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Original Article

Association between CTLA-4 polymorphisms and osteosarcoma susceptibility

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Abstract: Purpose: This study aimed to detect the association between cytotoxic T lymphocyte antigen-4 (*CTLA-4*) gene polymorohisms (-1722T/C and -318C/T) and osteosarcoma (OS) susceptibility. Haplotypes of the two polymorphisms were also explored in OS. Methods: Genotypes of -1722T/C and -318C/T polymorphisms of *CTLA-4* gene in 97 OS patients and 120 healthy controls were tested with polymerase chain reaction-restriction fragment polymorphism (PCR-RFLP) method. Linkage disequilibrium and haplotype analysis of the two polymorphisms were conducted with Arlequin software. χ^2 test was utilized to compare the differences of genotypes, alleles and haplotypes frequencies between case and control groups. Results: Age and gender had no significant association in OS. Genotype analysis indicated that both -1722CC genotype and -1722C allele were obviously increased the OS susceptibility (*P*=0.034, OR=2.19, 95% CI=1.07-4.45; *P*=0.030, OR=1.53, 95% CI =1.04-2.25). The result indicated that allele C was a susceptible factor for OS. However, there were no obvious differences in distributions of genotypes and alleles of -318C/T between case and control groups. There existed linkage disequilibrium between the two polymorphisms. Haplotype C-C was significantly higher in case group than that in control group, denominated that C-C haplotype might increase the susceptibility of OS (OR=1.60, 95% CI=1.05-2.44). Conclusion: -1722CC genotype and -1722C allele of *CTLA-4* gene promoter region can increase the risk of OS. C-C haplotype composed by -1722T/C and -318C/T polymorphisms in *CTLA-4* gene is an important factor for the occurrence of OS.

Keywords: *CTLA-4*, osteosarcoma, polymorphisms, haplotype

Instruction

Osteosarcoma (OS) is a common osteoblastic malignant tumor which originated from mesenchyme, and about accounts for 35% of primary bone malignancies [1]. It is featured by high malignancy and quick development. Adolescents are the main target of this disease and the morbidity in men is higher than in women [2-4]. In the early years after the discovery of OS, its mortality is very high, and the prognosis is very poor. With the improvement of chemotherapy, surgical techniques and tumor classification methods, 80-85% primary bone sarcoma patients could be treat through limb-salvage surgery [5]. However, there are also a plenty of patients dying of tumor metastasis. Combined treatment improved the 5-year tumor-free survival rate, but it is only about 50%-70% [6]. Various studies show that OS is a complex disease could effects by genetic, environment factors and physical injury [7-11]. But the etiology of OS is still unclear.

Since the find of cytotoxic T lymphocyte antigen-4 (*CTLA-4*) gene, previous studies indicate that it is strongly associated with autoimmune diseases [12, 13], plays an important part in the final stage of T lymphocyte activation. At present, a number of studies have shown that *CTLA-4* gene is closely associated with many diseases including OS [14-17]. Multiple polymorphisms existing in *CTLA-4* gene can affect *CTLA-4* expression. Besides, the abnormal functions and/or expressions of *CTLA-4* gene can result in the occurrence and development of some tumors [17-21]. -1722T/C and -318C/T are single nucleotide polymorphisms (SNPs) in the promoter region of *CTLA-4* gene. Some studies have suggested that the two SNPs were associated with many tumors [20, 22, 23]. However, there was no research on the association between *CTLA-4* polymorphisms (-1722T/C and -318C/T) with OS susceptibility.

So we carried out this study to analyze the association between *CTLA-4* polymorphisms

(-1722T/C and -318C/T) and OS in Chinese Han population. This research may be certified the etiology of OS.

Materials and methods

Research objects

97 OS patients, including 43 men and 54 women, who were diagnosed in Affiliated hospital of Jilin Medical University, were enrolled in this study. All the patients didn't receive radiotherapy or chemotherapy before operations. 120 healthy individuals (50 males and 70 females) who were take a healthy check-up in the same hospital at the same period, were recruited as controls. Controls were matched with the cases in terms of age, gender and ethnicity. People were excluded if they suffered from diabetes, coronary heart disease or tumors. All subjects were unrelated Chinese Han population and signed the written informed consent. Besides, this study had been approved by the Ethics Committee of Affiliated hospital of Jilin Medical University, The data and blood samples were collected under the accordance with the regulations of the ethics.

DNA extraction and polymerase chain reaction (PCR)

Genomic DNA was extracted from the peripheral blood of the fasting subjects. Its extraction and purification was used the DNA extraction kit (Dalian TaKaRa).

Primers of *CTLA-4* -1722 were designed by literature [13], and the primers sequences were as follows: forward primer: 5'-CTA AGA GCA TCC GCT TGC ACC T-3'; reverse primer: 5'-TTG GTG TGA TGC ACA GAA GCC TTT T-3'. PCR primer sequences of -318C/T were designed by Shanghai Sangon Biotech Co,. Ltd, and the up-/down stream primers were 5'-AAA TGA ATT GGA CTG GAT GGT-3' and 5'-TTA CGA GAA AGG AGG CCG TG-3' respectively. The primers of the two polymorphisms were synthesized by Shanghai Sangon Biotech Co,. Ltd.

PCR reaction was performed in a 25 μl volume system, including 2 μl 10 × PCR buffer, 0.1 μg template DNA, 2.5 mmol/L $MgCl_2$, 0.2 μmol/L dNTP (Shanghai RuiQI BioTech Co,. Ltd), 20 pmol of forward and reverse primers and 1.5 U Tag DNA polymerase (Beijing Shengke Boyuan Biotech Co,. Ltd.), and completed with double distilled water. For *CTLA-4*-1722 polymorphims, PCR amplification conditions were: denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 45 s; finally 8 min extension at 72°C. When it came to *CTLA-4* -318, PCR amplification conditions were as followed: 5 min denaturation at 94°C, followed by 35 cycles of 94°C for 45 s, 60°C for 45 s and 72°C for 1 min; finally 10 min extension at 72°C.

Polymorphisms analysis of amplified fragments

486 bp PCR product of -1722 T/C could be digested into two fragments (270 bp and 216 bp) by *Bbv*Ienzyme when the -1722 allele was C. PCR products of -318 C/T was 247 bp, could be digested into three fragments (130 bp, 96 bp and 21 bp) by *Mse I* enzyme, when cytosine (C) existed in -318C/T polymorphism. The results were identified by agarose gel electrophoresis.

Statistical methods

Statistical analysis was conducted by SPSS 18.0 software package. Adjustments to gender and age were done by non-conditional logistic regression. Hardy-Weinberg equilibrium (HWE) was used to examine whether the distributions of genotype and allele were representative. Additionally, Arlequin software was applied to do linkage disequilibrium and haplotype analysis of the SNPs. χ^2 test was adopted to compare the genotypes and alleles frequencies between case and control groups. Relative risk of OS was denoted by odds ratios (ORs) with 95% confidence intervals (95% CIs). Test criterion is P=0.05.

Results

Non-conditional logistic regression and HWE analysis

Logistic regression results showed there were no statistical differences in distribution of age (t=-0.100, P=0.920) and gender (χ^2=0.06, P=0.803) between the two groups, which indicated that the two groups had similar basic features and were comparable. HWE was utilized to further evaluate the reliability of the group investigation data. Genotype and allele distri-

Table 1. Genotypes and alleles distribution of -1722T/C and -318C/T in case and control groups

SNP	Cases (n=97) n (%)	Controls (n=120) n (%)	χ^2	*P* value	OR (95% CI)
Genotype					
-1722T/C					
TT	35 (36.1)	51 (42.5)	-	-	1.00
TC	32 (33.0)	49 (40.8)	0.025	0.875	0.95 (0.51-1.77)
CC	30 (30.9)	20 (16.7)	4.721	0.034	2.19 (1.07-4.45)
-318C/T					
CC	76 (78.4)	88 (73.3)	-	-	1.00
CT	20 (20.6)	30 (25.0)	0.623	0.430	0.77 (0.41-1.47)
TT	1 (1.0)	2 (1.7)	0.201	0.654	0.58 (0.05-6.51)
Allele					
-1722T/C					
T	102 (52.6)	151 (62.9)	-	-	1.00
C	92 (47.4)	89 (37.1)	4.717	0.030	1.53 (1.04-2.25)
-318C/T					
C	172 (88.7)	206 (85.8)	-	-	1.00
T	22 (11.3)	34 (14.2)	0.763	0.383	0.78 (0.44-1.38)

Table 2. Linkage disequilibrium and haplotype analysis of -1722T/C and -318C/T n (%)

Haplotype	Cases (2n=194)	Controls (2n=240)	χ^2	*P* value	OR (95% CI)
T-C	99 (67.0)	141 (58.8)	-	-	1.00
T-T	3 (4.1)	10 (4.5)	1.693	0.193	0.43 (0.12-1.59)
C-C	73 (37.6)	65 (27.1)	4.794	0.029	1.60 (1.05-2.44)
C-T	19 (6.2)	24 (8.0)	0.129	0.719	1.13 (0.59-2.17)

butions of *CTLA-4*-1722 T/C and -318C/T in the controls were fit with HWE, illustrating that the controls were representative.

Genotype distributions of CTLA-4-1722T/C and -318T/C

Genotype distributions of *CTLA-4*-1722 T/C and -318C/T were shown in **Table 1**. Compared with the control group, the frequency of -1722CC genotype was higher in the case group (*P*=0.034, χ^2=4.721, OR=2.19, 95% CI=1.07-4.45). As for the distribution of alleles, there was also apparent difference between the two groups (for allele C, *P*=0.030, χ^2=4.717; OR=1.53, 95% CI=1.04-2.25). However, in -318C/T SNP, the frequencies of genotype and allele had no apparent difference between the two groups.

Linkage disequilibrium and haplotypes in OS

Linkage disequilibrium and haplotype analysis on -1722T/C and -318 C/T showed that linkage equilibrium displayed statistical significance (*P*<0.05). The result suggested that there was linkage disequilibrium between the two polymorphisms. Haplotype distribution frequencies between case and control groups were calculated (**Table 2**). The frequency of C-C haplotype was higher in case group than that in control group (*P*=0.029). It suggested that C-C was a susceptible haplotype to OS (OR=1.60, 95% CI=1.05-2.44).

Discussion

OS is the most common malignant tumor in a bone, often occurs in adolescents and children, and ultimately results in amputation, pulmonary metastasis and death. So far, the pathogenesis of OS has not been clear, and studies hold the point that to a certain extent, OS is a complex disease which associated with genetic and environmental factors. In recent years, studies have discovered that SNPs of some genes are correlated with the occurrence, treatment and even prognosis of OS. Wang et al

showed that *CTLA-4* +49G/A polymorphism has relationship with the onset of OS [24].

CTLA-4 gene was first discovered by Brunet et al. in 1987 when they were screening the cDNA library of cytotoxic T lymphocyte in mouse [25]. With the development of modern molecular biology, *CTLA-4* gene is found to locate in 2q33 and includes 4 exons and 3 introns. *CTLA-4* gene is a member of the immunoglobulin superfamily, and the variants of it have been found associated with many diseases. *CTLA-4* polymorphisms frequently studied include promoter -318T/C (rs5742909), promoter -1722T/C (rs733618), extron 1 +49A/G (rs231775) and 3'UTR terminal codon CT60 (rs3087243) [26-31]. Many SNPs of *CTLA-4* were reported had correlation with OS risk [24, 32]. Therefore, *CTLA-4* gene was considered as a candidate gene for OS. However, the association between *CTLA-4* gene and OS risk still unclear. Up to date, there had a few studies detect the association between -1722T/C and -318 C/T SNPs of *CTLA-4* gene with OS risk, and no study focus on the haplotype of the two SNPs in OS.

So we implemented this study to explore the correlation of the genotypes, alleles and haplotypes of -1722T/C and -318C/T of *CTLA-4* gene and OS risk. The two SNPs of *CTLA-4* gene were compared between OS patients and healthy controls in this study. The results showed that *CTLA-4* -1722T/C polymorphism had a wide spread among populations. Frequencies of -1722CC genotype and -1722C allele were significantly higher in case group than that in control group. Both the genotype and allele increased the OS susceptibility about 2.19 and 1.53 times. That was accorded with the result in chronic Chagas disease [33], but no correlation existed in systemic lupus erythematosus and many cancers [20, 22, 34]. Allele and genotype distributions of -318T/C polymorphism of *CTLA-4* gene had no distinct difference between case and control groups. The result was similar to previous studies in Ewing's sarcoma [35], however, the -318TT genotype and -318T allele could increase the risk of rheumatoid arthritis [36]. Afterwards, we performed the linkage disequilibrium and haplotype analysis on the alleles of the two polymorphisms in *CTLA-4* promoter region. Frequencies of T-T and C-T haplotypes were relatively lower in cases and controls, and had no statistical difference between the two groups. Haplotype C-C distribution was significantly different between case and control groups, suggested that it might act as a susceptible haplotype of OS.

Although we obtained a meaningful result, the study was still in exploratory stage and had many limitations. Firstly, the sample size was small, and only involved one ethnicity. Secondly, the results were not adjusted. Thirdly, the effects of other factors, such as the gene-environment interactions and gene-gene interactions, were not involved in our study. All of the limitations maybe cause an imperfect result, and then offer an insufficient evidence for the research of OS etiology. We suggested that a well designed study focus on the OS pathogenesis should include a large sample size, more ethnicity and sufficient factors, so as to obtain more reliable results.

Disclosure of conflict of interest

None.

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