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Original Article

ESR2 polymorphism associated with the incidence and prognosis of hepatocellular carcinoma

Wei Zhang[1], Menghui Li[2], Jianping Gong[2], Mingxin Ye[2], Xiaoli Yang[2]

[1]Department of General Surgery, The People's Hospital of Jianyang City, Sichuan 641400, China; [2]Department of Hepatobiliary Surgery, The Second Affiliated Hospital of Chongqing Medical University, Chongqing 400016, China

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Abstract: Aim: To investigate the role of ESR2 polymorphism in incidence and prognosis of patients with hepatocellular carcinoma (HCC). Methods: The DNA from patients with HCC and hepatitis type B virus carriers was used to observe the distribution of ESR2 variations and carry out the clinical correlation analysis. Results: Single nucleotide polymorphisms (SNPs) in ESR2 gene (rs1256049, rs4986938) were significantly correlated with HCC risk. Individuals carrying AA/GA genotype of rs1256049 were associated with lower HCC risk when compared with GG genotype (OR 0.399; 95% CI 0.256-0.621; P<0.001). The opposite result was found in individuals with the rs4986938 AA/GA genotype (OR 2.154; 95% CI 1.370-3.385; P=0.001). The LL genotype (frequency n≤20) of CA repeat allele was associated with moderate-well differentiated degree (p=0.001) and early stage of HCC (p=0.001), and mean OS time was 46.2 (95% CI: 39.3-53.1) months for patients with LL genotype and 29.4 (95% CI: 24.3-34.5) months for patients with SS (frequency n>20) genotype (P=0.005). Conclusions: Findings indicated SNPs in ESR2 gene (rs1256049, rs4986938) play a different role in HCC risk and SS genotype lead to poor prognosis of HCC patients underwent hepatectomy. It provided a potential reference for the diagnosis and treatment of HCC.

Keywords: ESR2, gene polymorphism, HCC, incidence, overall survival, prognosis

Introduction

Liver cancer ranks third in the causes of global cancer death. The risk of liver cancer in males is 2-7 times that of females [1, 2]. Thus gender is an influencing factor in the occurrence of liver cancer obviously. Gender-related hormones, especially estrogen, may play an important role in liver cancer, but the specific role of these hormones in hepatocellular carcinoma (HCC) is still not very clear [3].

ERs are often significantly overexpressed in many cancers, such as mammary adenocarcinoma, prostatic carcinoma, endometrial cancer, esophageal carcinoma and bronchogenic carcinoma [4-8]. There are two main ERs that mediate the classical actions of estrogen, ESR1 and ESR2 [9]. Changes of ESR1 expression level, mutation and variation are closely related to occurrence and progression of malignant tumors [10]. On the contrary, ESR2 modulates the expression of genes regulated by it and reduces the migration of tumor cells [11]. The lack of expression of ESR2 gene is closely related to the occurrence and progression of cholangiocarcinoma [12]. Ryan J reported single nucleotide polymorphism (SNP), (1082 G/A, rs1256049), in ESR2 gene [13]. SNP (1730 G/A, rs4986938) was found by Keyes Y [14]. ESR2 genes had C (cytosine) and A (adenine) repeated polymorphism in No.5 intron. The rs4986938 (G1730A) variant is located in exon 8 of the ESR2, a 3' untranslated area and the rs4986938 A allele correlates with a lower expression of ESR2 [15, 16]. The differences of CA repeated frequency are connected with diseases related to estrogen levels [17]. Sunakawa Y found some SNPs located in a promoter region were related to the survival of patients with gastric carcinoma [18]. Recently, the T allele of ESR2 gene SNPs was found to be a risk factor for primary biliary cirrhosis [19]. Our research on distribution of ESR2 gene polymorphism in HCC group and hepatitis B virus carriers group was aimed to explore the relationship between ESR2 gene polymorphism and HCC from molecule genetics perspective.

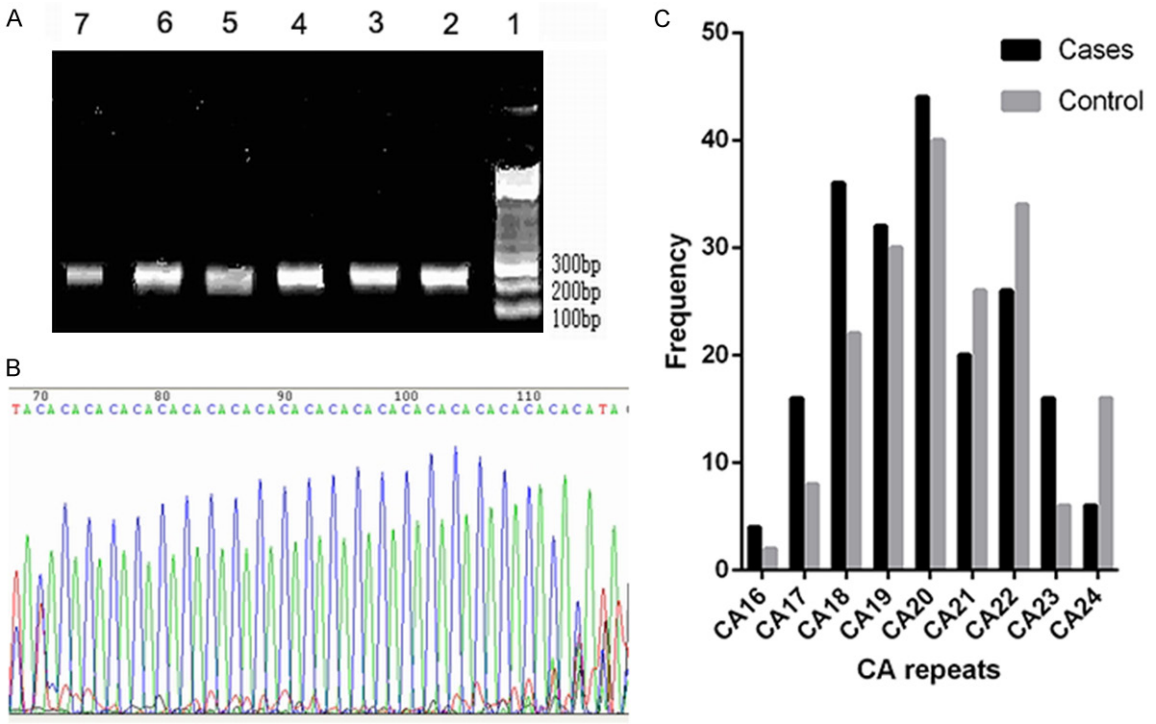

Figure 1. CA repeated sequence of ESR2 gene. A. Gel of PCR amplification. The bands located between 200 bp-300 bp. B. ESR2 gene CA repeat analyze. The gram shows the CA sequence repeated 23 times. C. Distribution of CA repeats. 9 alleles (n=16~24) were identified in CA repeated sequence polymorphism of ER-β genes. No significant differences were seen in distribution frequency of CA repeated sequence alleles in experimental and control groups (P=0.520).

Patients and methods

Eligible patients

200 cases of HCC patients underwent hepatectomy were recruited as cases group in the First Affiliated Hospital of Chongqing Medical University from October 2009 to May 2012. The diagnosis had been confirmed pathologically and the blood samples were taken before treated. 200 hepatitis B virus carriers without other liver diseases in the same period were selected as control group. Patients were excluded in this study with heart or kidney diseases, HCV infection, high blood pressure, diabetes, alcoholic liver disease, NAFLD/NASH, autoimmune hepatitis, primary biliary cirrhosis and sclerosing cholangitis, metabolic diseases or HIV-infection.

DNA extraction and genotyping

Whole blood samples from all subjects were collected and used to extract genomic DNA by phenol chloroform method. DNA concentration was determined spectrophotometrically. Polymorphism of ESR2 genes rs1256049 and rs4986938: one pair of estrogen receptor gene primers was applied to amplify estrogen receptor gene, specificity a section of target DNA in the fifth and eighth exon of ESR2 genes. Primer and reaction conditions were based on reference [20]. PCR amplification products were enzyme digested by *RsaI* or *AluI* separately for 4 hours at 37°C. The products were extracted to make electrophoresis in 2% agarose gel containing ethidium bromide. CA repeated sequence polymorphism of ESR 2 Genes: PCR primer was synthesized by Sangon Bioengineering Technology Services Limited in Shanghai. Primer and reaction conditions were based on reference [17]. Finally 1 μl PCR products was extracted to make electrophoresis in 2% agarose gel containing ethidium bromide. Its purity and integrity were appraised and observed under ultraviolet and photographed. The fragment with the length about 274 bp (**Figure 1A**) which included CA repeated sequences of ESR2 genes was sent to Shanghai Sangon Biological Engineering and Technology Services Company

Table 1. Baseline clinical characteristics of cases and controls

	Cases (n=200) No. (%)	Control (n=200) No. (%)	OP (95% CI)	*P* value
Sex			0.051	0.821
Men	146 (73)	148 (74)		
Women	54 (27)	52 (26)		
Ages (years)			0.078	0.779
<45	31 (15.5)	29 (14.5)		
≥45	167 (83.5)	171 (85.5)		
HBV infection			0.297	0.586
HBsAg (+)	148 (74)	200 (100)		
HBsAg (-)	52 (26)	0 (0)		
Alcohol			0.181	0.914
Never to occasional	105 (52.5)	108 (54)		
Daily (<150 g/week)	26 (13)	27 (13.5)		
Daily (≥150 g/week)	69 (34.5)	65 (32.5)		

Limited to make GeneScan analysis (**Figure 1B**).

Statistical analysis

χ^2-test was used to examine differences in basic characteristics between cases and controls. The allele distribution of SNPs was detected by exact test, and deviated from Hardy-Weinberg equilibrium. Logistic regression was applied to analyze allele relative risk. For CA repeated sequence polymorphism differences, Fisher's exact test was applied in comparing cases and control group. Period from surgery or diagnosis to death was counted as the overall survival. The Kaplan-Meier method was used to estimate overall survival and recurrence-free survival and the log-rank test was used to compare differences. Univariate and multivariate analysis was performed using the Cox proportional hazards model. IBM SPSS Statistics 22.0 was used to analysis the processing data.

Results

The baseline characteristics between cases and control group have no significant differences

From January 2009 to May 2010, 200 patients underwent hepatectomy for HCC in the First Affiliated Hospital of Chongqing Medical University were selected as cases group with 146 male cases and 54 female cases aging 29~71 with average 50 years old. 200 controls, free of cancer history at the time when the case was diagnosed, were matched with each case on sex, age (within three years), HBsAg status, drinking history and date of blood drawn (within three months). Compared to control group, there were no significant deviation in cases group (**Table 1**).

GG genotype of rs1256049 and AA/GA genotype of rs1256049 were closely related to high risk of HCC

The distributions of SNPs rs1256049 and rs4986938 in the control group were meets the Hardy Weinberg equilibrium (p>0.05). Both SNPs (rs1256049, rs4986938) in ESR2 gene were significantly related to HCC risk (**Table 2**). Individuals carrying AA/GA genotype of rs1256049 were associated with lower HCC risk when compared with GG genotype carriers (OR 0.399, 95% CI 0.256-0.621, P<0.001; **Table 2**). The inverse association was significant in individuals who carried the genotype of rs4986938 AA/GA (OR=2.154, 95% CI 1.370-3.385, P=0.001; **Table 2**). Distribution frequency of CA repeated sequence allele in cases and controls showed no conspicuous difference (X^2=7.155, P=0.520, **Figure 1C**). Distribution frequency of SS type and LL type was compared again with a significant difference when repeated frequency n≤20 was made as SS type and n20 as LL type. LL genotype covered 34% and 49% in cases and controls, respectively. LL genotype was associated with lower risk of HCC (OR=0.536, 95% CI 0.358-0.802, P=0.002, **Table 2**).

GG genotype of rs1256049 and AA/GA genotype of rs4986938 carriers showed worse clinical pathological feature in cases group

As ESR2 polymorphisms were visibly related to the risk of HCC, we performed further analyses to determine the relationship between clinical pathological characteristics and ESR2 polymorphisms in HCC cases. The AA/GA genotype of rs1256049 was positively correlated with single tumor number (**Table 3**). The AA/GA genotype of rs4986938 was associated with low differentiated degree in HCC (**Table 3**). The LL genotype of CA repeat allele was associated with moderate-well differentiated degree and early stage of HCC (**Table 3**).

Table 2. Association between ESR2 polymorphisms and HCC

	Genotype	Cases (n=200) No. (%)	Control (n=200) No. (%)	OP (95% CI)	*P* value
(rs1256049)	GG	80 (40.0)	42 (21.0)	Reference	
	GA	100 (50.0)	112 (56.0)	0.228 (0.120-0.435)	0.001
	AA	20 (10.0)	46 (23.0)	0.469 (0.296-0.743)	<0.001
	AA+GA	120 (60.0)	158 (79.0)	0.399 (0.256-0.621)	<0.001
(rs4986938)	GG	130 (65.0)	160 (80.0)	Reference	
	GA	58 (29.0)	36 (18.0)	1.983 (1.232-3.191)	0.005
	AA	12 (6.0)	4 (2.0)	3.692 (1.163-11.720)	0.027
	AA+GA	70 (35.0)	40 (20.0)	2.154 (1.370-3.385)	0.001
CA repeat	SS	132 (66.0)	102 (51.0)	Reference	
	LL	68 (34.0)	98 (49.0)	0.536 (0.358-0.802)	0.002

Table 3. Relationship between clinical pathological features and ESR2 gene polymorphism in cases

Variables	rs1256049				rs4986938				CA repeat		
	AA (20) No. (%)	AG (100) No. (%)	GG (80) No. (%)	*P* value	AA (12) No. (%)	AG (58) No. (%)	GG (130) No. (%)	*P* value	LL (68) No. (%)	SS (132) No. (%)	*P* value
Gender				0.697				0.983			0.830
Male	16 (80.0)	71 (71.0)	59 (73.8)		9 (75.0)	42 (72.4)	95 (73.1)		49 (72.1)	97 (73.5)	
Female	4 (20.0)	29 (29.0)	21 (26.3)		3 (25.0)	16 (27.6)	35 (26.9)		19 (27.9)	35 (26.5)	
Age (years)				0.972				0.993			0.310
<45	3 (15.0)	15 (15.0)	13 (16.3)		2 (16.7)	9 (15.5)	20 (15.4)		13 (19.1)	18 (13.6)	
≥45	17 (85.0)	85 (85.0)	67 (83.8)		10 (83.3)	49 (84.5)	110 (84.6)		55 (80.9)	114 (86.4)	
Differentiated degree				0.540				0.001			0.001
Well	3 (15.0)	8 (8.0)	4 (5.0)		0 (0.0)	1 (1.7)	14 (10.8)		15 (22.1)	0 (0.0)	
Moderate	10 (50.0)	60 (60.0)	45 (56.3)		2 (16.7)	37 (63.8)	76 (58.5)		43 (63.2)	72 (54.5)	
Low	7 (35.0)	32 (32.0)	31 (38.8)		10 (83.3)	20 (34.5)	40 (30.8)		10 (14.7)	60 (45.5)	
Cirrhosis				0.727				0.374			0.464
Yes	13 (65.0)	68 (68.0)	58 (72.5)		9 (75.0)	44 (75.9)	86 (66.2)		45 (66.2)	94 (71.2)	
No	7 (35.0)	32 (32.0)	22 (27.5)		3 (25.0)	14 (24.1)	44 (33.8)		23 (33.8)	38 (28.8)	
Tumor size				0.803				0.726			0.550
<5 cm	11 (55.0)	51 (51.0)	38 (47.5)		5 (41.7)	31 (53.4)	64 (49.2)		36 (52.9)	64 (48.5)	
≥5 cm	9 (45.0)	49 (49.0)	42 (52.5)		7 (58.3)	27 (46.6)	66 (50.8)		32 (47.1)	68 (51.5)	
Tumor number				0.004				0.984			0.085
Single	16 (80.0)	84 (84.0)	50 (62.5)		9 (75.0)	44 (75.9)	97 (74.6)		46 (67.6)	104 (78.8)	
Multiple	4 (20.0)	16 (16.0)	30 (37.5)		3 (25.0)	14 (24.1)	33 (25.4)		22 (32.4)	28 (21.2)	
Stage				0.383				0.086			0.001
I/II	15 (75.0)	69 (69.0)	49 (61.3)		5 (41.7)	36 (62.1)	92 (70.8)		35 (51.5)	98 (74.2)	
III/VI	5 (25.0)	31 (31.0)	31 (38.8)		7 (58.3)	22 (37.9)	38 (29.2)		33 (48.5)	34 (25.8)	
HBsAg				0.807				0.802			0.111
Negative	5 (25.0)	28 (28.0)	19 (23.8)		4 (33.3)	14 (24.1)	34 (26.2)		13 (19.1)	39 (29.5)	
Positive	15 (75.0)	72 (72.0)	61 (76.3)		8 (66.7)	44 (75.9)	96 (73.8)		55 (80.9)	93 (70.5)	

SS gene type is related to poor prognosis

We evaluated the ESR2 gene polymorphism to prognosis in the cases. At the time of last follow-up, 110 patients had recurrence and 84 patients died in 200 patients. Kaplan-Meier analysis indicated the mean OS time was 46.2 (95% CI: 39.3-53.1) months for patients with LL genotype and 29.4 (95% CI: 24.3-34.5) months for patients with SS genotype (P=0.005, **Figure 2A**). The average recurrence time was 36.7 (95% CI: 29.6-43.8) months for patients with LL genotype and 23.1 (95% CI: 18.3-30.0) months for patients with SS genotype (P=0.016, **Figure 2B**). Furthermore, no survival and recurrence difference were found among patients with different rs1256049 and rs4986938 genotypes (data not shown).

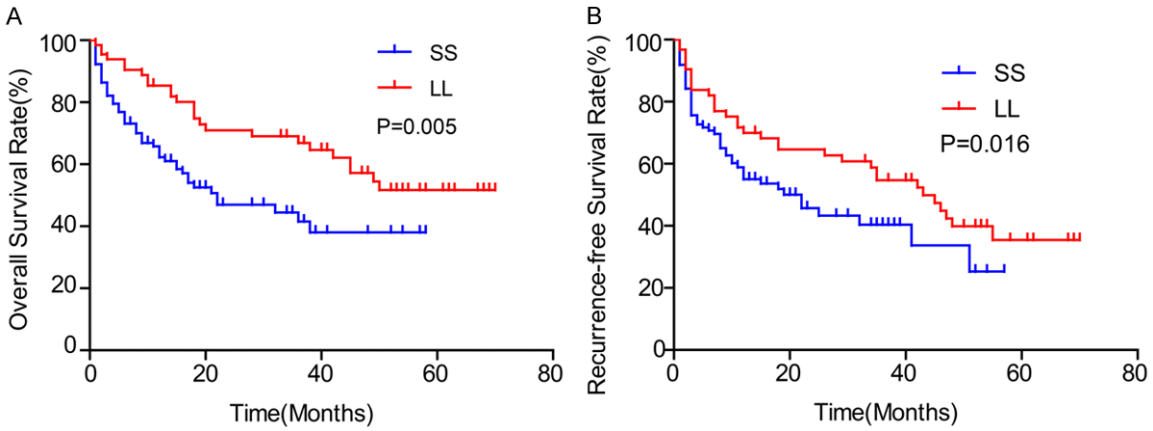

Figure 2. Prognosis of HCC according to CA repeated sequence. A. Overall survival. B. Recurrence free survival time.

Table 4. Multivariate analysis of different prognostic variables in overall survival of HCC by Cox proportional hazard model

Variables	Univariate analysis model			Multivariate analysis model		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Sex (male/female)	2.135	1.202-2.792	0.010	1.852	1.023-3.352	0.042
Age (<45/≥45)	1.050	0.581-1.897	0.871			
AFP (ng/ml) (≤400/>400)	1.739	1.052-2.875	0.031	0.895	0.395-2.026	0.790
Liver Cirrhosis (yes/no)	1.012	0.643-1.594	0.958	0.954	0.596-1.527	0.844
Tumor size (cm) (≤5/>5)	1.075	0.700-1.649	0.742	1.068	0.672-1.699	0.780
Multiplicity (single/multiple)	2.324	1.261-4.286	0.007	2.107	0.801-5.541	0.131
TNM stage (I-II/III-VI)	0.531	0.342-0.826	0.005	0.661	0.392-1.116	0.121
Differentiated degree						
High	1.000	Reference		1.000	Reference	
Middle	1.389	0.592-3.261	0.450	1.122	0.438-2.875	0.810
Low	2.687	1.101-6.556	0.030	1.632	0.546-4.881	0.381
HbsAg (negative/positive)	1.394	0.884-2.197	0.153			
rs1256049						
GG	1.000	Reference				
AG	1.362	0.851-2.179	0.198			
AA	1.414	0.686-2.912	0.348			
rs4986938						
GG	1.000	Reference				
AG	1.093	0.672-1.780	0.719			
AA	0.982	0.393-2.455	0.967			
CA repeat (LL/SS)	0.517	0.321-0.834	0.007	0.575	0.321-1.031	0.063

Univariate and multivariate analysis indicated that SS genotype was an independent risk factor for poor prognosis

To determine the association of LL genotype in prognosis patients with HCC, multivariate analysis was performed using Cox proportional-hazard regression. We found that LL genotype was a marginal protective factor for overall survival (HR, 0.575; 95% CI; 0.321-1.031, P=0.063) (**Table 4**) and an independent protective factor for recurrence-free survival (HR, 0.579; 95% CI, 0.346-0.971; P=0.038) (**Table 5**).

Discussions

Male-female ration of HCC cancer sufferers is 6.3:1 with the male covering much, while the

Table 5. Multivariate analysis of different prognostic variables in disease free survival of HCC by Cox proportional hazard model

Variables	Univariate analysis model			Multivariate analysis model		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Sex (male/female)	1.661	1.048-2.633	0.031	1.560	0.967-2.514	0.068
Age (<45/≥45)	1.001	0.589-1.701	0.998			
AFP (ng/ml) (≤400/>400)	1.538	1.01-2.344	0.045	0.835	0.400-1.746	0.633
Liver Cirrhosis (yes/no)	0.959	0.641-1.434	0.837			
Tumor size (cm) (≤5/>5)	1.176	0.808-1.710	0.397	1.260	0.837-1.896	0.268
Multiplicity (single/multiple)	1.867	1.149-3.035	0.012	1.529	0.657-3.561	0.325
TNM stage (I-II/III-VI)	0.601	0.407-0.887	0.010	0.652	0.412-1.033	0.068
Differentiated degree						
High	1.000	Reference		1.000	Reference	
Middle	1.129	0.560-2.277	0.734	1.009	0.46-2.211	0.983
Low	1.792	0.855-3.756	0.122	1.130	0.456-2.801	0.791
HbsAg (negative/positive)	1.188	0.787-1.795	0.412			
rs1256049						
GG	1.000	Reference		1.000	Reference	
AG	1.542	1.012-2.350	0.044	1.481	0.949-2.310	0.084
AA	1.985	1.095-3.597	0.024	2.119	1.143-3.930	0.017
rs4986938						
GG	1.000	Reference				
AG	1.012	0.658-1.558	0.956			
AA	0.847	0.368-1.947	0.696			
CA repeat (LL/SS)	0.612	0.405-0.925	0.020	0.579	0.346-0.971	0.038

ration of HCC sufferers with liver cirrhosis is 11:1 [21]. The relationship between HCC and sex hormone aroused people's attentions. Some clinical phenomenon such as long-term taking contraceptive drugs leading to HCC and male covering much suggested that relationship between sex hormone and HCC might exist. Researches indicated that estrogen receptor expressions of HCC patients were different from those with non-liver diseases [22]. Genotypes TTGG and TCGG of ESR1 polymorphisms were associated with high risk of postmenopausal osteoporosis [23]. There are some common target genes between ESR1 and ESR2, but their function were different in breast cancer [24]. Sundarrajan C suggested that the two common polymorphism sites of ESR2 genes: ligand binding area *RsaI* (-1082G/A, rs12560-49) of the fifth exon and 3'noncoding region *AluI* (-1730A/G, rs4986938) of the eighth exon were closely related with hormonal dependent diseases [20].

Our finding found that mutation of ESR2 gene has a significant effect on the incidence of HCC. The risk of HCC in patients carrying AA/GA genotype of rs1256049 was lower than those carried the GG genotype. In addition, 3'UTR A1730G genotype of ESR2 genes and allele frequency showed significantly differences between case and control groups and the risk of HCC for A allele was 2.154 times of that of G allele, indicating that A allele might be a risk factor for HCC. Rezende indicated the variants of rs4986938 modulated the severity of breast cancer, but didn't change the risk of sporadic mammary adenocarcinoma [25]. In this study, the mutations of rs4986938 not only had influence on the incidence, but also associated with the differentiated degree of HCC.

Short tandem repeat (here it appeared as CA repeated sequence), the second generation heredity marker, aroused attentions of many scholars. As for generation mechanism of nucleotide repeated sequence, it was generally held that gliding during DNA replication or a base mispairing of gliding strand and complementary strand during DNA duplication and reparation led to insertion or depletion of one or several repeating units [26]. We identifying 9 alleles (n=16~24) by analyzing the CA repeat-

ed sequence polymorphism of ESR2 genes. No significant differences were seen in distribution frequency of CA repeated sequence alleles in cases and control groups (P=0.520). But the data showed that SS allele frequency in case group was higher than control group, and the risk of suffering HCC for LL alleles was 0.536 times that of SS alleles (P=0.002), signifying that CA repeated sequence polymorphism of ESR2 genes was related to occurrence of HCC and SS type might increase its risk.

In addition, we found that ESR2 SNPs were significantly related to OS among HCC patients by univariate analysis. Moreover, we found the AA/GA genotype of rs1256049 was positively correlated with single tumor number, and AA/GA genotype of rs4986938 was associated with low differentiated degree in HCC. The LL genotype of CA repeat allele was associated with moderate-well differentiated degree and early stage of HCC. These indicate that AA/GA genotype of rs1256049 may be the risk factor of tumor malignancy, and AA/GA genotype of rs4986938 was just the opposite. This study demonstrates Baldissera's conjecture that ESR2 was associated with the risk of HCC [3]. Further investigation found overall survival and recurrence-free survival of patients with LL genotype is much higher than SS genotype. Univariate and multivariate analysis indicate that LL genotype was an independent protective factor for good prognosis.

In summary this research indicated SNPs in ESR2 gene (rs1256049, rs4986938) play a different role in HCC risk and SS genotype lead to poor prognosis of HCC patients underwent hepatectomy. It provided a potential reference for the diagnosis and treatment of HCC.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xiaoli Yang, Department of Hepatobiliary Surgery, The Second Affiliated Hospital of Chongqing Medical University, Linjiang Road 74#, Chongqing, China. Tel: +86-15086878251; Fax: +86 02363693532; E-mail: 344920646@qq.com

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