

RESEARCH ARTICLE

Association Between the c.3751G>A Genetic Variant of *MDR1* and Hepatocellular Carcinoma Risk in a Chinese Han Population

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Abstract

The objective of this study was to evaluate the influence of a genetic variant in the multidrug resistance 1 gene (*MDR1*) on hepatocellular carcinoma (HCC) risk. This case-control study was conducted in a Chinese population of 645 HCC cases and 658 cancer-free controls. The genotype of the c.3751G>A genetic variant in the *MDR1* gene was investigated by created restriction site-polymerase chain reaction (CRS-PCR) and DNA sequencing methods. Our data demonstrated significantly differences detected in the allelic and genotypic frequencies between HCC cases and those of cancer-free controls. Association analyses indicated that there were statistically increased risk of HCC in the homozygote comparison (AA versus (vs.) GG: OR = 2.22, 95% CI 1.51-3.27, $\chi^2 = 16.90$, $P < 0.001$), dominant model (AA/GA vs. GG: OR = 1.25, 95% CI 1.00-1.55, $\chi^2 = 3.98$, $P = 0.046$), recessive model (AA vs. GA/GG: OR = 2.14, 95% CI 1.47-3.09, $\chi^2 = 16.68$, $P < 0.001$) and allele comparison (A vs. G: OR = 1.33, 95% CI 1.13-1.57, $\chi^2 = 11.66$, $P = 0.001$). The allele-A and genotype-AA may contribute to HCC susceptibility. These preliminary findings suggest that the c.3751G>A genetic variant in the *MDR1* gene is potentially related to HCC susceptibility in a Chinese Han population, and might be used as a molecular marker for evaluating HCC susceptibility.

Keywords: Hepatocellular carcinoma - *MDR1* gene - genetic variant - susceptibility - molecular biomarker

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer globally and the third cause of cancer-related deaths worldwide (Parkin et al., 2001; Llovet et al., 2003; Parkin et al., 2005; Parikh et al., 2007). It represents a major and constantly rising health burden throughout the world (Li et al., 2012). Approximately 650,000 people die from HCC each year, and > 75% of these cases occur in the Asia-Pacific region (Parkin et al., 2005; Yuen et al., 2009; Zhang et al., 2012). It is indicated that China has a very high incidence with about 55% of annual new cases of HCC worldwide (Parkin et al., 2005; Schutte et al., 2009; Zhang et al., 2012). HCC has been one of the most common causes of cancer-related deaths in China (Chen et al., 2010; Zeng et al., 2012). The pathogenesis of HCC was related to comorbidities such as chronic hepatitis B (HBV) or hepatitis C (HCV) viral infections, cigarette smoking, alcohol consumption, and diabetes mellitus, environmental factors, and genetic factors (Thorsteirsson et al., 2002; Wang et al., 2003; Bosch et al., 2004; Suriawinata et al., 2004; Marrero et al., 2005; Farazi et al., 2006; Gomaa et al., 2008; Yuen et al., 2009; Nault et

al., 2011; Zhang et al., 2012). It is generally accepted that the genetic factors play key roles in hepatocarcinogenesis (Thorsteirsson et al., 2002; Nault et al., 2011). Nowadays, the exact mechanism of hepatocarcinogenesis still remains incompletely understood. Previous studies indicated that the multidrug resistance 1 gene (*MDR1*) is one of the most important candidate genes which have potentially influence on HCC risk (Wu et al., 2007; Vander Borghet et al., 2008; Chen et al., 2009; Chen et al., 2011; Yu et al., 2011; Ren et al., 2012; Gao, 2013; Yang et al., 2013). *MDR1* is a polymorphic gene and several genetic variants, such as G159T, T335C, C1236T, C1465T, G2677A/T, C3435T, A3073C, and A4125C, have been reported to have potential association with the risk of HCC (Cavaco et al., 2003; Pechandova et al., 2006; Wu et al., 2007; Vander Borghet et al., 2008; Chen et al., 2009; Chen et al., 2011; Yu et al., 2011; Ren et al., 2012; Gao, 2013; Yang et al., 2013). Among of these studies, several *MDR1* genetic polymorphisms have been approved to play an important genetic affection on HCC risk (Wu et al., 2007; Chen et al., 2009; Chen et al., 2011; Ren et al., 2012; Gao, 2013; Yang et al., 2013). Up to now, there are no similar studies which reported the association between c.3751G>A

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genetic variant of MDR1 gene and HCC risk. Therefore, considering the importance of MDR1 genetic variants for influencing HCC risk, in this case-control study, we aimed to investigate the distribution of c.3751G>A genetic variant of MDR1 gene in Chinese Han population and evaluate whether this genetic variant could influence on the risk of HCC.

Materials and Methods

Study subjects

The subjects in this case-control study were unrelated Chinese Han nationality, including 645 HCC patients and 658 cancer-free controls from the Yiwu Central Hospital (Zhejiang, China) between March 2009 to November 2012. All HCC patients recruited in this study were diagnosed by doctors and histologically confirmed according to the standards established by Chinese Society of Liver Cancer (CSLC). Clinical characteristics, including gender, age, tobacco smoking, alcohol drinking, diabetes mellitus, hypertension, serum a-FP levels, HBV serological markers, family history of HCC, are summarized in Table 1. The controls were selected from health screening program participants and matched with cases by age and gender. Those with medical history of surgery, cancer and other diseases were excluded. The protocol of this study was approved by the Ethics Committee of the Yiwu Central Hospital and informed consents from all subjects were obtained.

PCR amplification

The peripheral venous blood was collected from each participant. Genomic DNA was extracted using the Axygen DNA isolation kit (Axygen, CA). The specific polymerase chain reaction (PCR) primers were designed by Primer Premier 5.0 software (PREMIER Biosoft International, Palo Alto, CA). Table 2 shows the primers sequences, annealing temperature, fragment size and region. The PCR reactions were carried out in a total volume of 20 μ L solution containing 50ng template DNA, 1 \times buffer (Tris-HCl 100 mmol/L, pH 8.3; KCl 500 mmol/L), 0.25 μ mol/L primers, 2.0 mmol/L MgCl₂, 0.25 mmol/L dNTPs, and 0.5U Taq DNA polymerase (Promega, Madison, WI, USA). The PCR conditions were followed as 94°C for 5 minutes, followed by 32 cycles of 94°C for 30 seconds, 58.2°C for 30 seconds, and 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. The PCR amplified products were separated by 2.0% agarose gel electrophoresis containing ethidium bromide and observed under UV light.

Genotyping

The genotyping of c.3751G>A genetic variant of MDR1 gene was determined by created restriction site-PCR (CRS-PCR) method with one of the primers containing 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; Yuan et al., 2013; Yuan et al., 2013). Aliquots of 5 μ L PCR amplified products were digested with 2U selected restriction enzyme (MBI Fermentas, St.

Leon-Rot, Germany, shown in Table 2) at 37°C for 10 hours following with the supplier's manual. The digested products were separated by electrophoresis in 2.5% agarose gel containing ethidium bromide staining and observed under UV light to indentify the genotyping of c.3751G>A genetic variant of MDR1 gene. To confirm the concordance of CRS-PCR genotyping results, about 10% of random samples which showing different genotyping of c.3751G>A genetic variant were selected to analyze by DNA sequencing method at TaKaRa Biotechnology Co., Ltd (Dalian, China).

Statistical analyses

All statistical analyses were analyzed using the Statistical Package for Social Sciences software (SPSS, Windows version release 15.0; SPSS Inc.; Chicago, IL, USA). The chi-squared (χ^2) test was used to assess the Hardy-Weinberg equilibrium (HWE) in allele and genotype frequencies, and to compare the differences of general characteristics between cases and controls. The strength of associations between allele/genotype of genetic variant in MDR1 gene and HCC risk were evaluated by the odds ratios (ORs) with 95% confidence intervals (95% CIs) under unconditional logistic regression. P-values less than 0.05 were defined as statistically significant level.

Results

General characteristics of the subjects

A total of 1303 Chinese Han subjects were enrolled in this case-control study, which consisting of 645 HCC cases and 658 cancer-free controls. Table 1 shows the general characteristics of the studied subjects. The χ^2 test indicated

Table 1. Clinical Characteristics of Hepatocellular Carcinoma (HCC) Cases and Healthy Controls

Characteristics	Cases (n)	%	Controls (n)	%	χ^2 -value	P-value
Number	645	49.5	658	50.5		
Gender (n)					2.5663	0.1092
Male	409	63.41	445	67.63		
Female	236	36.59	213	32.37		
Age (years)					2.5456	0.1106
Mean \pm SD	58.59 \pm 14.45		59.13 \pm 13.52			
< 55	391	60.62	427	64.89		
\geq 55	254	39.38	231	35.11		
Alcohol drinking					2.9171	0.0876
Yes	378	58.6	416	63.22		
No	267	41.4	242	36.78		
Tobacco smoking					2.9651	0.0851
Yes	333	51.63	371	56.38		
No	312	48.37	287	43.62		
Diabetes mellitus (n)					0.9846	0.3211
Yes	123	19.07	140	21.28		
No	522	80.93	518	78.72		
Hypertension (n)					2.3078	0.1287
Yes	112	17.36	136	20.67		
No	533	82.64	522	79.33		
Serum a-FP levels						
< 400 ng/ml	202	31.32	-	-		
> 400 ng/ml	443	68.68	-	-		
HBV serological markers (n)						
HBs Ag (+)	159	24.65	-	-		
HBs Ag (-)	486	75.35	-	-		
Family history of HCC (n)						
Yes	55	8.53	-	-		
No	590	91.47	-	-		

Table 2. The PCR and CRS-PCR Analysis for c.3751G>A Genetic Variant of MDR1 Gene

Primer sequences	Annealing temperature (°C)	Amplification fragment (bp)	Amplification region	Restriction enzyme	Genotype (bp)
5'-GTGGTGTTCAGAAATGGCTGA-3' 5'-TCTTCATAATTCTGTAAGTGTTC-3'	58.2	209	Exon29	DdeI	GG:191,18 GA:209, 191,18 AA:209

PCR, polymerase chain reaction; CRS-PCR, created restriction site-PCR; Underlined nucleotides mark nucleotide mismatches enabling the use of the selected restriction enzymes for discriminating sequence variations

Table 3. The Genotypic and Allelic Frequencies of c.3751G>A Genetic Variant of MDR1 Gene in Hepatocellular Carcinoma (HCC) Cases and Healthy Controls

Groups	Genotypic frequencies (%)			Allelic frequencies (%)		χ^2	P
	GG	GA	AA	G	A		
Cases (n = 645)	283(43.87)	271(42.02)	91(14.11)	837(64.88)	453(35.12)	3.9232	0.1406
Controls (n = 658)	325(49.39)	286(43.47)	47(7.14)	936(71.12)	380(28.88)	2.2278	0.3283
Total (n = 1303)	608(46.66)	557(42.75)	138(10.59)	1773(68.04)	833(31.96)	0.3844	0.8251
	$\chi^2 = 17.2063, P = 0.0002$			$\chi^2 = 11.6670, P = 0.0006$			

Table 4. The Association of c.3751G>A Genetic Variant of MDR1 Gene with Hepatocellular Carcinoma (HCC) Risk

Comparisons	Test of association		
	OR (95% CI)	χ^2 -value	P-value
Homozygote comparison (AA vs. GG)	2.22(1.51-3.27)	16.9	<0.001
Heterozygote comparison (GA vs. GG)	1.09(0.86-1.37)	0.52	0.472
Dominant model (AA/GA vs. GG)	1.25(1.00-1.55)	3.98	0.046
Recessive model (AA vs. GA/GG)	2.14(1.47-3.09)	16.68	<0.001
Allele contrast (A vs. G)	1.33(1.13-1.57)	11.66	0.001

OR, odds ratio; CI, confidence interval; vs., versus

that there were no significant differences between HCC cases and cancer-free controls with regard to gender and age distribution. Besides, for other general characteristics, such as alcohol drinking, tobacco smoking, diabetes mellitus and hypertension, there were no significant differences between HCC cases and cancer-free controls ($P > 0.05$, Table 1).

Genotyping of XRCC1 genetic variant

The genotyping of c.3751G>A genetic variant of MDR1 gene was detected through CRS-PCR and DNA sequencing methods. Based on the results of DNA sequence analysis, the c.3751G>A genetic variant is a non-synonymous mutation, which caused by G to A mutations in exon29 of human MDR1 gene and led to the valine (Val) to isoleucine (Ile) amino acid replacement (p.Val1251Ile, Reference sequences GenBank IDs: NG_011513.1, NM_000927.4 and NP_000918.2). The PCR amplified products was digested with DdeI restriction enzyme. Three different genotypes were found, GG (191 and 18 bp), GA (209, 191 and 18 bp) and AA (209 bp, Table 2).

Allelic and genotypic frequencies

The allelic and genotypic frequencies of c.3751G>A genetic variant site were corresponded to HWE in HCC cases and cancer-free controls (all P -values > 0.05 , Table 3). As shown in Table 3, the allele-G and genotype-GG were predominant in the studied subjects. There were significant differences between the allelic frequencies of HCC cases (G, 64.88%; A, 35.12%) and those of cancer-

free controls (G, 71.12%; A, 28.88%, $\chi^2 = 11.6670, P = 0.0006$). Besides, we also detected that the genotypic frequencies in HCC cases (GG, 43.87%, GA, 42.02%, AA, 14.11%) were statistically significant different from those of healthy controls (GG, 49.39%, GA, 43.47%, AA, 7.14%, $\chi^2 = 17.2063, P = 0.0002$, Table 3).

Association between MDR1 gene and HCC risk

The potential association between the c.3751G>A genetic variant of MDR1 gene and HCC risk are shown in Table 4. There were statistically increased risk of HCC in the homozygote comparison (AA versus (vs.) GG: OR = 2.22, 95% CI 1.51-3.27, $\chi^2 = 16.90, P < 0.001$), dominant model (AA/GA vs. GG: OR = 1.25, 95% CI 1.00-1.55, $\chi^2 = 3.98, P = 0.046$), recessive model (AA vs. GA/GG: OR = 2.14, 95% CI 1.47-3.09, $\chi^2 = 16.68, P < 0.001$) and allele comparison (A vs. G: OR = 1.33, 95% CI 1.13-1.57, $\chi^2 = 11.66, P = 0.001$).

Discussion

In recent years, emerging evidence suggests that HCC is a global health problem and multi-factorial malignant solid cancers resulting from complex interactions between environmental and genetic factors (Marrero et al., 2005; Farazi et al., 2006; El-Serag et al., 2007; Amarpurkar et al., 2008; Yu et al., 2012). Previous studies have approved that genetic factors play key roles in the susceptibility to HCC (Thorgeirsson et al., 2002; Nault et al., 2011; Bayram et al., 2012; Ning et al., 2012; Sumbul et al., 2012). It has been reported that the MDR1 gene is regarded as one of the most important candidate genes for influencing on the susceptibility to HCC. The potential associations between several MDR1 genetic polymorphisms and HCC susceptibility have been assessed (Wu et al., 2007; Chen et al., 2009; Chen et al., 2011; Ren et al., 2012; Gao, 2013; Yang et al., 2013). Wu and his colleagues reported that the MDR1 gene polymorphisms (such as C1236T, G2677A/T, and C3435T) could be valuable molecular markers for HCC recurrence after liver transplantation (Wu et al., 2007). Chen et al., (2009) demonstrated that the MDR1 gene G2677T/A polymorphisms were associated

with HCC risk, and the 2677A could be a protective allele of HCC. The risk of suffering HCC was decreased significantly in individuals carrying at least one allele of A ($P < 0.05$) (Chen et al., 2009). Chen et al., (2011) detected that the C3435T polymorphism was significant associated with the prognosis of HCC, and no significant association was detected in the C1236T polymorphism. Therefore, Chen et al., indicated that the C3435T polymorphism may be a positive candidate molecular marker for the prognosis of HCC (Chen et al., 2011). Yang et al. (2013) found that the c.1465C>T polymorphism may contribute to the risk of HCC, while no significantly increased HCC risk was detected in c.159G>T polymorphism (Yang et al., 2013). However, results from these observations remain conflicting rather than conclusive. In the present study, we detected the distribution of c.3751G>A genetic variant of MDR1 gene through the CRS-PCR and DNA sequencing methods and evaluated whether this genetic variant could influence on the risk of HCC in Chinese Han population by association analysis. Our data suggested that the statistical significant difference were shown in the allelic and genotypic frequencies between HCC patients and cancer-free controls (all P -values < 0.01 , Table 3). The AA genotype was significantly associated with the increased risk of HCC compared to GG genotype and GA/AA carriers (all P -values < 0.001 , Table 4). The allele-A and genotype-AA could contribute to increase the risk of HCC in Chinese Han population. Sequence analyses indicate that this genetic variant results into the p.Val1251Ile amino acid replacement. It could affect the function of MDR1 protein, which significantly associated with the susceptibility to HCC. To the best of our knowledge, this is the first report about the potential association between c.3751G>A genetic variant of MDR1 gene and the susceptibility to HCC. Our data indicate that the c.3751G>A genetic variant is significantly associated with the increased susceptibility to HCC. Our findings support that the MDR1 genetic variants may be used as molecular markers for assessing the susceptibility to HCC. Results from this study provide more evidence to evaluate the biological function role of MDR1 gene on HCC susceptibility. Further prospective studies on large and different ethnic populations will be necessary to confirm these findings and elucidate the underlying molecular mechanism for the development of HCC.

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The author(s) declare that they have no competing interests.

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