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Original Article

Single nucleotide polymorphisms of *HER2* related to osteosarcoma susceptibility

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Abstract: Aims: The purpose of this study was to investigate the correlation between single necleotide polymorphisms (SNPs) of human epidermal growth factor receptor-2 (*HER2*) gene with osteosarcoma susceptibility in Chinese Han population. Methods: 90 patients with osteosarcoma and 100 healthy controls who were frequency-matched with the former by age and gender were enrolled for a case-control study. 5 SNPs of *HER2*, namely rs2952155, rs1810132, rs2952156, rs1136201 and rs1058808, were tested by Sequenom time of flight mass spectrometry technique. The linkage disequilibrium and haplotype were analyzed using haploview software. The risk intensity of osteosarcoma was expressed by odds ratio (OR) with 95% confidence interval (CI) which was calculated by chi-squared text. Hardy-Weinberg equilibrium (HWE) was also evaluated by chi-squared text. Results: *HER2* gene rs1136201 and rs1058808 polymorphisms were associated with the increased risk of osteosarcoma (*P*=0.04 and 0.02, respectively). Allele G in rs1136201 was 1.67 higher risk for osteosarcoma in cases than the control group (OR=1.67, 95% CI=1.11-2.51) and G allele of rs1058808 polymorphism also significantly increased osteosarcoma susceptibility (OR=2.06, 95% CI=1.27-3.22). The haplotype analysis showed that haplotype C-T-G-G might be a susceptible haplotype to osteosarcoma (OR=1.74, 95% CI=1.01-3.00). HWE test was eligible in controls (*P*>0.05). Conclusion: *HER2* gene rs1136201 and rs1058808 polymorphisms and haplotype C-T-G-G may be related to osteosarcoma susceptibility in Chinese Han population, indicating that the interaction of gene polrmorphism plays an role in osteosarcoma risk.

Keywords: Osteosarcoma, *HER2,* single nucleotide polymorphism

Introduction

Osteosarcoma is one of the common osteogenic malignant tumors, accounting for about 20% of total bone tumors, and is featured by high malignancy, rapid development, prevalence in juveniles and poor prognosis with an initial mortality of 80% [1-3]. With the improvement of chemotherapy, surgical techniques and tumor classification, most of patients with osteosarcoma can be treated by limb-salvage surgery and even healed. But there are still many patients dying of neoplasm metastasis and the 5-year tumor-free survival rate is only about 65% [4-8]. So the hot area of researches both at home and abroad currently lies in how to detect high-risk group of osteosarcoma early and how to early diagnose and treat timely. There are abundant studies exploring the pathogenesis of osteosarcoma, especially genetic variant has attracted more attention. Genetic variants in microRNA, DNA repair, GST and VEGF polymorphisms and so on have been verified that have influences on the occurrence of osteosarcoma risk [9-11].

Epidermal growth factor receptor-2 (HER2) is a member of human epidermal growth factor receptor family. It is encoded by *HER2* proto-oncogene, also called *ERBB2*, located on chromosome 17q21 and related to the development and progression of multiple tumors [12, 13]. Ile655Val polymorphism caused by the mutation of A→G in transmembrane transduction domain 655 site of *HER2* gene represents one of the differences between *HER2* proto-oncogene and oncogene [14, 15]. The majority of clinical observations show that the metastasis and invasion of gastric cancer are strong when *HER2* is expressed [16]. Some reports have

Table 1. Primer sequences of *HER2* polymorphisms

SNP	Primer Sequences	PCR product length
rs2952155	Forward 5'-CTGGCAGCAGGGCGTTATTT-3'	201 bp
	Reverse 5'-CAAAATAACGCCCTGCTGCC-3'	
rs1810132	Forward 5'-TGCCTCTTCATCTCTGGGGT-3'	352 bp
	Reverse 5'-GAACCCCAGAGATGAAGAGG-3'	
rs2952156	Forward 5'-ACTTTGGGGAGAAAAACAGA-3'	289 bp
	Reverse 5'-GTTTTTCTCCCCAAAGTCCT-3'	
rs1136201	Forward 5'-AGGCAGGTTTTAGAGTAGGA-3'	355 bp
	Reverse 5'-TACTCTAAAACCTGCCTTGG-3'	
rs1058808	Forward 5'-TAATGGGTCACCTTCTCTTG-3'	300 bp
	Reverse 5'-CAAGAGAAGGTGACCCATTA-3'	

demonstrated the close relationship of Ile655Val polymorphism with breast cancer risk [17].

To ascertain the correlation of *HER2* single necleotide polymorphisms (SNPs) with the pathogenesis of osteosarcoma in Chinese Han population, 5 SNPs of *HER2* gene were tested using time of flight mass spectrometry technique in patients with osteosarcoma. 90 patients with osteosarcoma and 100 healthy controls were enrolled to explore the effect of SNPs on osteosarcoma in present study. We hope that our study can provide experimental basis for searching drug targets related to the pathogenesis of osteosarcoma.

Materials and methods

Clinical data

Ninty patients with osteosarcoma including 53 males and 37 females were collected in the case group. They were aged 16-53 with a median age of 19.6, and were histopathologically confirmed with osteosarcoma through needle or open biopsy. All patients without the history of genetic cancer syndromes did not experience radiothe rapy or chemotherapy before operation. 100 healthy people were frequency-matched by gender and age with cases as the controls. They were enrolled in regular physical examination center included 59 males and 41 females aged 12-51 with a median age of 20.3. People were excluded from controls if they suffered from diabetes, coronary heart disease or tumors. Samples were collected in accordance with the national ethics criteria for human genome research. All subjects were unrelated by blood.

Primary reagents and instruments

DNA extraction kit and Taq enzyme were purchased from Beijing Aidelai Biological Science and Technology Co. Ltd., meanwhile alkaline phosphatase, iPLEX enzyme and cation exchange resin were from Shanghai North Connaught Biotechnology Co. Ltd. SpectroCHIP and MassARRAY-Typer software system were provided by Shanghai Pan Ke Industrial Co. Ltd.

Primer design and synthesis

Primers were designed by Primer 5.0 software and synthesized by Shanghai Genecore Biotechnologies Co. Ltd. and primer sequences are listed in **Table 1**.

DNA extraction

2~3 mL peripheral venous blood was collected in morning from every subject with an empty stomach, and was conducted anticoagulation using 20 g/L EDTA 200 μL. Genome DNA was extracted using Qiagen genome DNA extraction kit following the operating manual, standardized the concentration to 50 μg/L, and preserved in freezer at -20°C for later.

PCR system

PCR reaction system is a volume of 20 μL solution, including 1 μL of DNA sample diluted to 5 μg/L previously, 0.5 μL forward and reverse primers, respectively, 2.0 μL PCR buffer (containing 15 mmol/L $MgCl_2$), 0.2 L of 2.5 mmol/L dNTP, 0.1 μL HotStarTaq enzyme and 15.7 μL ddH_2O. PCR conditions were as follows: 94°C for 15 min; 45 cycles of 94°C for 20 s, 56°C for 30 s and 72°C for 1 min; 72°C for 3 min. Residual dNTP was digested through dephosphorylation after PCR amplification, containing 1.53 μL water, 0.17 μL SAP buffer and 0.3 U alkaline phosphatase. The reaction was proceeded at 37°C for 40 min and then at 85°C for 5 min to make enzyme inactive. The primer extension reaction was conducted according to Sequenom program.

Genotyping analysis

SNP genotyping was operated by Shanghai Pan Ke Industrial Co. Ltd. utilizing MassARRAY system of American Sequenom company. The final

Table 2. Genotype and allele frequencies of *HER2* polymorphisms

SNP	Genotype (n)			*P*	Allele (2n)		*P*
rs2952155	CC	CT	TT	0.28	C	T	0.15
Case	33	31	26		97	83	
Control	26	41	33		93	107	
rs1810132	CC	CT	TT	0.26	C	T	0.09
Case	19	39	32		77	103	
Control	31	41	28		103	97	
rs2952156	GG	GA	AA	0.18	G	A	0.97
Case	47	26	17		120	60	
Control	46	41	13		133	67	
rs1136201	AA	AG	GG	0.04	A	G	0.01a
Case	20	47	23		87	93	
Control	38	46	16		122	78	
rs1058808	CC	CG	GG	0.02	C	G	3.00×10^{-3} b
Case	48	28	14		124	56	
Control	69	26	5		164	36	

Note: a represents OR=1.67 and 95% CI=1.11-2.51; b means OR=2.06 and 95% CI=1.27-3.22.

Table 3. Linkage disequilibrium and haplotype analysis of *HER2* rs2952155, rs1810132, rs2952156 and rs1508808

Haplotype	Cases, 2n	Controls, 2n	OR (95% CI)	*P* value
T-C-A-C	60 (34.48)	67 (34.18)	1.00 (Ref.)	-
C-T-G-C	41 (22.99)	57 (29.08)	0.80 (0.47-1.37)	0.42
C-T-G-G	56 (32.76)	36 (18.37)	1.74 (1.01-3.00)	4.60×10^{-2}
T-C-G-C	17 (9.77)	36 (18.37)	0.53 (0.23-1.03)	0.06

reactant was added with 6 mg cation exchange resin for desalination and mixed with 25 μL water for suspension. The final typing products were operated spotting to a spectroCHIP with 384 holes using MassARRAY Nanodispenser system and were analyzed by matrix assisted laser desorption ionizing time of flight mass spectrometry. Final results were read in real time by MassARRAYRT software system and conducted genotyping analysis through MassARRAYTyper software system.

Statistical analysis

Hardy-Weinberg equilibrium (HWE) was tested by chi-squared test in the control group. Statistical analysis was operated using SPSS 18.0 software. The distributions of allele and genotype in *HER2* SNPs both in cases and controls were calculated by χ^2 test. The linkage disequilibrium and haplotype were analyzed using haploview software. Odds ratio (OR) and 95% confidence interval (CI) were calculated to evaluate the effects of genotypes, alleles and haplotypes in *HER2* SNPs on the risk of osteosarcoma. $P<0.05$ represents statistical significance.

Results

Test of Hardy-Weinberg equilibrium

Genotype distributions of *HER2* rs2952155, rs1810132, rs2952156, rs1136201 and rs1058808 polymorphisms in the control group were all consistent with Hardy-Weinberg equilibrium ($P>0.05$), indicating that the study objects came from the same community and had the representativeness of regional population.

Correlation analysis between genotypes and alleles of HER2 SNPs with the pathogenesis of osteosarcoma

Table 2 lists the distribution of genotypes and alleles of rs2952155, rs1810132, rs2952156, rs1136201 and rs1058808 polymorphisms in *HER2* gene. The genotypes frequencies of *HER2* rs1136201 and rs1058808 polymorphisms were significantly different between in cases and the control group ($P=0.04$ and 0.02, respectively). They might be associated with the increased risk of osteosarcoma. Allele G in rs1136201 was 1.67 higher risk for osteosarcoma in cases compared with the control group (OR=1.67, 95% CI=1.11-2.51). Similarly, G allele of rs1058808 polymorphism was also a risk factor for osteosarcoma (OR=2.06, 95% CI=1.27-3.22).

Haplotype analysis

Linkage disequilibrium and haplotype analysis of rs2952155, rs1810132, rs2952156 and rs1058808 polymorphisms (**Table 3** and **Figure 1**) were conducted with haploview software. The correlation analysis of these four haplotypes with osteosarcoma susceptibility demonstrated that haplotype C-T-G-G had significant differences between cases and controls ($P<0.05$) and might be a susceptible factor to osteosarcoma (OR=1.74, 95% CI=1.01-3.00).

Discussion

Osteosarcoma is a connective tissue malignant tumor which tumor cells can directly produce

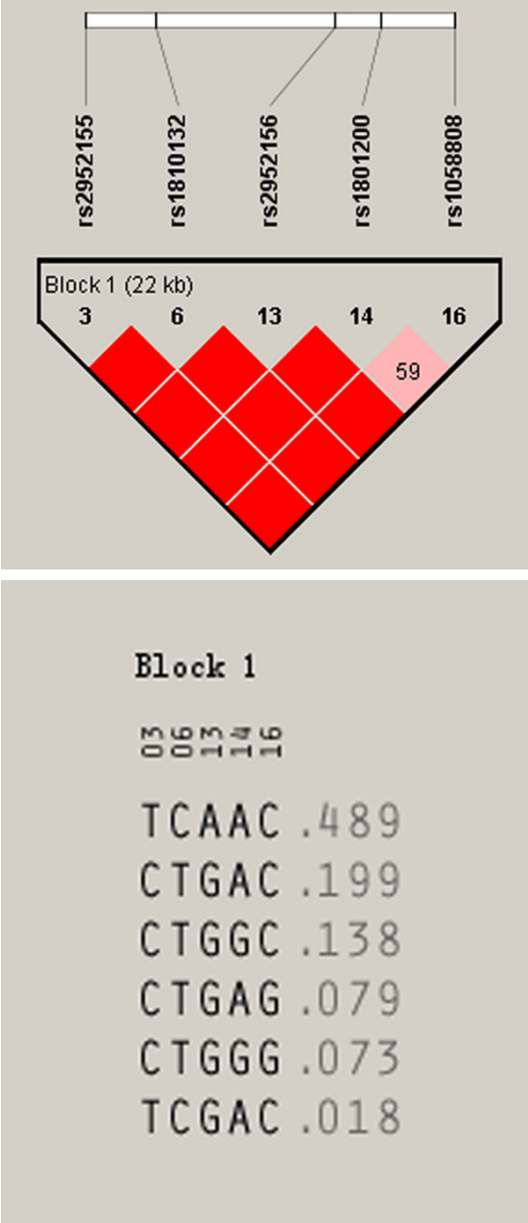

Figure 1. Haplotype analysis diagram. Darker block represent a stronger linkage disequilibrium, while the lighter one stands for a weaker linkage disequilibrium.

tumor bones and osteoid tissues and its morbidity ranks the first in all primary malignancies [18, 19]. It has taken place mostly on distal femur and proximal tibia, accounting for 75% of all osteosarcoma patients [20]. Distant metastasis is the main cause of treatment failure and death in the majority of patients with osteosarcoma [21]. In recent years, with the development of tumor molecular biology, it has been realized that cell carcinogenesis is mainly caused by the changes of genetic information [22-24]. According to the study of Chang et al. in a meta-analysis, genetic variants of *CD 152* can significantly increased the development of osteosarcoma risk in Chinese population [25]. As far as the study of Wang et al., *MDM2* plays a vital role in the carcinogenesis of osteosarcoma, the polymorphism rs2279744 of it is associated with the increased risk of osteosarcoma [26].

HER2 participates in the regulation of cell proliferation, apoptosis, metastasis and invasion through various signal transduction pathways, including Ras/Raf/MAPK, PI3K/Akt and STAT [27]. Studies have shown that Ile655Val polymorphism caused by the conversion of A to G in transmembrane transduction domain of HER2 protein can significant activate similar ligand mediated receptor and tyrosine kinase and improve the autophosphorylation, tyrosine kinase activity and cell transformation [28]. What is more, multiple studies have reported the relationship of *HER2* gene polymorphism Ile655Val with the risk of breast cancer [29]. Recently, with the increasing number of studies on the association of *HER2* with gastric cancer risk, monoclonal antibody Herceptin targeting at *HER2* is applied to treating advanced gastric cancer patients, which demonstrates promising effects. At the moment, observed results mainly show that *HER2*/neu is related to tumor size, serosal invasion degree and lymph node metastasis [30]. Lots of researches suggest that *HER2* is closely related to multiple cancers. The high expression of HER2 protein affects the malignant biological behavior of osteosarcoma and always predict a poor prognosis. Scotlandi et al. researched 84 cases of osteosarcoma using immunohistochemical staining and found that *HER2* gene was overexpressed in 32% of the cases [31].

In present study, 5 SNPs from *HER2* were analyzed and their functions were different. Two of them rs1136201 and rs1508808 have demonstrated that either genotypes or alleles are associated with the increased risk of osteosarcoma. But the other three SNPs rs1810132, rs2952155 and rs2952156 had no significant relevance with osteosarcoma susceptibility. Further haplotype analysis manifested that haplotype C-T-G-G constituted by rs2952155, rs1810132, rs2952156 and rs1508808 had significant differences between cases and con-

trols, being the potential susceptible haplotype to osteosarcoma. Additionally, as haplotype C-T-G-G contains susceptible allele G, further evidence has been offered to prove that G allele at rs1508808 may be the susceptible factor for osteosarcoma.

There are more than 20 SNPs in human *HER2* gene, and in this study we only primarily analyzed the association between the incidence of osteosarcoma with the SNPs of minor allele frequency (MAF) larger than 0.05. Based on the present and previous studies, we speculate genetic variant of *HER2* affect the development of osteosarcoma through regulating the expression level of HER in patients with osteosarcoma. Rs2952155 and rs2952156 are the mutations in intron domain, which may be a reason that both of two don't make woke in the pathogenesis of osteosarcoma. Additionally, the sample size of the study was relatively small. To fully understand the functions of *HER2* SNPs on the onset of osteosarcoma in Chinese Han population, more efforts should be made to seek more SNPs and expand the scale of samples in studies so as to provide experimental evidence for the pathogenesis, procession and early diagnosis of osteosarcoma.

Disclosure of conflict of interest

None.

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