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Association analysis between genetic variants of *MDM2* gene and osteosarcoma susceptibility in Chinese

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Abstract. Osteosarcoma (OS) is the most common pediatric bone malignancy worldwide. The *MDM2* gene is an important candidate gene for influencing the susceptibility to OS. The objective of this study aimed to detect the potential association between *MDM2* genetic variants and OS susceptibility in Chinese Han population. We recruited 415 OS patients and 431 cancer-free controls in this case-control study. The c.44C>T and c.1002T>C genetic variants in *MDM2* gene were investigated using created restriction site-polymerase chain reaction (CRS-PCR) and PCR-restriction fragment length polymorphism (PCR-RFLP), respectively. We found that the genotypes/alleles of c.44C>T and c.1002T>C were statistically associated with the increased risk of OS (for c.44C>T, TT versus (vs.) CC: OR = 2.43, 95% CI 1.49-3.95, $p < 0.001$; T vs. C: OR = 1.36, 95% CI 1.11-1.67, $p = 0.003$; for c.1002T>C, CC vs. TT: OR = 2.38, 95% CI 1.37-4.13, $p = 0.002$; C vs. T: OR = 1.27, 95% CI 1.02-1.56, $p = 0.030$). The T allele and TT genotype of c.44C>T and C allele and CC genotype of c.1002T>C could be increased risk factors for the susceptibility to OS. Results from this study suggest that *MDM2* genetic variants are potentially related to OS susceptibility in Chinese Han population, and might be used as molecular markers for assessing OS susceptibility.

Key words: Osteosarcoma, *MDM2* gene, Genetic variants, Molecular markers, Susceptibility

OSTEOSARCOMA (OS) is the most common pediatric bone malignancy that occurs primarily in adolescents and young adults in the world [1-5]. The exact mechanism of development of OS carcinogenesis still remains poorly understood. Patients with localized OS at presentation have a 60–80% rate of long-term survival, while metastatic disease carries a poorer prognosis [6, 7]. It is estimated that the annual incidence rate for individuals less than 20 years old is 8.7 per million children, and account for ~6% of childhood cancer [8, 9]. Several studies suggest that multiple factors such as genetic mutations and environment factors could affect the OS pathogenesis. However, the genetic factors could play key roles in the pathogenesis of OS [9-15]. There are several studies indicate that the *MDM2* gene is an important candidate

gene for influencing the susceptibility to OS [16-21]. The single nucleotide polymorphisms (SNPs) in *MDM2* gene (such as rs2279744 (T309G) and rs1690916) have been analyzed the potential association with OS susceptibility and might have influences on the development of OS [16-19]. However, the association of *MDM2* genetic variants with OS occurrence is not well understood. Up to now, the potential association of c.44C>T and c.1002T>C genetic variants in *MDM2* gene with the susceptibility to OS have not been reported. Thus, the objective of this study aimed to investigate the distribution of *MDM2* c.44C>T and c.1002T>C genetic variants and to assess whether these genetic variants influence on OS susceptibility in Chinese Han population.

Materials and Methods

Study population

A total of 415 diagnosed OS patients (aged 10–68 years old) and 431 healthy subjects (aged 12-67 years old) who had no history of OS as controls were recruited

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from the Second Xiangya Hospital Affiliated with Central South University between January 2004 and December 2012. All subjects were unrelated Chinese with Han nationality and lived in Changsha city of China. The cancer-free controls were matched with OS patients with regards to gender, age and residence area (urban or rural). Table 1 shows the general characteristics, including gender, sex, tumor location, and metastasis. The written informed consent was obtained from each participant. The protocol of this study was approved by the Ethics Committee of Second Xiangya Hospital Affiliated with Central South University.

DNA extraction and PCR amplification

Genomic DNA was extracted from peripheral venous blood using the blood genomic DNA miniprep kit

(Axygen Biosciences) according to the manufacturer's instructions. Based on the mRNA sequences (GenBank ID: NM_002392.4) and DNA sequences (GenBank ID: NG_016708.1) of the human *MDM2* gene, two pair of primers were designed by the Primer Premier 5.0 software (PREMIER Biosoft International, Palo Alto, CA). Table 2 shows the primers sequences, annealing temperature, fragment sizes and region. The polymerase chain reaction (PCR) reaction was performed 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 cycle conditions were as followed: an initial 94°C for 5 minutes, then followed by 34 cycles of 94°C for

Table 1 General characteristics of the subjects

Characteristics	Osteosarcoma cases (n)	%	Cancer-free controls (n)	%	χ^2 -value	<i>p</i> -value
Number	415	49.05	431	50.95		
Age (years)					2.3954	0.1217
< 20	316	76.14	308	71.46		
$\geq$ 20	99	23.86	123	28.54		
Mean $\pm$ SD	27.15 $\pm$ 16.53		29.26 $\pm$ 18.66			
Gender (n)					2.6718	0.1021
Male	237	57.11	222	51.51		
Female	178	42.89	209	48.49		
Tumor location						
Long tubular bones	325	78.31				
Axial skeleton	90	21.69				
Metastasis						
Yes	111	26.75				
No	304	73.25				

Table 2 The primers, CRS-PCR and PCR-RFLP analysis for *MDM2* genetic variants

SNPs	Primer sequences	Amplification fragment (bp)	Region	Annealing temperature (°C)	Genotype method	Restriction enzyme	Genotype (bp)
c.44C>T	5'-TGTGCAATACCAACATGTCTGGAC-3' 5'-GCCAGAGGTAGCACACTTTAAGCT-3'	218	Exon2	62.5	CRS-PCR	<i>Ava</i> II	CC:218 CT:218,197,21 TT:197,21
c.1002T>C	5'-GCAAAGTTGCTAGCATTCTGTG-3' 5'-CCCTCTTCAGCTTGTGTTGAGTT-3'	243	Exon11	62.0	PCR-RFLP	<i>Tsp</i> EI	TT:243 TC:243,157,86 CC:157,86

Note: SNPs, single nucleotide polymorphisms; PCR, polymerase chain reaction; CRS-PCR, created restriction site-PCR; PCR-RFLP, PCR-restriction fragment length polymorphism; Underlined nucleotides mark nucleotide mismatches enabling the use of the selected restriction enzymes for discriminating sequence variations.

30 seconds, annealing at the corresponding temperature (given in Table 2) for 30 seconds and 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. The PCR products were separated by electrophoresis in 2.0% agarose gel containing ethidium-bromide and observed under UV light.

Genotyping of *MDM2* genetic variants

The c.44C>T genetic variant in *MDM2* gene was genotyped by the 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 [22-26]. The genotyping of c.1002T>C genetic variant in *MDM2* gene was detected by the PCR-restriction fragment length polymorphism (PCR-RFLP) method. According to the supplier's manual, aliquots of 5 µL PCR products were digested with 2U selected restriction enzyme (MBI Fermentas, St. Leon-Rot, Germany, shown in Table 2) at 37°C for 10 hours. The digested products were separated by 2.5% agarose gel electrophoresis containing ethidium-bromide and observed under UV light. To confirm the genotyping results from CRS-PCR and PCR-RFLP methods, 15% of random samples were re-analyzed by DNA sequencing method (ABI3730xl DNA Analyzer, Applied Biosystems, Foster City, CA).

Statistical analyses

The chi-squared (χ^2) test was utilized to assess the Hardy-Weinberg equilibrium (HWE) in allele and genotype distribution, and general characteristics between OS patients and cancer-free controls. The odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated by unconditional logistic regression and utilized to evaluate the potential associations between *MDM2* genetic variants and OS risk. All statistical analyses were analyzed by the Statistical Package for Social Sciences software (SPSS, Windows version release 15.0; SPSS Inc.; Chicago, IL, USA). *P*-values less than 0.05 were considered as significant level.

Results

General characteristics of subjects

In this case-control study, we enrolled 846 Chinese Han subjects. Table 1 shows the general characteristics of OS patients and cancer-free controls. Our data indicate that there are no significant differences between OS patients and cancer-free controls with regard to

gender, sex, tumor location and metastasis.

Identification of *MDM2* genetic variants

Two *MDM2* genetic variants (c.44C>T and c.1002T>C) were confirmed through CRS-PCR, PCR-RFLP and DNA sequencing methods. As for c.44C>T, DNA sequence analyses indicate that this genetic variant is a non-synonymous mutation, which causes by C to T mutations in exon2 of the human *MDM2* gene. This mutation has resulted into the proline (Pro) to leucine (Leu) amino acid replacement (p.Pro15Leu, reference sequences GenBank IDs: NG_016708.1, NM_002392.4 and NP_002383.2). The *AvaII* restriction enzyme was selected to digest the PCR amplified products of c.44C>T, and three genotypes were detected consisted of CC genotype (218 bp), CT genotype (218,197 and 21bp) and TT genotype (197 and 21bp, Table 2). As for c.1002T>C, DNA sequence analyses suggest that this genetic variant is a synonymous mutation, which causes by T to C mutations in exon11 of the human *MDM2* gene (p. asparagine (Asn) 334Asn, reference sequences GenBank IDs: NG_016708.1, NM_002392.4 and NP_002383.2). The PCR amplified products of c.1002T>C were digested by *TspEI* restriction enzyme and three genotypes were found, TT genotype (243 bp), TC genotype (243,157 and 86 bp) and CC genotype (157 and 86 bp, Table 2).

Genotype and allele frequencies

The genotype and allele frequencies of c.44C>T and c.1002T>C genetic variants in *MDM2* gene are performed in Table 3. The C allele and CC genotype frequencies of c.44C>T and T allele and TT genotype frequencies of c.1002T>C were maximums in OS patients and cancer-free controls. As for c.44C>T, we found significant differences between the allele frequencies of OS patients (C, 64.34%; T, 35.66%) and those of cancer-free controls (C, 71.00%; T, 29.00%, $\chi^2 = 8.5822$, $p = 0.0034$), and the genotype frequencies in OS patients were statistically significant different from those of cancer-free controls ($\chi^2 = 13.6661$, $p = 0.0011$, Table 3). As for c.1002T>C, the allele frequencies of OS patients (T, 69.64%; C, 30.36%) were statistically significantly different from healthy controls (T, 74.36%; C, 25.64%, $\chi^2 = 4.6841$, $p = 0.0304$). The genotype frequencies of OS patients were not consistent with cancer-free controls, the differences being statistically significant ($\chi^2 = 10.4809$, $p = 0.0053$, Table 3). The observed genotype frequencies of c.44C>T and c.1002T>C genetic variants

were corresponded to HWE in OS patients and cancer-free controls (All p -values > 0.05 , Table 3).

Association between *MDM2* genetic variants and osteosarcoma risk

Table 4 shows the potential association between *MDM2* genetic variants and OS risk. As for c.44C>T, we found statistically significantly increased risk of OS in the homozygote comparison (TT *versus* (vs.) CC: OR = 2.43, 95% CI 1.49-3.95, $\chi^2 = 13.24$, $p < 0.001$), recessive model (TT vs. CT/CC: OR = 2.34, 95% CI 1.47-3.73, $\chi^2 = 13.40$, $p < 0.001$) and allele comparison (T vs. C: OR = 1.36, 95% CI 1.11-1.67, $\chi^2 = 8.58$, $p = 0.003$). As for c.1002T>C, statistically increased risk of OS were detected in the homozygote comparison (CC vs. TT: OR = 2.38, 95% CI 1.37-4.13, $\chi^2 = 9.91$, $p = 0.002$), recessive model (CC vs. TC/TT: OR = 2.38, 95% CI 1.39-4.06, $\chi^2 = 10.47$, $p = 0.001$) and allele comparison (C vs. T: OR = 1.27, 95% CI 1.02-1.56, $\chi^2 = 4.68$, $p = 0.030$).

Discussion

OS is a primary bone tumor and causes from complex interactions between environmental factors and genetic factors. Recently, there are several genetic variants in *MDM2* gene have been reported the potential association with OS susceptibility, most of these studies were focused on the rs2279744 (T309G) and rs1690916 genetic variants [16-19]. Toffoli *et al.*, demonstrated that the *MDM2* T309G polymorphism was associated with an increased risk of developing osteosarcomas (GG vs. TT: OR = 2.09, 95% CI 1.15-3.78). It provides evidence supporting the association of *MDM2* SNP309 T>G with high-grade osteosarcoma risk in females (GG vs. TT: OR = 4.26; 95% CI 1.61-11.25) [16]. Mirabello *et al.*, observed that *MDM2* rs1690916 polymorphism was significantly associated with a decreased risk of OS after correction (OR = 0.62, 95% CI 0.45-0.85, p -adjust = 0.026). Three intronic SNPs were significantly associated with an increased risk of OS before correction (ORs = 1.6-1.8,

Table 3 The genotype and allele frequencies of *MDM2* genetic variants in the osteosarcoma (OS) cases and cancer-free controls

Groups	c.44C>T						c.1002T>C					
	Genotype frequencies (%)			Allele frequencies (%)			Genotype frequencies (%)			Allele frequencies (%)		
	CC	CT	TT	C	T	χ^2 -value p -value	TT	TC	CC	T	C	χ^2 -value p -value
Cases (n = 415)	179 (43.13)	176 (42.41)	60 (14.46)	534 (64.34)	296 (35.66)	2.3855 0.3034	208 (50.12)	162 (39.04)	45 (10.84)	578 (69.64)	252 (30.36)	2.4520 0.2935
Controls (n = 431)	210 (48.72)	192 (44.55)	29 (6.73)	612 (71.00)	250 (29.00)	2.8787 0.2371	231 (53.60)	179 (41.53)	21 (4.87)	641 (74.36)	221 (25.64)	3.4298 0.1800
Total (n = 846)	389 (45.98)	368 (43.50)	89 (10.52)	1146 (67.73)	546 (32.27)	0.0202 0.9899	439 (51.89)	341 (40.31)	66 (7.80)	1219 (72.04)	473 (27.96)	0.0004 0.9998
	$\chi^2 = 13.6661$, $p = 0.0011$			$\chi^2 = 8.5822$, $p = 0.0034$			$\chi^2 = 10.4809$, $p = 0.0053$			$\chi^2 = 4.6841$, $p = 0.0304$		

The chi-squared (χ^2) test was utilized to assess the Hardy-Weinberg equilibrium (HWE) in allele and genotype distribution, and general characteristics between OS patients and cancer-free controls.

Table 4 The association between osteosarcoma (OS) risk and *MDM2* genetic variants

SNPs	Comparisons	Test of association		
		OR (95% CI)	χ^2 -value	p -value
c.44C>T	Homozygote comparison (TT vs. CC)	2.43(1.49-3.95)	13.24	< 0.001
	Heterozygote comparison (CT vs. CC)	1.08(0.89-1.43)	0.25	0.618
	Dominant model (TT/CT vs. CC)	1.25(0.96-1.64)	2.66	0.103
	Recessive model (TT vs. CT/CC)	2.34(1.47-3.73)	13.40	< 0.001
	Allele contrast (T vs. C)	1.36(1.11-1.67)	8.58	0.003
c.1002T>C	Homozygote comparison (CC vs. TT)	2.38(1.37-4.13)	9.91	0.002
	Heterozygote comparison (TC vs. TT)	1.01(0.76-1.33)	0.00	0.972
	Dominant model (CC/TC vs. TT)	1.15(0.88-1.51)	1.02	0.312
	Recessive model (CC vs. TC/TT)	2.38(1.39-4.06)	10.47	0.001
	Allele contrast (C vs. T)	1.27(1.02-1.56)	4.68	0.030

SNPs, single nucleotide polymorphisms; OR, odds ratio; CI, confidence interval; vs., *versus*.

$p = 0.008-0.02$). The *MDM2* T309G polymorphism was marginally non-significantly associated with an increased risk of OS (OR = 1.31, 95% CI 0.97-1.77, $p = 0.07$). Mirabello and his colleagues indicated that *MDM2* gene was found to be significantly associated with OS ($p = 0.016$), but not after correction for multiple tests [17]. Naumov *et al.*, reported that the allele A of *MDM2* rs1690916 polymorphism decreased the disease risk and it could be regarded as protective factor (OR = 0.39, 95% CI 0.19-0.78). These findings confirmed that the *MDM2* gene was involved in bone tumor development [19]. Cai conducted a meta-analysis to systematically examine the association between these functional SNPs and sarcoma risk. Compared to TT genotype, carriers of TG and GG genotypes showed a 34% increased risk to develop sarcomas. Results from this meta-analysis indicate that the functional *MDM2* T309G genetic variant may play a more important role in carcinogenesis of sarcoma including OS [18]. In the present study, we demonstrate that the c.44C>T and c.1002T>C genetic variants in *MDM2* gene are statistically significant correlated with the susceptibility to OS through association analysis in 415 OS patients and 431 cancer-free controls. We found significant differences in allele and genotype frequencies between OS patients and cancer-free controls (All p -values < 0.05,

Table 3). As for c.44C>T, comparing with CC genotype and CT/CC carriers, the TT genotype was statistically associated with the increased risk of OS (p -values < 0.05). As for c.1002T>C, comparing with TT genotype and TC/TT carriers, the CC genotype was statistically associated with the increased risk of OS (p -values < 0.05). Our data indicate that the T allele and TT genotype of c.44C>T and C allele and CC genotype of c.1002T>C are increased risk factors for the susceptibility to OS in Chinese Han population (Table 4). Up to date, this is the first report about the potential association of c.44C>T and c.1002T>C genetic variants in *MDM2* gene with OS susceptibility. Evidence from this study indicates that *MDM2* genetic variants could be utilized as molecular biomarkers for assessing OS susceptibility. Our findings add to the growing literatures that suggest that the association between *MDM2* genetic variants and OS susceptibility is complex and incompletely understood. Future functional studies on different ethnic populations are essential to confirm these findings and to elucidate the underlying molecular mechanism of genetic influence on the pathogenesis of OS.

Conflict of interests

The authors have no conflict of interests.

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