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Original Article

Association of -308G/A and -238G/A polymorphisms of *TNF-α* and osteosarcoma risk

Zhongwei Zhao[1,2], Xiangyu Tang[1], Kai Song[1], Xiang Li[3], Yonggang Zhang[1]

[1]Department of Orthopaedics, Chinese People's Liberation Army General Hospital, 28 Fuxing Road, Beijing 100835, China; [2]Department of Orthopaedics, The Puren Hospital of Beijing, 100 Chongwai Street, Beijing 100062, China; [3]Department of Spine Surgery, Beijing Bo'ai Hospital, China Rehabilitation Research Center, Beijing 100068, China

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Abstract: Objective: As a proinflammatory cytokine, *TNF*-α is associated with increased risk of osteosarcoma (OS). Our study aimed to explore the association of *TNF*-α polymorphisms and OS susceptibility in the Han Chinese population. Methods: 80 OS patients and 99 healthy people, matched on the age and sex, participated in the study. Genotyping was conducted by the method of polymerase chain reaction-restricted fragment length polymorphisms (PCR-RFLP). Then logistic regression was used to evaluate the effects of *TNF*-α polymorphisms (-308G/A and -238G/A) on the pathology of OS. Results: The frequency of AA genotype in -308G/A locus in the cases was significantly higher than that of the healthy group (20.0% vs. 6.1%). Patients with OS were more likely to possess AA genotype of -308G/A locus (OR=4.00, 95% CI=1.41-11.38). For the patients with A allele, the risk for OS increased 0.62 fold (OR=1.62, 95% CI=1.04-2.50). There was no remarkable relationship of -238G/A polymorphisms and OS susceptibility. In addition, we found that patients with G-A and A-A haplotypes was much higher in the cases than that of control group (68.0% and 25.0%, 53.0% and 38.9%, respectively). A-G haplotype appeared to increase the risk for OS (OR=1.93, 95% CI=1.13-2.94). Conclusion: The AA genotype of -308G/A locus of *TNF-α* gene was a risk factor for OS, however there was no correlation between -238G/A of *TNF-α* and OS.

Keywords: *TNF-α,* polymorphisms, -308G/A, -238G/A, osteosarcoma, susceptibility

Introduction

Osteosarcoma is one of common malignant osteogenesis tumors, accounting for about 20% of all bone tumors, with the characteristics of high malignancy, rapid development, and frequent occurrence in young people. And the mortality once reached 80%. With the improvement in chemotherapy, surgical technology and tumor classification, most patients can be healed with the therapy of limb salvage surgery. However, there are still many patients dying from tumor metastasis. Thus, the 5-year survival rate is only about 65% [1]. Early diagnosis and early treatment can effectively increase the survival rate. In domestic and international, scientists have been exploring how to identify the population with high-risk for OS.

Tumor necrosis factor-α (*TNF-α*) is a potential immunomodulator and an important inflammatory cytokines, which usually appears during the early stage of inflammatory response to various physiological activities. Tumors associated with mutations in cancer susceptibility genes develop rapidly and it is generally considered that such tumors require no inflammatory stimuli [2]. However, Mori et al. reported that *TNF-α*, an inflammatory cytokine, is required for the tumorigenesis of osteosarcoma, which maintained osteosarcoma cells in an undifferentiated state in vitro [3]. Furthermore, the serum level of *TNF-α* was significantly increased in OS patients than in controls [4]. Further studies also showed close relationship of *TNF-α* polymorphisms and various cancer [5-10].

In this study, we investigated the role of *TNF-α* polymorphisms (-308G/A and -238G/A) in the OS pathogenesis. The study may help us understand the etiology of OS.

Materials and methods

Subjects

A total of 80 OS patients were enrolled from our hospital during 2008-2010. The cases included

Table 1. Primers of -308G/A and -238 G/A

Loci	Primer	Enzyme and fragment
-308 (G→A)	F: 5'-CCGTGCTTGGTGCTTTGGACTA-3'	Msp I
	R: 5'-AGAGCTGGTGGGGACATGTCTG-3'	133bp and 19bp
-238 (G→A)	F: 5'-GCAATAGGTGAGGGCCAT-3'	Nco I
	R: 5'-TGGGGACACACAAGCATCAA-3'	130bp and 20bp

F: Former primer; R: Reverse primer.

Table 2. Genotypes and alleles distribution of -308G/A and -238G/A

Genotype/allele	Case n (%)	Control n (%)	χ^2	*P*	OR (95% CI)
-308G/A					
GG	30 (37.5)	45 (45.4)	-	-	1
GA	34 (42.5)	48 (48.5)	0.352	0.852	1.06 (0.56-2.01)
AA	16 (20.0)	6 (6.1)	7.307	0.007	4.00 (1.41-11.38)
G	94 (58.8)	138 (69.7)	-	-	1
A	66 (41.2)	60 (30.3)	4.649	0.031	1.62 (1.04-2.50)
- 238G / A					
GG	62 (77.5)	74 (74.7)	-	-	1
GA	16 (20)	24 (24.2)	0.391	0.532	0.80 (0.39-1.63)
AA	2 (2.5)	1 (1.1)	0.525	0.469	2.39 (0.21-26.95)
G	140 (87.5)	172 (86.9)	-	-	1
A	20 (12.5)	26 (13.1)	0.031	0.859	0.94 (0.51-1.76)

57 males and 23 females, with the average age of 14.68 (5-22 years). All patients were required no radiation therapy or chemotherapy before the operation. 99 healthy controls with 54 males and 46 females were obtained from health examination center of the hospital. They were aged from 12 to 24, with the average of 15.6. The person suffering diabetes, coronary heart disease or tumors were excluded. In addition, they were required normal blood routine, liver and kidney functions and heart and abdominal ultrasounds test.

Methods

DNA was extracted from 5ml peripheral venous blood with EDTA anticoagulation, then was stored under -70°C for spare. 25 μl PCR amplification reaction were prepared using 10×PCR Buffer (without $MgCl_2$) 2.5 μl, dNTPs 3 μl, each primer 0.5 μl, $MgCl_2$ 2 μl, Taq polymerase 0.3 μl, template 2.0 μl and double-distilled water. The PCR amplification was performed under the following conditions: 5min 94°C initial denaturation followed by 36 cycles at 94°C for 45 s, 63°C for 45 s, 72°C for 60 s, then 72°C for 7 min. PCR products were identified with 1.5% agarose gel electrophoresis. The primers were listed in **Table 1**.

Enzyme digestion reaction was 30 μL including PCR product 25 μl, restriction enzyme (MspI or NcoI) 1 μl and added double-distilled water and then were stored under 37°C for 12 h. Enzyme digestion products was detected by 1.5% agarose gel and the gel was dyed with EB.

Statistics

All data were analyzed by SPSS 18.0. X^2 test was used to conduct Hardy-Weinberg equilibrium (HWE) and compare genotypes and allele frequencies between groups. *P*-values< 0.05 were considered statistically significant.

Results

Genotypes analysis of -308G/A and -238G/A

PCR product of -308G/A was 117 bp. According to the enzyme digestion results, there were 3 genotypes including GG (2 bands, 97 bp and 20 bp), GA (3 bands, 117 bp, 97 bp and 20 bp) and AA (1 band, 117 bp). For -238 G/A, there were also 3 genotypes including the genotypes of GG (133 bp and 19 bp), GA (152 bp, 133 bp and 19 bp)and AA (152 bp).

Association between genotypes of TNF-α polymorphisms (308G/A and -238G/A) and OS

In the study, AA genotype of -308 G/A was a susceptibility factor for OS (**Table 2**). The genotypes distribution of *TNF-α* polymorphisms (-308 G/A and -238G/A) were consistent with HWE (*P* = 0.14, 0.53). The AA genotype (-308G/A) frequency (20.0%) was found higher in the cases than that of control group (6.1%). And we also found that the OS patients were more likely to possess AA genotype (OR=4.00, 95% CI=1.41-11.38). For the patients with A allele of -308 G/A, the risk to OS increased 0.62 fold. The polymorphisms of -238G/A showed no effect on OS susceptibility.

Table 3. Association of haplotypes of -308G/A and 238G/A and OS

Site 1 - site 2 -308--208	Case (2n=160) n (%)	Control (2n=198) n (%)	*P* value	OR (95% CI)
G-G	84 (52.5)	126 (63.6)	-	1
G-A	10 (2.6)	12 (6.1)	0.620	1.25 (0.52-3.02)
A-A	10 (2.6)	14 (7.1)	0.875	1.07 (0.46-2.52)
A-G	56 (35.0)	46 (23.2)	0.013	1.93 (1.13-2.94)

Association of haplotypes and OS susceptibility

With the analysis of haplotypes, we found that the frequency of A-G haplotype was much higher in the cases compared to that of controls (35.0% vs. 23.2%), then concluded that A-G haplotype increased the risk to for OS (OR =1.93,95% CI=1.13-2.94) (**Table 3**).

Discussion

Due to high malignancy and metastasis rate, the main cause of death and treatment failure in the majority of patients was the distant metastasis [11, 12]. In recent years, with the development of tumor molecular biology, people have already realized that the cell carcinogenesis is mainly due to the changes of genetic information. It is carcinogenic activation of gene expression and gene loss or loss of function of anti-oncogene that leads to carcinogenesis [13]. Therefore, it is helpful to diagnose, treat and find out the high-risk groups of OS early for improving the survival rate and life quality [14]. *TNF-α* is a peptide regulatory factor secreted by mononuclear phagocytic system and endothelial cells which plays an important role in immune regulation [15-17].

TNF-α is a cytokine produced by the macrophage [18-20] in the stationary state which was stimulated and activated by endogenous interferon, bacteria with endotoxin and virus. *TNF-α*, together with interleukin-1 and other cytokines, can induce each other and stimulate the cascade reaction of the inflammatory medium [4, 21]. As an important endogenous cytokines, *TNF-α* has extensive biological function. *TNF-α* in moderate concentration with anti-infectious immunity can remove pathogens and their products.

-308G/A loci are located in the promoter of *TNF-α*. With studying the effect of *TNF-α*-308G/A polymorphisms on TNF-transcription, Kroeger found that the transcription level of recombinant including -308G/A A allele were double higher than that including -308G/A G allele [22-24]. In addition, a study on *TNF-α* expression and regulation mechanism found that a 10 bp DNA fragment with *TNF-α* -308 may be the recognition sequence of the transcription factor AP-2. When the -308 site was G allele, AP-2 could identify the sequence and combined with it. If there appeared G→A substitution, AP-2 can identify the sequence. So *TNF-α* gene promoter polymorphisms might influence the downstream of *TNF-α* by expression products, and were related with the susceptibility to infectious diseases, development and prognosis [25, 26].

Base G of *TNF-α* -238G/A was replaced by base A in the promoter region of upstream of the transcriptional start site 238th. Base G gene locus is more common, and base A gene loci is relatively rare. This mutation caused a deletion sites of restriction endonuclease MsP I, and that is the nucleotide sequence replaced by A cannot recognized and cut off by MsP I, namely the emergence of restriction fragment length polymorphism, which was called TNF-238. Due to the special position and important biological function of TNF gene, the TNF gene polymorphisms and disease were attracted more attention. TNF genetic polymorphisms and relative diseases have received widespread attention, due to the special position and its important biological functions.

Our research showed that the frequency of AA genotype of -308G/A in the cases was significantly higher than that in control group (20.0% vs. 6.1%), which indicated that the AA genotype was a susceptibility factor for OS (OR=4.00, 95% CI=1.41-11.38). The distribution differences of GA genotype in the cases and controls were not significant (P=0.852). Furthermore, we found that the A allele of -308G/A could increased the risk to OS (OR=1.62, 95% CI=1.04-2.50). There was no remarkable correlation of -238G/A polymorphisms and OS susceptibility. Haploview software was used to conduct the analysis of linkage disequilibrium and haplotypes distribution. The results

showed that A-G haplotype increased the risk to for OS (OR=1.93, 95% CI=1.13-2.94).

Due to different genetic background, there may be variances in *TNF-α* polymorphisms in different populations. Further studies with more ethnics and larger population group are needed to clarify the relationship between *TNF-α* polymorphisms and OS susceptibility.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yonggang Zhang, Department of Orthopaedics, Chinese People's Liberation Army General Hospital, 28 Fuxing Road, Beijing 100835, China. E-mail: zhaozw1976@aliyun.com

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