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Association between the c.1564A>T genetic polymorphism of the *MDR1* gene and hepatocellular carcinoma in Chinese population

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ABSTRACT. The objective of this study was to evaluate the influence of c.1564A>T genetic polymorphisms in the multidrug resistance 1 gene (*MDR1*) on hepatocellular carcinoma (HCC) susceptibility through association analysis. A total of 632 HCC patients and 645 cancer-free controls were enrolled in this study. The c.1564A>T genetic polymorphisms were genotyped by created restriction site-polymerase chain reaction (CRS-PCR) and confirmed using DNA sequencing methods. The potential associations of c.1564A>T genetic polymorphisms with the risk of HCC were analyzed by different genetic models. Statistically significantly increased risks of HCC were detected in the homozygote comparison (TT versus AA: OR = 1.70, 95%CI = 1.17-2.45, χ^2 = 7.99, P = 0.005), recessive model (TT versus AT/AA: OR = 1.64, 95%CI = 1.15-2.33, χ^2 = 7.66, P = 0.006), and allele contrast (T versus A: OR = 1.23, 95%CI = 1.04-1.45, χ^2 = 6.09, P = 0.014). Our data suggest that the genotypes/alleles from c.1564A>T genetic polymorphisms are

statistically associated with HCC risk. The allele-T and genotype TT may contribute to susceptibility to HCC in the Chinese Han population.

Key words: *MDR1* gene; Genetic polymorphisms; Susceptibility; Hepatocellular carcinoma; Risk factors

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant cancers and is the third leading cause of cancer-related deaths worldwide (Llovet et al., 2003; Parkin et al., 2005; Parikh and Hyman, 2007). More than 600,000 people die from HCC each year, and about >75% of these HCC cases occur in the Asia-Pacific region (But et al., 2008; Yuen et al., 2009). China has a very high HCC incidence, contributing to approximately 55% of all new annual cases of HCC in the world (Parkin et al., 2001, 2005; Schutte et al., 2009). HCC has been the second leading cause of cancer deaths in China since the 1990s (Chen et al., 2010). Previous studies reported that the multidrug resistance 1 gene (*MDR1*) is an important candidate gene influencing HCC (Wu et al., 2007; Vander Borght et al., 2008; Chaijaroenkul et al., 2011; Chen et al., 2009, 2011; Geng et al., 2011; Yu et al., 2011; Ren et al., 2012; Sun et al., 2012; Gao, 2013; Li et al., 2013; Yang et al., 2013). *MDR1* is a polygenetic gene and many genetic polymorphisms, such as G159T, T335C, C1236T, C1465T, G2677A/T, C3435T, A3073C, and A4125C, have been reported to have possible associations with the risk of HCC (Cavaco et al., 2003; Pechandova et al., 2006; Wu et al., 2007; Vander Borght et al., 2008; Chen et al., 2009, 2011; Yu et al., 2011; Ren et al., 2012; Gao, 2013; Yang et al., 2013). Several studies suggested that *MDR1* genetic polymorphisms influenced HCC risk (Wu et al., 2007; Chen et al., 2009, 2011; Ren et al., 2012; Gao, 2013; Yang et al., 2013). However, the potential association between the c.1564A>T genetic polymorphism and HCC risk was not analyzed. Thus, this study aimed to evaluate whether this genetic polymorphism could influence HCC risk.

MATERIAL AND METHODS

Study population

In this case-control study, 1277 subjects were enrolled from The Huai'an First People's Hospital (Nanjing Medical University, Huai'an, Jiangsu, China) between January 2009 to December 2012, including 632 HCC patients and 645 cancer-free controls. All subjects were unrelated Han Chinese and lived in Huai'an city, Jiangsu Province of China. All HCC patients involved in this study were pathologically confirmed. Clinical characteristics are shown in Table 1, including gender, age, tobacco smoking, alcohol drinking, hypertension, diabetes mellitus, hepatitis B virus (HBV) serological markers, serum alpha-fetoprotein (a-FP) levels, and family history of HCC. The control group was matched with the case group with regard to gender and age. Those with a history of HCC and other medical diseases were excluded. The local ethics committee approved this study and all subjects enrolled in this study gave written informed consent.

Table 1. Characteristics of hepatocellular carcinoma (HCC) in cases and controls.

Characteristics	Cases (N)	%	Controls (N)	%	χ^2 value	P
Number	632	49.49	645	50.51		
Gender (N)					2.62	0.106
Male	399	63.13	435	67.44		
Female	233	36.87	210	32.56		
Age (years)					2.41	0.120
Means ± SD	57.73 ± 13.22		58.66 ± 14.18			
< 55	382	60.44	417	64.65		
≥ 55	250	39.56	228	35.35		
Diabetes mellitus (N)					0.65	0.421
Yes	119	18.83	133	20.62		
No	513	81.17	512	79.38		
Hypertension (N)					0.19	0.663
Yes	103	16.30	111	17.21		
No	529	83.70	534	82.79		
Tobacco smoking					3.23	0.072
Yes	322	50.95	361	55.97		
No	310	49.05	284	44.03		
Alcohol drinking					2.60	0.107
Yes	354	56.01	390	60.47		
No	278	43.99	255	39.53		
HBV serological markers (N)						
HBs Ag (+)	142	22.47	-			
HBs Ag (-)	490	77.53	-			
Serum a-FP levels						
<400 ng/mL	211	33.39	-			
>400 ng/mL	421	66.61	-			
Family history of HCC (N)						
Yes	39	6.17	-			
No	593	93.83	-			

Genotyping

Peripheral blood was collected from each subject. Genomic DNA was isolated from blood using the standard protocol (Daly et al., 1996). The polymerase chain reaction (PCR) primers were designed through the Primer Premier 5.0 software (Premier Biosoft International; Palo Alto, CA, USA). The sequences of primers, amplification region, annealing temperature, and amplification fragment size, are shown in Table 2. One of the primers contained a nucleotide mismatch, which enables the use of restriction enzymes for discriminating sequence variations (Haliassos et al., 1989; Zhao et al., 2003; Yuan et al., 2012, 2013a,b). The c.1564A>T genetic variants of *MDR1* gene were genotyped by the created restriction site-PCR (CRS-PCR) method. To confirm the concordance of the CRS-PCR results, about 10% of the random samples were selected to verify the genetic variants by DNA sequencing method (ABI3730xl DNA Analyzer, Applied Biosystems, Foster City, CA, USA). PCR were carried out in a total volume of 20 µL solution, including 50 ng template DNA, 1X buffer (Tris-HCl 100 mM, pH 8.3; 500 mM KCl), 0.25 µM primers, 2.0 mM $MgCl_2$, 0.25 mM dNTPs (Takara; Dalian, China), and 0.5 U Taq DNA polymerase (Promega; Madison, WI, USA). PCR cycle conditions were as follows: initial denaturation at 94°C for 5 min, followed by 32 cycles at 94°C for 30 s, 60.7°C for 30 s, at 72°C for 30 s, and a final extension at 72°C for 5 min. PCR amplified products were digested with 5 units selected *Mae*III restriction enzyme (MBI Fermentas; St. Leon-Rot, Germany) at 37°C for 10 h, and then electrophoresed on 2.0% agarose gel containing ethidium-bromide and observed under UV light for analyzing the genotyping.

Table 2. Primers and CRS-PCR analysis for c.1564A>T genetic polymorphisms of the *MDR1* gene.

Primer sequences	Annealing temperature (°C)	Amplification fragment (bp)	Amplification region	Restriction enzyme	Genotype (bp)
5'-GGGTTTTCTGTGGTAGAAATTTGTC-3' 5'-AAATCAGGTTGGTTTGAACTAAGC-3'	60.7	220	Exon15	*Mae*III	AA:198,22 AT:220,198,22 TT:220

PCR = polymerase chain reaction; CRS-PCR = created restriction site-polymerase chain reaction. Underlined nucleotide marks nucleotide mismatches enabling the use of the selected restriction enzymes for discriminating sequence variations.

Statistical analysis

The Hardy-Weinberg equilibrium of genotype frequencies, and the differences of clinical characteristics between HCC patients and cancer-free controls were evaluated using the chi-squared (χ^2) test. The odds ratios (ORs) with 95% confidence intervals (CIs) of the correlation with allele/genotype frequencies of HCC risk were evaluated by unconditional logistic regression models. All statistical analyses were analyzed by the Statistical Package for Social Sciences software (SPSS, Windows version release 14.0; SPSS Inc.; Chicago, IL, USA). A P value < 0.05 was considered to be statistically significant.

RESULTS

General characteristics

A total of 632 HCC patients and 645 cancer-free controls were recruited in this study. Table 1 shows the clinical characteristics of subjects. No statistically significant difference between HCC patients and cancer-free controls were found in regards to gender and age (P = 0.106 and P = 0.120, respectively). Furthermore, there were no statistically significant differences in other clinical characteristics, for example, tobacco smoking, alcohol drinking, diabetes mellitus, and hypertension, between HCC patients and cancer-free controls (P = 0.072, P = 0.107, P = 0.421, and P = 0.663, respectively).

Genotyping of *MDR1* genetic polymorphism

We detected the c.1564A>T genetic polymorphism through the CRS-PCR method. The sequence analyses indicated that this genetic polymorphism was a non-synonymous mutation, which was caused by A to T mutations in exon15 of *MDR1*, leading to the threonine (Thr) to Serine (Ser) amino acid replacement (p.Thr522Ser). The amplified PCR products were digested with *Mae*III restriction enzyme and divided into 3 genotypes: AA (198 and 22 bp), AT (220, 198, and 22 bp), and TT (220 bp; Table 2). Table 3 shows the allele and genotype frequencies in HCC patients and cancer-free controls. The frequencies of allele-A and genotype-AA were predominant in the populations studied. The allele frequencies of HCC cases (A = 65.03%; T = 34.97%) were significantly different from those of cancer-free controls (A = 69.61%; T = 30.39%; χ^2 = 6.09, P = 0.014). The genotype frequencies in HCC cases (AA = 43.99%; AT = 42.09%; TT = 13.92%) were not consistent with cancer-free controls (AA =

48.22%; AT = 42.79%; TT = 8.99%), the differences being statistically significant (χ^2 = 8.07, P = 0.018). The distributions of genotype for this genetic polymorphism in the studied subjects did not significantly deviate from Hardy-Weinberg equilibrium (all P values > 0.05).

Table 3. Genotype and allele frequencies of c.1564A>T genetic polymorphisms in the *MDR1* gene in cases and controls.

Groups	Genotype frequencies (%)			Allele frequencies (%)		χ^2	P
	AA	AT	TT	A	T		
Cases (N = 632)	278 (43.99)	266 (42.09)	88 (13.92)	822 (65.03)	442 (34.97)	3.5162	0.1724
Controls (N = 645)	311 (48.22)	276 (42.79)	58 (8.99)	898 (69.61)	392 (30.39)	0.0843	0.9587
Total (N = 1277)	589 (46.12)	542 (42.44)	146 (11.44)	1720 (67.35)	834 (32.65)	1.5646	0.4573
		χ^2 = 8.07, P = 0.018		χ^2 = 6.09, P = 0.014			

Association between *MDR1* genetic polymorphism and HCC risk

Table 4 shows the association between *MDR1* genetic polymorphisms and the risk of HCC. We found a statistically significantly increased risk of HCC in the homozygote comparison (TT versus AA: OR = 1.70, 95% CI = 1.17-2.45, χ^2 = 7.99, P = 0.005), recessive model (TT versus AT/AA: OR = 1.64, 95% CI = 1.15-2.33, χ^2 = 7.66, P = 0.006) and allele contrast (T versus A: OR = 1.23, 95% CI = 1.04-1.45, χ^2 = 6.09, P = 0.014, Table 4). However, we failed to detect a statistically significantly increased risk of HCC in the heterozygote comparison (AT versus AA: OR = 1.08, 95% CI = 0.85-1.36, χ^2 = 0.40, P = 0.528) or the dominant model (TT/AT versus AA: OR = 1.19, 95% CI = 0.95-1.48, χ^2 = 2.30, P = 0.130) (Table 4).

Table 4. Association of c.1564A>T genetic polymorphisms in the *MDR1* gene with hepatocellular carcinoma (HCC) risk.

Comparisons	Test of association		
	OR (95%CI)	χ^2 value	P
TT *vs* AA (Homozygote comparison)	1.70 (1.17-2.45)	7.99	0.005
AT *vs* AA (Heterozygote comparison)	1.08 (0.85-1.36)	0.40	0.528
TT/AT *vs* AA (Dominant model)	1.19 (0.95-1.48)	2.30	0.130
TT *vs* AT/AA (Recessive model)	1.64 (1.15-2.33)	7.66	0.006
T *vs* A (Allele contrast)	1.23 (1.04-1.45)	6.09	0.014

OR = odds ratio; CI = confidence interval.

DISCUSSION

HCC is a complex and multi-factorial process, in which both environmental and genetic factors interfere and contribute to malignant solid cancers (Marrero et al., 2005; Farazi and DePinho, 2006; El-Serag and Rudolph, 2007; Amarapurkar et al., 2008; Yu et al., 2012). Emerging evidence suggests the genetic factors play key roles in HCC susceptibility (Thorgeirsson and Grisham, 2002; Nault and Zucman-Rossi, 2011; Bayram et al., 2012; Ning et al., 2012; Sumbul et al., 2012). In recent years, several studies reported that the *MDR1* gene is regarded as one of the most important candidate genes for HCC susceptibility, and the potential associations between *MDR1* genetic polymorphisms and HCC risk have been analyzed (Wu et al., 2007; Chen et al., 2009, 2011; Ren et al., 2012; Gao, 2013; Yang et al., 2013). However,

the findings from these observations remain conflicting rather than conclusive. In this study, we investigated the distribution of the c.1564A>T genetic polymorphism through the CRS-PCR method and evaluated the influence of this genetic polymorphism on HCC risk in the Chinese Han population by association analysis. Our data indicated statistically significant differences in allele and genotype frequencies between HCC cases and healthy controls (Table 3). The TT genotype was significantly associated with increased risk of HCC compared to the AA genotype and AT/AA carriers (Table 4). The allele T and genotype TT might contribute to HCC risk in the Chinese Han population. Our sequence analyses indicated that the c.1564A>T genetic polymorphism caused a p.Thr522Ser amino acid replacement. This amino acid replacement might affect the function of the *MDR1* protein, which is significantly associated with susceptibility to HCC. The findings from the present study might add more evidence to evaluate the influence of the *MDR1* gene on HCC risk.

In conclusion, this is the first report regarding the association of the c.1564A>T genetic polymorphism in the *MDR1* gene with the risk of HCC. Our findings support the use of *MDR1* genetic polymorphisms as molecular marker for evaluating susceptibility to HCC. Further studies will be needed to confirm these findings in large, different populations, and to help explain the molecular mechanisms.

Conflicts of interest

The authors declare no conflict of interests.

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