149	Primer	F: CACCCAACCTGGTTCTCCTTT
		R: CCATGCTTCCATTCCCAGAA
	Probe	C: FAM-TCAGCAAGCCCCAGGACTG -TAMRA
		T: HEX-TGTCAGCAAGCCTCAGGACTG -TAMRA
rs9909601	Primer	F: CACGGTACGATAGTCTCTTGATCTG
		R: TCCGCTGCCTTGTTCAAAAC
	Probe	G: FAM-AGCTCCTGAGCCAGTCACCC -TAMRA
		A: HEX-CAGCTCCTGAACCAGTCACCC -TAMRA
rs2004079	Primer	F: CTTCAGGCCCACAAATCACA
		R: TCTTCCTCTATACTAACATGAGTGTGGAT
	Probe	G: FAM-TTACAGTCGGATGCTTCAAAGCA -TAMRA
		T: HEX-TTTACAGTCGGATTCTTCAAAGCAA -TAMRA
rs9889937	Primer	F: AGACTAGCCCGGTTCTGAAGTG
		R: TCCTGAAAGCAGGGTCTCAATATAA
	Probe	T: FAM-TGCCAGAGCCTTCCGCC -TAMRA
		C: HEX-CCAGAGCCCTCCGCCC -TAMRA
rs6913881	Primer	F: TCCAGTTTTCTCTGCATAAGTAATTAAAATAGACTT
		R: GAACTAGTTCAGGCGCCTGT
	Probe	G: FAM-CTGGATGCAAATAA -MGB
		A: HEX-TTTTATCTGGATACAAATAA -MGB