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Original Article

Single nucleotide polymorphisms in VEGF gene are associated with an increased risk of osteosarcoma

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Abstract: We aimed to assess whether the five common SNPs can affect the risk of osteosarcoma, and its association with demographic characteristics of osteosarcoma. 165 osteosarcoma patients and 330 cancer-free controls were enrolled into our study. Five common SNPs in VEGF gene, -2578C/A (rs699947), -1156G/A (rs1570360), +1612G/A (rs10434), +936C/T (rs3025039) and -634G/C (rs2010963), were detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Conditional logistic regression analyses found that individuals with AA genotype and A allele of rs699947 were associated with an increased risk of osteosarcoma. Individuals with GG genotype and G allele of rs2010963 were associated with an increased risk of osteosarcoma. By stratified analysis, AA genotype of rs699947 was associated with an increased risk of osteosarcoma in those with shorter age, males and a family history of cancer, and GG genotype of rs2010963 was correlated with an increased risk of osteosarcoma in those with shorter age, females and a family history of cancer. Our study suggests that rs699947 and rs2010963 polymorphisms may play a role in the pathogenesis of osteosarcoma.

Keywords: Single nucleotide polymorphism, vascular endothelial growth factor, osteosarcoma

Introduction

Osteosarcoma is an aggressive malignant tumor arising from mesenchymal tissues, which most often occurs in the long bones, such as distal femur, proximal tibia and humeral metaphysis. Osteosarcoma most often affects children between 10 and 25 years of age. It is estimated that the annual incidence of osteosarcoma is about 4-5/10^5 worldwide [1-3].

It is well known that development of osteosarcoma is a complex, multistep and multifactorial process, and many environmental and genetic factors are involved in the process [4-6]. Several previous studies are reported that cancer stem cells can play an important role in causing tumors [7, 8]. Recently, many studies have conducted and reported that many genetic factors are involved in the development of osteosarcoma, such as GSTs, RECQL5, CTLA-4 and MDM2 gene polymorphisms [9-12].

It is reported that vascular endothelial growth factors (VEGF) is a common pro-angiogenic growth factor, and it is one of the most potent endothelial cell mitogens [13, 14]. The stimulation of VEGF under hypoxic conditions is involved in prolonging the lifetime of malignant cell which play a critical role in tumor growth and invasion, and also play an important role in the development of metastases of malignant tumor. It is well known that single nucleotide polymorphisms (SNPs) in VEGF can affect the expression of this gene. Previous studies showed that -2578C/A (rs699947), -1156G/A (rs1570360), +1612G/A (rs10434), +936C/T (rs3025039) and -634G/C (rs2010963) are five common SNPs in the VEGF, and they are reported to have a role in VEGF protein synthesis [15].

Several previous studies reported that polymorphisms in VEGF have an important role in the development of cancers [16-19]. However, only one study reported the association between

Table 1. Demographic and clinical characteristics of osteosarcoma cases and control subjects

Variables	Osteosarcoma cases	%	Control subjects	%	χ^2-test	*P* value
Age						
≤ 20	92	55.6	170	51.6		
> 20	73	44.4	160	48.4	0.79	0.37
Sex						
Male	108	65.3	215	65.3		
Female	57	34.7	115	34.7	0.005	0.95
Family history of cancer						
No	20	12.4	27	8.2		
Yes	145	87.6	303	91.8	1.99	0.16
Tumor location						
Long tubular bones	118	71.3				
Axial skeleton	47	28.7				
Stage						
I-II	105	63.4				
III-IV	60	36.6				
Therapy						
Amputation	35	21.3				
Limb salvage	130	78.7				
Metastasis						
Yes	37	22.3				
No	128	77.7				

three VEGF polymorphisms and osteosarcoma risk in a Chinese population [20]. Therefore, the aim of this present study is to assess whether the five common SNPs can affect the risk of osteosarcoma, and its association with demographic characteristics of osteosarcoma.

Materials and methods

Study population

All patients in this hospital-based case-control study were histologically confirmed to be osteosarcoma at the Second Affiliated Hospital of Inner Mongolia Medical University between January 2011 and January 2013. The study population consisted of 165 osteosarcoma patients who were newly diagnosed and histologically confirmed to be osteosarcoma. The 330 cancer-free controls were randomly recruited from a pool of individuals who came to receive a health check-up in the health check-up center of the same hospitals, and the control subjects are free from any cancer, and two health control subjects were matched to one case by sex and age.

All the cases and control subjects signed an informed consent before participating into this study, and the protocol of this study was approved by the institutional ethnics committee of the First Affiliated Hospital of Chongqing Medical University and the Second Affiliated Hospital of Inner Mongolia Medical University.

Data collection

We collected data regarding demographic and clinical characteristics from a self-designed questionnaire or medical records, including sex, age, family history of cancer, tumor location, metastasis and tumor stage as well as therapy.

Blood samples and genotyping

After participating into this study, cases and control subjects were asked to provide 5 mL blood sample. 0.5 mg/ml EDTA was used for anticoagulant of blood and stored in -70°C until use. Genomic DNA was isolated from peripheral blood with TIANamp Blood DNA Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. Five SNPs of the VEGF gene were detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) according to manufacturer's instructions, including VEGF-2578C/A (rs699947), -1156G/A (rs1570360), +1612G/A (rs10434), +936C/T (rs3025039) and -634G/C (rs2010963).

The primers and probes of rs699947, rs1570360, rs10434, rs3025039 and rs2010963 in VEGF were designed using Sequenom Assay Design 3.1 software (Sequenom, San Diego, CA). The cycling programme of PCR reaction condition involved preliminary denaturation at 95°C for 10 min, followed by 35 cycles of denaturing at 95°C for 30 s, 62°C for 30 s and 72°C for 30 s, and performing a final exten-

Table 2. Genotype distribution of five SNPs in VEGF gene between cases and controls

SNPs	Genotype	Osteosarcoma group	%	Control group	%	HWE	OR (95% CI)[1]	*P* value
rs699947	CC	64	38.6	159	48.2		1.0 (Ref.)	-
	CA	72	43.7	136	41.3		1.32 (0.86-2.02)	0.19
	AA	29	17.7	35	10.5	0.46	2.06 (1.11-3.49)	0.01
Allele	C	199	60.45	454	68.85		1.0 (Ref.)	-
	A	131	39.55	206	31.15		1.45 (1.09-1.92)	0.008
rs1570360	AA	90	54.6	203	61.5		1.0 (Ref.)	-
	AG	45	27.3	81	24.6		1.25 (0.78-1.99)	0.32
	GG	30	18.1	46	13.9	< 0.001	1.47 (0.84-2.55)	0.15
Allele	A	225	68.25	487	73.8		1.0 (Ref.)	-
	G	105	31.75	173	26.2		1.31 (0.97-1.77)	0.06
rs10434	CC	68	41.5	151	45.7		1.0 (Ref.)	-
	CT	76	46.3	146	44.2		1.16 (0.76-1.76)	0.48
	TT	20	12.2	33	10.1	0.79	1.35 (0.68-2.62)	0.35
Allele	C	213	64.65	447	67.8		1.0 (Ref.)	-
	T	117	35.35	213	32.2		1.15 (0.86-1.54)	0.32
rs3025039	CC	111	67.2	232	70.4		1.0 (Ref.)	-
	CT	39	23.5	74	22.4		1.10 (0.68-1.76)	0.67
	TT	15	9.3	24	7.2	< 0.001	1.31 (0.61-2.71)	0.44
Allele	C	261	78.95	539	81.6		1.0 (Ref.)	-
	T	69	21.05	121	18.4		1.18 (0.83-1.66)	0.33
rs2010963	CC	42	25.3	120	36.3		1.0 (Ref.)	-
	CG	80	48.4	151	45.7		1.51 (0.95-2.43)	0.07
	GG	43	26.3	59	18	0.34	2.08 (1.19-3.65)	0.006
Allele	C	163	49.5	390	59.15		1.0 (Ref.)	-
	G	167	50.5	270	40.85		1.48 (1.12-1.95)	0.004

HWE: Hardy-Weinberg equilibrium. [1]Adjusted for sex, age and family history of cancer.

sion at 72°C for 10 minutes. We also randomly selected 5% of the cases and control subjects to repeat genotyping the five SNPs, and the results were confirmed with the previous results.

Statistical analysis

All statistical analyses were conducted using the STATA version 9.0 statistical software. Continuous and categorical variables were expressed shown as the mean ± SD and analyzed by student t test. Categorical variables were expressed as frequencies and percentage of study participants and analyzed by χ^2-test, respectively. The Hardy-Weinberg equilibriums of rs699947, rs1570360, rs10434, rs3025039 and rs2010963 genotype frequencies between groups in control subjects were compared by χ^2-test. The difference in demographic and clinical factors between groups was compared by χ^2-test. Unconditional logistic regression was conducted to assess the effects of rs699947, rs1570360, rs10434, rs3025039 and rs2010963 on the risk of osteosarcoma. The results were expressed with odds ratio (OR) and their corresponding 95% confidence intervals (CI). All *P*-values were two sided, and a *P*-value was regarded as statistically significant when it less than 0.05.

Results

There were 57 females and 108 males in cases with osteosarcoma, and there were 115 females and 215 males in control subjects (**Table 1**). The mean age of cases and control subjects were 19.1 ± 6.7 years and 19.6 ± 7.2 years. Of osteosarcoma patients, 118 patients (71.3%) had tumors at long tubular, and 47 (28.7%) had tumors at axial skeleton. 105 patients (63.4%) were at stage I-II, 60 (36.6%) were at stage III-IV, 35 (21.3%) received amputation, and 37 (22.3%) showed metastasis.

Table 3. Stratification analysis on the association between rs699947 and rs2010963 and demographic characteristics in osteosarcoma risk

Variables	rs699947 Cases/Controls			OR (95% CI)	*P*	OR (95% CI)	*P*	rs2010963 Cases/Controls			OR (95% CI)	*P*	OR (95% CI)	*P*
	CC	CA	AA	CA vs CC		AA vs CC		CC	CG	GG	CG vs CC		GG vs CC	
Age														
≤ 20	36/82	39/72	17/16	1.23 (0.68-2.23)	0.46	2.42 (1.02-5.73)	0.03	23/63	44/76	26/32	1.59 (0.83-3.05)	0.13	2.23 (1.04-4.78)	0.02
> 20	28/77	33/65	12 (19)	1.42 (0.74-2.71)	0.26	1.74 (0.67-4.33)	0.2	19/57	36/75	17/27	1.44 (0.72-2.95)	0.27	1.89 (0.78-4.52)	0.12
Sex														
Male	43/102	48/94	17/19	1.21 (0.71-2.06)	0.45	2.12 (0.93-4.77)	0.04	30/75	52/96	26/44	1.35 (0.76-2.42)	0.27	1.48 (0.74-2.95)	0.23
Female	21/57	24/42	12 (16)	1.55 (0.72-3.35)	0.22	2.04 (0.74-5.46)	0.12	12 (45)	28/55	17/15	1.91 (0.82-4.59)	0.002	4.25 (1.50-12.13)	0.002
Family history of cancer														
No	7 (12)	9 (11)	4 (4)	1.40 (0.33-6.15)	0.6	1.71 (0.23-12.45)	0.53	6 (9)	10 (13)	4 (5)	1.15 (0.26-5.37)	0.83	1.20 (0.16-8.51)	0.83
Yes	57/147	63/125	25/31	1.30 (0.83-2.05)	0.23	2.08 (1.07-3.99)	0.02	36/111	70/138	39/54	1.56 (0.95-2.59)	0.06	2.23 (1.23-4.04)	0.005

The rs699947, rs1570360, rs10434, rs3025039 and rs2010963 genotype frequencies in cases and control subjects, and their association with osteosarcoma risk were shown in **Table 2**.

The genotype distributions of rs699947, rs10434, and rs2010963 were in Hardy-Weinberg equilibrium in the control subjects, but distributions of rs1570360 and rs3025039 were not. Conditional logistic regression analyses found that individuals with AA genotype of rs699947 were associated with an increased risk of osteosarcoma compared to CC genotype (OR = 2.06, 95% CI = 1.11-3.49), and A allele had an increased cancer risk (OR = 1.45, 95% CI = 1.09-1.92). Individuals with GG genotype of rs2010963 were associated with an increased risk of osteosarcoma compared to CC genotype (OR = 2.08, 95% CI = 1.19-3.65), and G allele was correlated with an enhanced risk of cancer (OR = 1.48, 95% CI = 1.12-1.95). However, we did not find significant association between rs1570360, rs10434 and rs3025039 and osteosarcoma risk.

Moreover, we conducted the analysis on the association between rs699947 and rs2010963 in VEGF and demographic characteristics in osteosarcoma patients (**Table 3**). The stratification analysis with variables of age, sex and family history of cancer were shown in **Table 3**. We found that AA genotype of rs699947 was associated with an increased risk of osteosarcoma in those with shorter age, males and a family history of cancer. Moreover, GG genotype of rs2010963 was correlated with an increased risk of osteosarcoma in those with shorter age, females and a family history of cancer.

Discussion

VEGF is considered as a growth factor that regulates angiogenesis in human cancer, and this gene plays an important role in the process of carcinogenesis. The underlying mechanism of VEGF gene polymorphisms on the risk of osteosarcoma is still not very clear. It is well known that angiogenesis is an important factor for the development and prognosis of tumors, VEGF expression can regulates angiogenesis, and thus VEGF can have be involved in promoting endothelial cell proliferation and regulating the extracellular matrix in the blood vessels [21, 22].

Several functional SNPs in the VEGF gene are associated with altering the expression levels of VEGF protein in cancer cells, and thus influence the tumor angiogentic activity and susceptibility of several kinds of cancers, such as bladder cancer, breast cancer, lung cancer and renal cell carcinoma [16-19]. Only one recent study reported the association between VEGF genetic polymorphisms and susceptibility of osteosarcoma [20]. Wang et al. reported that TT genotype and T allele of +936C/T could increase the risk of osteosarcoma, and VEGF genetic variants are potentially related to risk of osteosarcoma [20]. Therefore, we conducted this case-control study to explore the role of five SNPs of VEGF gene on the risk of osteosarcoma.

In our study, we found that AA genotype and A allele of rs699947 and GG genotype and G allele of rs2010963 were associated with increased risk of osteosarcoma. Previous studies also reported that rs699947 polymorphism was associated with risk of cancers [23-27]. Zhao et al. conducted a meta-analysis with 12 studies, and reported that AA genotype of rs699947 and CC genotype of rs2010963 are associated with a risk of colorectal cancer in Asian populations [23]. Song et al. conducted a meta-analysis and found that rs699947 polymorphism may increase lung cancer risk, especially in smoker patients [24]. Xu et al. conducted a meta-analysis with eight studies, and found that rs699947 polymorphism was associated with prostate cancer risk [25]. However, several previous studies reported that the common SNPs of VEGF gene were not associated with risk of cancers [26, 28, 29]. Wang et al. conducted a meta-analysis on the association between VEGF polymorphisms and risk of breast cancer, and found that rs699947 and rs2010963 were not association with risk of breast cancer [28]. Supic et al. conducted a case-control study and found that rs699947 and rs2010963 polymorphisms were not associated with risk of oral squamous cell carcinoma [29]. The results are inconsistent. For the association between VEGF polymorphism and risk of osteosarcoma, only one previous study found that T allele of rs3025039 was associated with increased risk of osteosarcoma, but no association between rs2010963 polymorphism and cancer risk [20]. The results of our study were not in line with previous studies. The discrepancy of these results may be caused by

differences in ethnicities, study design, tumor types, and sample size.

Several limitations should be considered in our study. First, cases and controls were selected from one hospital, which may not be representative of other populations. However, the controls were a random sample from a pool of individuals who came to receive a health check-up, which may well represent the general population. Second, since the rarity of osteosarcoma, the sample size of osteosarcoma patients is relatively small. The small sample size could limit the statistical power to find the association between groups. Third, the risk of osteosarcoma could be modified by many other genetic factors in the angiogenesis pathway except for VEGF. Therefore, further studies with more subjects are needed to confirm the association between *VEGF* gene polymorphisms and risk of osteosarcoma.

In this case-control study, we suggest that rs699947 and rs2010963 in VEGF gene may play a role in the pathogenesis of osteosarcoma. Further large sample studies are needed to confirm these associations.

Disclosure of conflict of interest

None.

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