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ORIGINAL ARTICLE

Association between EZH2 Genetic Variants and Hepatocellular Carcinoma in a Chinese Han Population

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SUMMARY

Background: **Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. Studies have shown that EZH2, as the member of the Polycomb groups (PcGs) family, plays an important biological role in the occurrence and development of HCC. The association between the genetic variants of EZH2 and HCC is not yet fully established.**

Methods: **In this study, we used 175 patients with HCC and 209 healthy volunteers' blood samples of Chinese Han population to further analyze the relationship between EZH2 variants and HCC susceptibility.**

Results: **The results showed significant differences in distribution of alleles rs2302427 and rs3757441 between patients and the controls ($p < 0.05$). The three SNPs of EZH2 investigated show significant association with the elevated risk of HCC ($p < 0.05$) in addition to the overdominant model of rs3757441 and recessive model of rs41277434 ($p > 0.05$). The haplotype analysis of the three EZH2 SNPs revealed that the CCA and GTA haplotypes were associated with a higher risk of HCC ($p < 0.05$).**

Conclusions: **The results of these experiments indicated that the presence of EZH2 variants was significantly associated with HCC, and these variants could be useful genetic markers for predicting susceptibility to HCC in a Chinese Han population.**

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies and the third leading cause of cancer related death worldwide [1]. HCC carcinogenesis is a complex multifactor and multistep process, associated with multiple risk factors, including chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, liver cirrhosis, and a variety of genetic factors, but the exact molecular mechanism remains unclear [2-6]. Meanwhile, the process involves accumulation of multiple epigenetic alternations in the hepatocyte genomes leading to activation of oncogenes and inactivation of

tumor suppressor genes which are closely related to HCC development [7].

Polycomb group genes (PcGs) form two major distinct protein complexes: polycomb repressive complex (PRC) 1 and PRC2 [8]. PcG proteins achieve transcriptional repressor machineries by changing chromatin structures. There have been many studies which confirmed that PcGs are important factors to maintain stable performance of the Hox gene in the development of Drosophila, and they play an important role in embryonic development, tumor occurrence and metastasis, and maintaining stem cells [9-11]. EZH2 is a subunit of PRC2 and a histone H3-specific histone methyltransferase (HMTases). In the previous study, we found that EZH2 occupancy at chromatin coincided with H3K27me3 at promoters and directly silenced the transcription of certain tumor suppressors in HCC, including E-cadherin, RUNX3, BRCA1, as well as cyclin-dependent kinase inhibitors [12]. There was clear evidence that increased global levels of H3K27me3 and related histone H3-specific (HMTases), such as EZH2, were activated in human primary HCC [13-15].

As an important epigenetic regulation factor, mutations in EZH2 gene were also involved in certain types of tumors [12]. For example, Morin RD et al. [16] found that sporadic point mutations affecting the Y641 and A677 residues in the SET domain of EZH2 have been identified in lymphoma and myeloid neoplasms. Breyer et al. [17] found rs2302427 (D185H) of EZH2 had a minor allele frequency of 3.7% in cases and 5.2% in controls, conferring significant association with prostate cancer. In addition, Yoon et al. [18] demonstrated EZH2 genetic polymorphisms rs6950683 and rs3757441 contributed to significant associations with lung cancer risk, indicating protective effects of the variants on lung cancer risk. In order to clarify the relationship between EZH2 variants and HCC, this study used a large number of blood samples of Chinese Han population for SNP genetic analysis, including 175 patients with HCC and 209 healthy volunteers, to further analyze the relationship between EZH2 variants and susceptibility to HCC.

MATERIALS AND METHODS

Study subjects and specimen collection

We recruited 175 patients with HCC and 209 healthy, age-matched volunteers. All samples were from a Chinese Han population. The healthy control subjects were matched to the case subjects by gender and did not have a positive family history of HCC. All subjects gave written informed consent before participating in this study, which was approved by the Ethics Committee of the School of Public Health, Jilin University. Demographic information on gender, age, ethnicity and other measures were collected using a case investigation.

DNA extraction and genotyping

We selected three EZH2 SNPs (rs2302427, rs3757441, and rs41277434) based on previous studies' documented associations. The minor allele frequency (MAF) of all three SNPs was more than 0.05 in the Chinese Han population.

All subjects provided 5 mL of blood for biochemical analysis. Peripheral blood samples were collected in the morning using non-anticoagulant plexiglas tubes and stored at -20ºC. Genomic DNA was then extracted from the peripheral blood lymphocytes using a commercial DNA extraction kit (Kangwei Biotech Company, Beijing, China). SNP genotyping was performed using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS).

Statistical analysis

All statistical analysis were conducted using the SPSS program (version 19.0; SPSS, Chicago, IL, USA) and the online SNP Stats (http://bioinfo.Iconcologia.net/SNPStats) program. The minor allele frequency of the SNPs was compared between subjects with and without HCC using the Chi-square (χ^2) test. For each SNP, a Hardy-Weinberg disequilibrium (HWD) test was conducted in the case and control groups. The χ^2 test was also used to compare SNP allele frequencies and genotype distributions between cases and controls. A one-way ANOVA or nonparametric test was used to evaluate the association of SNPs with physical/biochemical parameters. P-values less than 0.05 were considered to be statistically significant.

RESULTS

Demographic and biochemical characteristics of study population

The study involved 384 participants, including 175 patients with HCC and 209 healthy volunteers. Participants were all from the Chinese Han population. Table 1 displayed the demographic characteristics and summarized the clinical and biochemical features of study participants. 175 patients with HCC included 147 males and 26 females, mean age (± S.D.), 56.83 ± 13.37 years old; 209 healthy volunteers included 167 males and 42 females, mean age (± S.D.), 53.89 ± 11.81 years old. There was no significant difference in the gender distribution between cases and controls ($p > 0.05$). The genotype distributions of the three SNPs in the control group did not deviate from the Hardy-Weinberg equilibrium ($p > 0.05$, Table 2).

Differences in allele and genotype frequencies in case and control groups

In this study, we detected three SNP sites (rs2302427, rs3757441, and rs41277434) of EZH2. As shown in Table 3, we compared genotype distributions and allele frequencies of all polymorphisms studied between cases and controls. The rs2302427 C allele, rs3757441 C al-

Table 1. Demographic characteristics of controls and patients with HCC.

Variable	Case (n = 175)	Control (n = 209)	p-value
Age (years)	56.83 ± 13.37 [a]	53.89 ± 11.81 [a]	> 0.05
Gender	(%)	(%)	
Male	147 (84.97%)	167 (79.90%)	
Female	26 (15.03%)	42 (20.10%)	
AFP (ng/mL)	191.30 (3893.5 - 5.99) [b]		
ALP (u/g)	107.35 (460.60 - 75.35) [b]		
ALT (u/L)	30.95 (53.63 - 20.15) [b]		
AST (u/L)	38.00 (78.38 - 24.65) [b]		
PB (umol/L) DBIL (umol/L)	13.95 (20.30 - 10.00) [b] 5.90 (8.93 - 4.18) [b]		
IBIL (umol/L) ALB (g/L)	7.30 (10.53 - 5.00) [b] 38.71 (43.10 - 33.88) [b]		
PT (s)	14.51 ± 2.32 [a]		

AFP - alpha fetoprotein, ALP - alkaline phosphatase, ALT - alanine aminotransferase, AST - aspartate transaminase, PB - preoperative bilirubin, DBIL - direct bilirubin, IBIL - indirect bilirubin, ALB – albumin, PT - prothrombin time, [a] - Mean ± S.D, [b] - M (75% - 25%).

Table 2. The Hardy-Weinberg equilibrium results by goodness-of-fit Chi-square tests.

Tag SNPs	Cases				Controls			
	H_0	H_e	X^2	p	H_0	H_e	X^2	p
rs2302427	0.2840	0.3018	0.5657	0.4520	0.1667	0.1609	0.2621	0.6087
rs3757441	0.4277	0.4003	0.7434	0.3886	0.3197	0.2903	1.2447	0.2646
rs41277434	0.0479	0.0468	0.1006	0.7511	0.1086	0.1128	0.2459	0.6200

Table 3. Comparison of genotype distributions and allele frequencies of polymorphisms in the EZH2 gene.

SNP	Allele	Case	Control	X^2	p	OR	95% CI	
							Lower	Upper
rs2302427	C/C	109 (67.3%)	169 (82.8%)	7.917	0.005 *	10.853	1.317	89.441
	C/G	46 (28.4%)	34 (16.7%)					
	G/G	7 (4.3%)	1 (0.5%)					
	C allele	264 (81.5%)	372 (91.2%)	14.896	0.000 *	2.348	1.509	3.655
	G allele	60 (18.5%)	36 (8.8%)					
rs3757441	T/T	81 (50.9%)	81 (66.4%)	8.608	0.003 *	0.200	0.042	0.941
	T/C	68 (42.8%)	39 (32.0%)					
	C/C	10 (6.3%)	2 (1.6%)					
	T allele	230 (72.3%)	201 (82.4%)	7.801	0.005 *	0.559	0.371	0.843
	C allele	88 (27.8%)	43 (17.6%)					
rs41277434	A/A	159 (95.2%)	155 (88.6%)	0.977	0.323	0.000		
	A/C	8 (4.8%)	19 (10.9%)					
	C/C	0 (0.0%)	1 (0.05%)					
	A allele	326 (97.6%)	329 (94.0%)	5.470	0.019 *	0.384	0.168	0.880
	C allele	8 (2.4%)	21 (6.0%)					

*** considered statistically significant.**

Table 4. Genotype distribution and allele frequency differences between HCC patients and healthy controls for three EZH2 SNPs.

SNP	Genotype	Inheritance Model	Case	Control	OR (95% CI)	p	AIC
rs2302427	C/C	Codominant	108 (67.5%)	169 (82.8%)	1.00	< 0.001 *	489.9
	C/G		45 (28.1%)	34 (16.7%)	2.10 (1.26 - 3.50)		
	G/G		7 (4.4%)	1 (0.5%)	10.44 (1.25 - 87.05)		
	C/C	Dominant	108 (67.5%)	169 (82.8%)	1.00	< 0.001 *	490.8
	C/G - G/G		52 (32.5%)	35 (17.2%)	2.34 (1.43 - 3.85)		
	C/C - C/G	Recessive	153 (95.6%)	203 (99.5%)	1.00	0.012 *	496.1
	G/G		7 (4.4%)	1 (0.5%)	8.77 (1.06 - 72.78)		
	C/C - G/G	Overdominant	115 (71.9%)	170 (83.3%)	1.00	0.0077 *	495.3
	C/G		45 (28.1%)	34 (16.7%)	1.99 (1.19 - 3.30)		
rs3757441	T/T	Codominant	79 (50.3%)	81 (66.4%)	1.00	0.0084 *	377.0
	C/T		68 (43.3%)	39 (32.0%)	1.76 (1.06 - 2.92)		
	C/C		10 (6.4%)	2 (1.6%)	5.64 (1.18 - 26.97)		
	T/T	Dominant	79 (50.3%)	81 (66.4%)	1.00	0.0008 *	377.5
	C/T - C/C		78 (49.7%)	41 (33.6%)	1.94 (1.18 - 3.18)		
	T/T - C/T	Recessive	147 (93.6%)	120 (98.4%)	1.00	0.03 *	379.9
	C/C		10 (6.4%)	2 (1.6%)	4.50 (0.95 - 21.21)		
	T/T - C/C	Overdominant	89 (56.7%)	83 (68.0%)	1.00	0.068	381.2
	C/T		68 (43.3%)	39 (32.0%)	1.59 (0.96 - 2.62)		
rs41277434	A/A	Codominant	158 (95.8%)	155 (88.6%)	1.00	0.041 *	467.4
	C/A		7 (4.2%)	19 (10.9%)	0.36 (0.15 - 0.89)		
	C/C		0 (0.0%)	1 (0.6%)	0.00 (0.00 - NA)		
	A/A	Dominant	158 (95.8%)	155 (88.6%)	1.00	0.014 *	465.8
	C/A-C/C		7 (4.2%)	20 (11.4%)	0.35 (0.14 - 0.85)		
	A/A - C/A	Recessive	165 (100%)	174 (99.4%)	1.00	0.33	470.8
	C/C		0 (0%)	1 (0.6%)	0.00 (0.00 - NA)		
	A/A - C/C	Overdominant	158 (95.8%)	156 (89.1%)	1.00	0.02 *	466.4
	C/A		7 (4.2%)	19 (10.9%)	0.36 (0.15 - 0.89)		

*** considered statistically significant.**

Table 5. The association between EZH2 haplotypes and HCC.

	rs2302427	rs3757441	rs41277434	Frequency			OR (95% CI)	p
				Total	Case	Control		
1	C	T	A	0.5894	0.5135	0.6757	1.00	-
2	C	C	A	0.2369	0.2780	0.1780	2.03 (1.29 - 3.19)	0.0025 *
3	G	T	A	0.1311	0.1846	0.0862	2.57 (1.61 - 4.11)	< 0.001 *
4	C	T	C	0.0425	0.0232	0.0580	0.43 (0.17 - 1.05)	0.065

*** - considered statistically significant.**

Table 6. Association of SNPs with clinical indexes.

SNP	Clinical Index	Allele frequency			F	p
rs2302427		CC	CG	GG		
	AFP	201.60 (10378.00 - 6.00) [a]	191.30 (2637.00 - 6.51) [a]	4.30 (139.50 - 3.48) [a]	0.073	0.930
	ALP	107.90 (159.00 - 75.80) [a]	103.00 (235.20 - 82.00) [a]	68.80 (111.00 - 62.00) [a]	2.252	0.109
	ALT	31.60 (54.50 - 20.25) [a]	30.90 (52.80 - 20.10) [a]	25.20 (45.00 - 21.60) [a]	0.073	0.930
	AST	41.70 (83.00 - 24.70) [a]	39.00 (83.00 - 27.70) [a]	21.70 (26.20 - 20.30) [a]	3.626	0.029 *
	PB	14.20 (20.50 - 9.70) [a]	14.20 (20.40 - 10.48) [a]	12.20 (21.50 - 10.20) [a]	0.161	0.851
	ALB	38.60 (42.57 - 34.60) [a]	40.47 (44.97 - 33.76) [a]	45.03 (45.84 - 34.10) [a]	1.096	0.337
	PT	14.62 ± 1.60 [b]	14.93 ± 2.09 [b]	14.44 ± 0.97 [b]	0.430	0.651
rS3757441		CC	TC	TT		
	AFP	515.00 (77759.25 - 17.37) [a]	327.20 (20140.50 - 6.02) [a]	144.45 (2221.75 - 5.31) [a]	0.800	0.451
	ALP	142.50 (186.88 - 103.48) [a]	108.30 (169.90 - 73.83) [a]	101.25 (145.33 - 68.65) [a]	2.009	0.138
	ALT	45.50 (69.68 - 21.63) [a]	35.80 (59.70 - 22.98) [a]	27.85 (52.70 - 18.98) [a]	1.905	0.152
	AST	79.50 (119.40 - 20.50) [a]	45.80 (93.08 - 25.93) [a]	34.90 (68.63 - 24.93) [a]	1.224	0.297
	PB	19.15 (24.48 - 16.15) [a]	12.65 (17.95 - 10.50) [a]	13.80 (20.85 - 9.45) [a]	2.934	0.056
	ALB	34.11 (38.45 - 30.88) [a]	38.84 (42.98 - 34.17) [a]	40.10 (44.07 - 35.68) [a]	2.719	0.069
	PT	14.58 ± 1.46 [b]	14.69 ± 1.37 [b]	14.65 ± 1.88 [b]	0.248	0.781
rs41277434		AA	AC	CC		
	AFP	211.70 (6905.00 - 6.05) [a]	49.10 (7420.25 - 2.50) [a]	-	0.764	0.383
	ALP	108.30 (159.83 - 74.45) [a]	104.30 (235.65 - 60.90) [a]	-	0.053	0.818
	ALT	31.60 (53.88 - 20.35) [a]	32.70 (69.00 - 21.73) [a]	-	0.057	0.812
	AST	39.05 (77.83 - 24.73) [a]	49.30 (87.25 - 25.58) [a]	-	0.013	0.909
	PB	14.10 (20.60 - 10.10) [a]	16.30 (17.25 - 13.63) [a]	-	0.146	0.702
	ALB	39.08 (43.17 - 34.18) [a]	38.05 (43.22 - 32.63) [a]	-	0.246	0.620
	PT	14.67 ± 1.63 [b]	14.38 ± 1.12 [b]	-	0.055	0.815

[a] - M (75% - 25%) [b], Mean ± S.D. [*] - considered statistically significant.

lele, and rs41277434 A allele frequencies were significantly higher in the cases than in the controls ($p < 0.05$). Then, we performed genotype distributions and odds ratio estimates. Our analysis using five inheritance models, the results showed that three SNPs of EZH2 investigated (rs2302427,rs3757441 and rs41277434) have significant association with the elevated risk of HCC ($p < 0.05$) in addition to over dominant model of rs3757441 and recessive model of rs41277434 ($p > 0.05$, Table 4). In the results of odds ratio estimates, patients with C/G and G/G genotypes at EZH2 rs2302427 showed 2.10-fold (95% CI: 1.26 - 3.50) and 10.44-fold (95% CI: 1.25 - 87.05); patients with C/T and T/T genotypes at EZH2 rs3757441 showed 1.76-fold (95% CI: 1.06 - 2.92) and 5.64-fold (95% CI: 1.18 - 26.97). Haplotype analysis showed that the haplotype CTA (in the order frequency: rs2302427, rs3757441, and rs41277434) accounted for 58.94% of the total sample and was the most common haplotype. The haplotype CCA (23.69%) was the second most common haplotype, its distribution was significantly different between cases and controls (OR = 2.03; CI, 1.29 - 3.19; $p < 0.05$, Table 5).

Association of SNPs with the clinical index of subjects with HCC

In the analysis of quantitative characters, Table 6 showed the results of the association between the three SNPs and some clinical indexes of subjects with HCC. We found that there was no significant association between clinical indexes and HCC ($p > 0.05$) except the rs2302427 variant with aspartate transaminase (AST) (GG<CG<CC, $p < 0.05$).

DISCUSSION

As evidence of the evolution of the human genome, SNPs represent a type of genetic mutation that has become the third generation of genetic markers [19]. In

complex diseases, some small alleles of SNPs could be used as risk factors, whereas others were protective [20]. In our previous studies, we discovered a highly conserved G553C point mutation, which was the SNP site rs2302427. The rs2302427 variant was found in both HCC and in paracancerous tissues by the EZH2 exon sequencing. Interestingly, the allele and genotype distributions of rs2302427 were different in HCC and paracancerous tissues, and they were significantly associated with overall and tumor-free survival when found in paracancerous tissues but not in HCC tissue. We speculated that the G553C mutation might be both a SNP (rs2302427) and a somatic mutation. Yu et al. [21] found that the rs2302427 was associated with urothelial cell cancer (UCC) in a Taiwan population. The patients carrying CG, GG, or CG+GG at rs2302427 showed a lower risk of UCC (all $p < 0.05$). In addition to the results of the clinical correlation analysis, the UCC risk for people carrying the G allele was lower than the C allele at rs2302427 in invasive tumor stage. In the study of Oral Squamous Cell Carcinoma (OSCC) in a Taiwan population, Su et al. [22] found that there was no association between rs2302427 and OSCC. These results suggested that EZH2 rs2302427 variants might be susceptibility markers linked to the tumor, but the genetics of EZH2 in different tumors remains to be further confirmed. In order to further clarify the genotype distribution in HCC, we examined the rs2302427 genotypes in 384 blood samples. The results showed significant differences in alleles and genotypes of rs2302427 between patients and the controls ($p < 0.05$). Importantly, in the analysis of quantitative characters, we found that AST levels in CG and GG genotypes were lower than in the CC genotype. Based on the above results, we concluded that the G allele of rs2302427 might function as a protective factor in regulation disorder of AST, tumor migration, and tumor survival of HCC.

Rs3757441 is an intronic SNP and as such might affect gene expression through several mechanisms, including changes in transcription-factor binding sites, microRNA-targeting sequences, and splicing variants [23,24]. Rs41277434, as another important variant of EZH2 which was located in intron 17, might impact gene expression by affecting promoter function [25]. Although the functional importance of rs3757441 and rs41277434 had not been tested experimentally, Cardoso C et al. [26] observed that individuals carrying C/C alleles at these two SNPs had a lower risk of lung cancer than those carrying the T wild-type allele. In the study of OSCC, Su et al. found rs3757441 of EZH2 had a minor allele frequency of 26.6% in cases and 30.7% in controls [22]. Then in this study, our results showed significant differences in alleles and genotypes of rs3757441 between cases and the controls ($p < 0.05$).The results of five Inheritance Models showed the significant differences in genotypes between cases and controls ($p < 0.05$) except over dominant model ($p > 0.05$). For the study of rs41277434 in malignant myeloid disorders, the results showed that there was no statistical difference between patients and controls [26]. However, in our results, significant differences were found in alleles of rs41277434 between HCC patients and controls in a Chinese Han population ($p < 0.05$). Table 4 showed that individuals carrying C alleles at rs41277434 had a lower risk of HCC than carrying other alleles. Therefore, we speculated that EZH2 rs41277434 variants might play a protective role in HCC.

Although many SNPs have no direct effect on gene products, they can be used as genetic markers to locate adjacent functional variants that contribute to disease [25]. In addition, it might not be apparent when looking at individual SNPs. Therefore, haplotype analysis is sometimes advantageous over analysis of individual SNPs for detecting an association between alleles and a disease phenotype [27]. Our haplotype analysis of the three EZH2 SNPs rs2302427, rs3757441, and rs41277434 revealed that the CCA and GTA haplotypes were associated with a higher risk of HCC ($p < 0.05$, Table 6). It was possible that these EZH2 SNPs were linked with other functional polymorphisms. However, further studies would be required to confirm this finding. Furthermore, functional studies would be also needed to understand the underlying mechanisms of the associations between HCC and these SNPs.

CONCLUSION

The results of these experiments showed that the presence of EZH2 variants was significantly associated with HCC and could be useful genetic markers for predicting susceptibility to HCC in a Chinese Han population. Because of the population heterogeneity, the association between EZH2 and HCC susceptibility would be further validated in the other populations.

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Declaration of Interest:
The authors declared no conflict of interests.

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