Int J Clin Exp Pathol 2016;9(10):10721-10727
www.ijcep.com /ISSN:1936-2625/IJCEP0037226

Original Article

Associations of Epstein-Barr virus-induced gene 3 polymorphisms and expression with osteosarcoma risk

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Received August 3, 2016; Accepted August 23, 2016; Epub October 1, 2016; Published October 15, 2016

Abstract: Background: No previous study reported the associations of Epstein-Barr virus-induced gene 3 (EBI3) polymorphisms with osteosarcoma risk to date. The aim of present study was to investigate these associations in Chinese population. Methods: The plasma EBI3 levels were measured by enzyme-linked immunosorbent assay. Three EBI3 variants, rs4740, rs9807813 and rs428253 were evaluated in a case-control study of 108 osteosarcoma patients and 216 health controls, by using SNaPshot method. Results: The plasma EBI3 levels were significantly higher in osteosarcoma patients than in controls, and also higher in patients with tumor metastasis than without metastasis (P<0.01). Genotypes of rs4740 GA and GA+AA were associated with the risk of osteosarcoma (AA: OR=1.97, 95% CI=1.22-3.19; GA+AA: OR=1.93, 95% CI=1.21-3.08). No associations were observed between rs9807813, rs428253 and osteosarcoma risk (P>0.05). Stratification analysis showed that there was association between rs4740 polymorphisms and the tumor metastasis (P<0.05). No associations between rs4740 polymorphisms and patients' gender, tumor location (P>0.05). Conclusion: Our data suggest that the elevated expression of EBI3 is involved in the carcinogenesis of osteosarcoma, and EBI3 rs4740 polymorphisms confer the susceptibility to osteosarcoma risk and the osteosarcoma metastasis.

Keywords: EBI3, osteosarcoma, polymorphism, association

Introduction

Osteosarcoma is the most common type of malignant bone tumor. It occurs mostly in adolescents and people over 50 years of age and usually affects more males than females [1]. The etiology of osteosarcoma remains to be elucidated. Many studies have suggested that osteosarcoma is caused by multiple factors, such as radiotherapy, age, genetic predisposition [2]. Among them, the genetic factors may contribute an important part to the carcinogenesis of osteosarcoma. Currently, some gene variants are reported to be associated with the risk of osteosarcoma, including CTLA-4, TGF-β1 and IL-27 [3, 4]. IL-27 has two subunits, including Epstein-Barr-induced gene 3 product (EBI3) and p28 [5]. It can inhibit tumor progression through several mechanisms, regardless of tumor immunogenicity, which potentially making it a valuable agent in the treatment of tumors [5].

EBI3 is a member of the IL-12 heterodimeric cytokine family. Besides IL-27, EBI3 is associates with the p35 to form IL-35 (EBI3/p35) [6, 7]. The expression of EBI3 is significant up-regulation in variety of cancers, such as breast cancer [8], gastric cancer [9] and pancreatic ductal adenocarcinoma [10]. EBI3 functions as a soluble α-receptor, and IL-27 can directly activate its target cells through a heterodimer of glycoprotein 130 and WSX-1 [11]. Importantly, the role of EBI3 in IL-27 is not identical to the IL-35. There was study reported that EBI3-deficient mice showed a phenotype of IL-27-deficiency rather than IL-35-deficiency during anti-tumor T-cell responses [12].

Previously, we found that the expression of IL-27 was decreased in osteosarcoma [4]. Because EBI3 forms IL-27 together with p28, it will be important to clarify whether EBI3 is involved in the pathogenesis of osteosarcoma. In addition, EBI3 polymorphisms are reported

Table 1. Primer sequences for genotyping EBI3 polymorphisms

Variants	Primer sequence
rs4740	F: 5'-CAGTGGCCCCTCTCCATCTTCT-3'
	R: 5'-CGAGGACCCAGGTGCTGTCTG-3'
rs9807813	R: 5'-AAATTAGCTGGGCATGTTGG-3'
	F: 5'-TCAAGTGATCCTCCCACCTC-3'
rs428253	F: 5'-AAAAGTAGCCGGGCATGGT-3'
	R: 5'-CAATGGTGGGATGGGGTTAC-3'

to be closely associated with susceptibility to several diseases, such as pulmonary tuberculosis [13], allergic rhinitis [14], and chronic rhinosinusitis [15]. However, to date, no study has explored the associations of EBI3 polymorphisms with osteosarcoma risk. Therefore, the present study was aimed to investigate the association between the EBI3 polymorphisms and osteosarcoma risk in Chinese population.

Materials and methods

Study population

The case-control population study was hospital-based. The study group included 108 newly diagnosed osteosarcoma patients recruited from the affiliated hospital of Youjiang Medical College for Nationalities between 2005 and 2012. All patients were diagnosed without a familial cancer history. A total number of 216 controls were recruited from healthy volunteers who visited the hospitals at the same time for general health exams. All the control subjects were matched with the patient population in terms of age, sex, and residence area (urban or rural). All subjects were unrelated ethnic Han Chinese. Written informed consent was obtained from each participant. The study was approved by the Review Boards of the affiliated hospital of Youjiang Medical College for Nationalities. Each study participant provided a peripheral blood sample.

Selection of SNPs

The selection of tagging SNPs (tag SNPs) of EBI3 gene was using the International Haplotype Mapping (HapMap) SNP databases, and the screened region was extended 2000 base pairs (bp) upstream of the annotated transcription start site and downstream at the end of the last exon in each gene. The data was analyzed using Haplo View 4.2 software. The minor allele frequencies (MAF) of these SNPs were greater than 5%, and the pair-wise r^2 values were greater than 0.8. Finally, we selected three SNPs for EBI3, including rs4740, rs9807813, rs428253.

DNA extraction

Genomic DNA was extracted from EDTA-anticoagulated peripheral blood leukocytes by the salting-out method [16]. Briefly, 3 mL of blood was mixed with Triton lysis buffer (0.32 M sucrose, 1% Triton X-100, 5 mM $MgCl_2$, H_2O, 10 mM Tris-HCl, pH 7.5). Leukocytes were spun down and washed with H_2O. The pellet was incubated with proteinase K at 56°C and subsequently salted out at 48°C using a saturated NaCl solution. Precipitated proteins were removed by centrifugation. The DNA in the supernatant fluid was dissolved in 300 mL of H_2O.

Genotype determination

The genotypes of EBI3 rs4740, rs9807813 and rs428253 were analyzed using the SNaPshot Multiplex Kit (Applied Biosystems Co., Ltd., Foster City, CA, USA). The PCR primer sequences were designed using online primer 3.0 (version 0.4.0) tool and shown in **Table 1**. The SNP genotyping procedure has been described previously [17]. Briefly, the SNaPshot reactions were executed in a 10 μL final volume containing 5 μL of the SNaPshot Multiplex Kit (ABI), 1 μL primer mix, 2 μL water, and 2 μL templates consisting of the multiplex PCR products. The response procedures were: initial denaturation at 96°C for 1 min; denaturation at 94°C for 20 s; annealing at 52°C for 5 s; extension at 60°C for 40 s; and for a total of 24 cycles. The purified products (0.5 μL) were mixed with 9 μL of Hi-Di and 0.5 μL of the Liz120 size standard (Applied Biosystems Co., Ltd.). The samples were incubated at 95°C for 5 min and then loaded onto an ABI 3130XL DNA sequence detector for capillary electrophoresis. The results were analyzed with GeneMapper 4.0 (Applied Biosystems Co., Ltd.). Randomly selected DNA samples from each genotype were analyzed in duplicate using ligation detection reaction and sequence analysis.

Measurement of plasma EBI3 levels

The plasma samples were collected before chemotherapy or other medications on the patients

Table 2. Characteristics of the study population

Variable	Osteosarcoma (n=108)	Controls (n=216)	*P*
Mean Age (years)	27.6±1.4	27.6±1.4	0.076
>20	36 (33.3)	58 (26.9)	0.225
≤20	72 (66.7)	158 (73.1)	
Gender n (%)			
Male	70 (64.8)	134 (62.0)	0.625
Female	38 (35.2)	82 (38.0)	
Smoker n (%)			
Never smoker=1	52 (48.2)	102 (47.2)	0.834
Former smoker=2	36 (33.3)	68 (31.5)	
Current smoker=3	20 (18.5)	46 (21.3)	
Alcohol consumption n (%)			
Drinkers	53 (49.1)	110 (50.9)	0.844
Non-drinkers	55 (40.9)	106 (49.1)	
Tumor location n (%)			
Long tubular bones	90 (83.3)		
Axial skeleton	18 (16.7)		
Metastasis			
Yes	34 (31.5)		
No	74 (68.5)		

Table 3. Genotype frequencies of EBI3 polymorphism in osteosarcoma and controls

Polymorphism	Osteosarcoma (n=108)	Controls (n=216)	OR (95% CI)
rs4740			
GG	49 (45.4)	133 (61.6)	1.00
GA	53 (49.1)	73 (33.8)	1.97 (1.22-3.19)
AA	6 (5.6)	10 (4.6)	1.63 (0.56-4.72)
GA+AA	59 (54.7)	83 (38.4)	1.93 (1.21-3.08)
rs9807813			
CC	51 (47.2)	122 (56.5)	1.00
CT	47 (43.5)	74 (34.3)	1.49 (0.91-2.43)
TT	10 (9.3)	20 (9.3)	1.19 (0.52-2.71)
CC+CT	57 (16.0)	94 (43.6)	1.42 (0.90-2.26)
rs428253			
CC	63 (58.3)	134 (62.0)	1.00
CG	42 (38.9)	73 (33.8)	1.22 (0.75-1.98)
GG	3 (2.8)	9 (4.2)	0.71 (0.19-2.71)
CG+GG	45 (73.6)	132 (62.9)	1.17 (0.73-1.87)

from venous blood at room temperature, and stored at -80°C until use. The quantity determination of plasma EBI3 was performed by enzyme-linked immunosorbent assay kits (Fermentas China Co., Ltd.) according to the manufacturer's instruction. Developed color reaction was measured as OD450 units on an enzyme-linked immunosorbent assay reader (BIO-RAD 680, Tokyo, Japan). The concentration of cytokines was determined using standard curve constructed with the kit's standards over the range of 0-10,000 pg/mL.

Statistical analysis

Demographic and clinical characteristics of patients and controls were presented as percentages or mean and standard deviation (SD), and differences between them were tested using the chi-square test or Student's t test as appropriate. Genotype of polymorphisms on EBI3 gene were compared between osteosarcoma patients and controls using the chi-square test and Fisher's exact test when appropriate, and odds ratios (OR) and 95% confidence intervals (CI) were calculated to assess the relative risk conferred by a particular genotype. The goodness of fit test for Hardy-Weinberg equilibrium (HWE), calculating the expected frequencies of each genotype and comparing them with the observed values for subjects, was performed using chi-square test. The relationship between polymorphisms and clinical characteristics was determined using chi-square test. Haplotypes and linkage disequilibrium (LD) were determined based on the expectation maximization algorithm using the SNPStats program [18]. All statistical analyses were performed using SPSS16.0. All statistical tests were considered significant with a level of $P<0.05$.

Results

Demographic and clinical characteristics of study participants

The demographic and clinical characteristics of the osteosarcoma patients and controls were shown in **Table 2**. There were no significant differences in age, gender distribution, smoking status and alcohol consumption between osteosarcoma patients and controls ($P>0.05$).

Table 4. Haplotype of EBI3 association with osteosarcoma (n=324)

rs4740	rs9807813	rs428253	Frequency	OR (95% CI)	*P*-value
G	C	C	0.7159	1.00	---
A	T	G	0.2145	1.24 (0.81-1.89)	0.321
G	T	C	0.0403	0.39 (0.13-1.17)	0.095
A	T	C	0.0261	17.26 (3.84-77.55)	<0.001

Table 5. Genotype frequencies of rs4740 in relation to clinical parameters

Variable	Osteosarcoma patients (n=108)		OR (95% CI)
Gender	Male (n=70)	Female (n=38)	
GG	32 (45.7)	17 (44.7)	1.00
GA	34 (48.6)	19 (50.0)	1.05 (0.47-2.37)
AA	4 (5.7)	2 (5.3)	0.94 (0.16-5.67)
GA+AA	38 (54.3)	21 (55.3)	1.04 (0.47-2.30)
Location	L (n=90)	A (n=18)	
GG	44 (48.9)	5 (27.8)	1.00
GA	42 (46.7)	11 (61.1)	2.31 (0.73-7.20)
AA	4 (4.4)	2 (11.1)	4.40 (0.67-13.02)
GA+AA	45 (50.0)	14 (77.8)	2.48 (0.82-7.56)
Metastasis	Yes (n=34)	No (n=74)	
GG	10 (29.4)	39 (52.7)	1.00
GA	20 (58.8)	33 (44.6)	7.80 (1.25-48.82)
AA	4 (11.8)	2 (2.7)	3.30 (0.55-19.68)
GA+AA	24 (60.6)	35 (47.3)	2.67 (1.12-6.37)

L, long tubular bones; A, axial skeleton.

Plasma EBI3 levels analysis

The plasma levels of EBI3 were significantly increased in osteosarcoma patients compared with healthy controls (768.2±203.6 pg/mL vs. 127.4±77.8 pg/mL, *P*<0.01). Patients with tumor metastasis showed a elevated plasma levels of EBI3 compared to those without tumor metastasis (988.5±317.4 pg/mL vs. 598.2± 224.1 pg/mL, *P*<0.01).

Genotype distribution of EBI3 polymorphisms

The genotype frequencies of EBI3 rs4740, rs9807813 and rs428253 polymorphisms among the osteosarcoma patients and the controls were shown in **Table 3**. The genotype distribution for each polymorphism was all in agreement with HWE (*P*>0.05).

The AA genotype and dominant model GA+AA of rs4740 were associated with risk of osteosarcoma compared with wild-type GG (GA: OR=1.97, 95% CI=1.22-3.19; GA+AA: OR=1.93, 95% CI=1.21-3.08). Genotype AA was not associated with risk of osteosarcoma compared with wild-type GG (*P*>0.05). However, genotypes of EBI3 rs9807813 and rs428253 were not associated with risk of osteosarcoma (*P*>0.05).

Haplotype analysis of the EBI3 gene

We performed the haplotype analysis for EBI3 rs4740, rs9807813 and rs428253, The LD were observed between locus rs4740 and locus rs9807813 (r=0.892, D'=0.9817), locus rs4740 and locus rs428253 (r=0.920, D'=0.9997), locus rs9807813 and locus rs428253 (r=0.890, D'=0.9996). Three haplotypes were identified, but only the haplotype of A-T-C was found to be significant confer decreased the risk of osteosarcoma (OR=0.06, 95% CI=0.01-0.26). See **Table 4**.

rs4740 polymorphisms and clinical parameters in osteosarcoma

Because the rs4740 polymorphisms were associated with osteosarcoma risk, we further evaluated the associations of rs4740 polymorphisms with clinical parameters in osteosarcoma patients. The stratification analysis including age, gender, tumor location, and metastasis, and the results were shown in **Table 5**. We observed that the frequency AA genotype and dominant model GA+AA of rs4740 was significantly different between patients with or without tumor metastasis (*P*<0.05). However, no association was found between rs4740 polymorphisms and patients' age, gender and tumor location (*P*>0.05).

Discussion

EBI3 is firstly identified by its induced expression in B lymphocytes in response Epstein-Barr virus infection. It encodes a secreted glycoprotein belonging to the hematopoietin receptor family and contains 2 fibronectin type III domains [19]. EBI3 is involved in many cancers and the high expression of EBI3 in blood or tissue is associated with the large tumor size or tumor metastasis and poor prognosis [8-10]. In this study, the plasma EBI3 levels were elevat-

ed significantly in osteosarcoma patients compared with controls. Moreover, the plasma EBI3 levels were higher in patients with tumor metastasis than those without tumor metastasis, suggesting that EBI3 participates in the pathogenesis of osteosarcoma and potential to be a diagnostic biomarker of osteosarcoma.

Previously, several studies reported that EBI3 gene polymorphisms are associated with pulmonary tuberculosis [13], allergic rhinitis [14], chronic rhinosinusitis risk [15]. Variant rs4740 is a nonsynonymous SNP (ns SNPs) and G allele replaced by A allele leads to substitution of valine with isoleucine at position 201 which located in fibronectin type III domains of EBI3. In a genome bioinformatics analysis of ns SNPs, Burke et al [20] found that rs4740 mutation was likely to affect the structure or function of the EBI3 gene. In another study, Van Bergen et al [21] identified that EBI3 gene rs4740 was a new minor histocompatibility antigen and this variation could influence T-cell recognition. More recently, a study using the Identify Candidate Causal SNPs and Pathways (ICSNPathway) to analyze the diabetic nephropathy GWAS data, they found that rs4740 altered the role of EBI3 in various pathways and processes [22].

In this study, we selected three variants of EBI3 gene and analyzed the association between them and osteosarcoma risk. We found that EBI3 gene rs4740 genotype GA and dominant model GA+AA was associated with the risk of osteosarcoma. This result is similar to the Zheng et al [13] reported, who found that EBI3 gene rs4740 was associated with risk of pulmonary tuberculosis. The AA genotype frequency was 5.7%, which is also similar to the Zheng et al [13] reported. Furthermore, the significant difference between the genotype of rs4740 and the tumor metastasis revealed that rs4740 is a functional variant of EBI3 and involves in the role of EBI3 in the pathogenesis of diseases. Taken together, these results tempting to speculate that the rs4740 polymorphism likely to affect the stability, structure or biological function of the EBI3 gene to affect the process of osteosarcoma, which is worth further study to clarify.

In this study, we did not find that variant of rs9807813 and rs428253 associated with osteosarcoma risk, suggesting that these two variants may not involve in the pathogenesis of osteosarcoma. In Zheng et al [13] study, EBI3 rs9807813 and rs428253 was not associated with susceptibility to pulmonary tuberculosis. However, rs428253 has been reported to be associated with risk of allergic rhinitis [14], chronic rhinosinusitis [15]. These results indicated that the role of rs9807813 and rs428253 is different in immunological diseases and tumor. However, the null association also possible caused by the small sample size of enrolled patients, which undermine the statistical power to identify a significant results, thus a larger sample size study is warranted to further confirm the relationship. Furthermore, the haplotype analysis revealed that haplotype of rs4740, rs9807813 and rs428253 was associated with the risk of osteosarcoma, suggesting that rs9807813 and rs428253 may form a haplotype with rs4740 to confer a decreased susceptibility to osteosarcoma.

To our knowledge, this is the first study to evaluate the association between the variants of EBI3 gene polymorphism and the risk of osteosarcoma in a Chinese population. We showed that rs4740 of EBI3 was associated with osteosarcoma risk, and the haplotype constructed from rs4740, rs9807813 and rs428253 was associated with the risk of osteosarcoma. However, there are several limitations of this study need to be noted. First, the sample size of in this study is relatively small, which may undermine the statistical power. Second, our sample is derived from the local region, the genetic background is different from other region, thus the conclusion should be interpreted with caution when apply to other ethnicity. Third, the selection of osteosarcoma patients was hospital based, while the controls were enrolled from the communities, thus, a selection bias may occur. Therefore, to further validate the association between EBI3 polymorphism and osteosarcoma risk, a larger sample size of population-base study with other ethnicities involved is mandatory.

In conclusion, increased of plasma EBI3 levels are associated with the development and progression of osteosarcoma. In addition, the EBI3 variant, rs4740, associated with susceptibility to osteosarcoma in a Chinese population. Taken together, our results identify EBI3 as a susceptibility gene in osteosarcoma and suggests a role for this protein in the pathogenesis of osteosarcoma.

Acknowledgements

This study was supported by National Natural Science Foundation (No. 81260315 and No. 81560461); the Foundation of the Nature Science Fund, Guangxi Province, China (No. 2012GXNSFAA053169); Foundation of the Education Department of Guangxi Province, China (No. KY2015ZD094); Foundation of Guangxi outstanding talent project on 2011; Foundation of open laboratory in Guangxi Medical University on 2011 (KFJJ2011-01).

Disclosure of conflict of interest

None.

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