



## Investigation of the role of *VEGF* gene polymorphisms in the risk of osteosarcoma

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**ABSTRACT.** We conducted a case-control study in a Chinese population, and assessed whether the *VEGF* -634G/C, +936C/T, and +1612G/A polymorphisms could affect the risk of osteosarcoma and its association with environmental factors. A total of 180 consecutive osteosarcoma patients and 360 controls were included in our study. The genotypes of *VEGF* -634G/C, +936C/T, and +1612G/A were determined by the polymerase chain reaction-restriction fragment length polymorphism assay. Conditional logistic regression analyses showed that subjects carrying the GG genotype and the G allele of *VEGF* -634G/C were significantly associated with increased risk of osteosarcoma compared to those with the CC genotype; the ORs (95% CIs) were 2.28 (1.31-3.96) and 1.51 (1.16-1.97) for the GG genotype and G allele, respectively. We found that the GG genotype of *VEGF* -634G/C was associated with a significantly increased risk

of osteosarcoma in patients of either gender with younger age and a family history of cancer. In summary, this study found that the *VEGF* -634G/C gene polymorphism was associated with an increased risk of osteosarcoma.

**Key words:** Single nucleotide polymorphism; Osteosarcoma; Vascular endothelial growth factor

## INTRODUCTION

Osteosarcoma is a rare bone cancer, and has a first peak of incidence in adolescence around 16 years and a second peak in later life after 60 years (Ritter and Bielack, 2010). The fundamental mechanism underlying the development of osteosarcoma is still not well understood. Previous studies have suggested that osteosarcoma is a complex disease, and that multiple factors play important roles in its development, including genetic and environmental factors (de Alava, 2007; Bovee and Hogendoorn, 2010; Powers et al., 2010). Of the former, polymorphisms in *IL-27*, *CD152*, *ITGA3*, *VEGF*, *RECQL5*, and Endothelin-1 (Chang et al., 2014; Tang et al., 2014; Wang et al., 2014; Yang et al., 2014a; Zhi et al., 2014; Zhou et al., 2014) are thought to contribute to osteosarcoma pathogenesis.

Vascular endothelial growth factor (VEGF) is one of the key initiator endothelial cell mitogens and the most potent; it is also reported to have a role in stimulating angiogenesis (Barbera-Guillem et al., 2002; Evensen et al., 2009). Previous experimental studies in rats have shown that VEGF expression can affect the growth and metastasis of malignant cells, and that inhibition of VEGF signaling can contribute to the control of tumor-induced angiogenesis and the growth of tumor cells (Ferrara, 2002; Hicklin and Ellis, 2005; Andersen et al., 2011). The *VEGF* gene is located on chromosome 6p21.3 and has a 14-kb coding region with 8 exons and 7 introns. There are over 30 variants in *VEGF*; three of the common polymorphisms in the *VEGF* gene can influence gene expression and alter the function of the protein, including -634G/C, +936C/T, and +1612G/A (Watson et al., 2000; Ruggiero et al., 2011). *VEGF* -634G/C, +936C/T, and +1612G/A are reported to have a role in VEGF protein synthesis and in the development of several kinds of cancers (Chen et al., 2014; Dong-Ju et al., 2014; Lau et al., 2014; Rahoui et al., 2014; Yang et al., 2014b; Yu et al., 2014). Only one previous study has reported an association between *VEGF* polymorphisms and the development of osteosarcoma (Wang et al., 2014). Therefore, we conducted a case-control study in a Chinese population, and assessed whether *VEGF* -634G/C, +936C/T, and +1612G/A can affect the risk of osteosarcoma and its association with environmental factors.

## MATERIAL AND METHODS

### Study population

A total of 180 consecutive patients with osteosarcoma from the First Affiliated Hospital of Chongqing Medical University and the Second Affiliated Hospital of Inner Mongolia Medical University seen between May 2011 and May 2013 were included in our study. All

patients were newly diagnosed and their osteosarcoma was histologically confirmed. Patients who had a history of tumors were excluded from our study. After a patient was selected, we selected one age- and gender-matched control from individuals who sought a health examination in the Health-check Center of our hospital. A total of 360 controls were enrolled in our study. Control subjects who had a history of cancer were excluded from the study. A written informed consent form was obtained from each patient and control subject.

This study was approved by the Ethics Committees of the First Affiliated Hospital of Chongqing Medical University and the Second Affiliated Hospital of Inner Mongolia Medical University.

Baseline characteristics of all patients and controls were obtained using a self-designed questionnaire and medical records, and included gender, age, family history of cancer, tumor location, metastasis, and tumor stage as well as applied therapies.

### Blood samples and genotyping

For DNA extraction, approximately 5 mL peripheral blood was obtained from each subject and was stored at  $-20^{\circ}\text{C}$  with a non-anticoagulant until use. Genomic DNA was extracted from peripheral blood using a TIANamp Blood DNA Kit (Tiangen, Beijing, China), and the isolated DNA was dissolved in water, according to manufacturer instructions.

Single nucleotide polymorphism (SNP) genotyping of the *VEGF*-634G/C, +936C/T, and +1612G/A polymorphisms were conducted by a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. Probes and primers for *VEGF* -634G/C, +936C/T, and +1612G/A were designed using the Primer 5.0 software (PREMIER Biosoft, Palo Alto, CA, USA). The primers for *VEGF* -634G/C were 5'-GTAGCAAGAGCTCCAGAGAGAAGT-3' and 5'-TGGACGAAAAGTTTCAGTGCGACG-3', forward and reverse, respectively. The primers for *VEGF* +936C/T were 5'-CTCGGTGATTTAGCAGCAAG-3' and 5'-CTCGGTGATTTAGCAGCAAG-3'. The primers for *VEGF*+1612G/A were 5'-CACATGCTGCACGCGCATCTCA-3' and 5'-ACCCCAGGAAGGGGAGCAGGA-3'. PCR was conducted in a 25- $\mu\text{L}$  reaction solution with 25 mM  $\text{MgCl}_2$ , 2 mM deoxynucleotide triphosphates, 1 mM  $\text{MgCl}_2$ , 1.25 U Taq polymerase and 0.5  $\mu\text{L}$  5X PCR buffer. The cycling program for PCR amplification was as follows: one 5-min initial denaturation step at  $94^{\circ}\text{C}$ , followed by 35 cycles of 45 s denaturation at  $94^{\circ}\text{C}$ , 60 s annealing at  $62^{\circ}\text{C}$ , and 60 s extension at  $72^{\circ}\text{C}$ , followed by a final extension at  $72^{\circ}\text{C}$  for 10 min. For quality control, 20% blood samples in cases and controls were randomly selected, and genotyping was repeated in 20% samples to check for accuracy.

### Statistical analysis

Continuous variables are reported as means  $\pm$  standard deviation (SD) and analyzed by the Student *t*-test. Categorical variables are shown as N (%) and analyzed by the  $\chi^2$  test. The  $\chi^2$  test was performed to verify whether *VEGF* -634G/C, +936C/T, and +1612G/A genotype distributions were in Hardy-Weinberg equilibrium. The associations between patients with osteosarcoma and controls were assessed using conditional logistic regression, and the results are reported as ORs and their CIs. Two-tailed P values  $<0.05$  were considered to represent

statistically significant differences. All statistical analyses were conducted using the STATA version 9.0 statistical software (Stata Corporation, USA).

## RESULTS

### Characteristics of patients and controls

The demographic and clinical characteristics of the study subjects are shown in Table 1. As expected, no significant differences were identified between the patients and control subjects in terms of age and gender ( $P > 0.05$ ). The patients with osteosarcoma were more likely to have a family history of cancer. Of the 180 patients, 128 (71.11%) had long tubular bones, 105 (58.33%) were at stage I-II, 136 (75.56%) had received limb salvage, and 145 (80.56%) did not show metastasis.

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

Variables	Osteosarcoma cases (N = 180)	%	Control subjects (N = 360)	%	$\chi^2$ test	P value
Age (years)						
$\leq 20$	123	68.33	236	65.56		
$> 20$	57	31.67	124	34.44	0.42	0.52
Gender						
Male	110	61.11	220	61.11		
Female	70	38.89	140	38.89	0.00	1.00
Family history of cancer						
No	17	9.44	11	3.06		
Yes	163	90.56	349	96.94	9.06	0.002
Tumor location						
Long tubular bones	128	71.11				
Axial skeleton	52	28.89				
Stage						
I-II	105	58.33				
III-IV	75	41.67				
Therapy						
Amputation	44	24.44				
Limb salvage	136	75.56				
Metastasis						
Yes	35	19.44				
No	145	80.56				

### Genotypes of eight gene polymorphisms

The genotype distributions of the *VEGF* -634G/C, +936C/T, and +1612G/A polymorphisms demonstrated Hardy-Weinberg equilibrium (Table 2). Furthermore, we found that the minor allele frequencies of the three gene polymorphisms in the control group were similar to those in dbSNP. Conditional logistic regression analyses showed that subjects carrying the GG genotype and the G allele of *VEGF* -634G/C had significant association with increased risk of osteosarcoma compared to those having the CC genotype; the ORs (95% CIs) were 2.28 (1.31-3.96) and 1.51 (1.16-1.97) for the GG genotype and the G allele, respectively. However, we did not find significant association between the *VEGF* +936C/T and +1612G/A polymorphisms and risk of osteosarcoma.

**Table 2.** Genotype distribution of three SNPs in the VEGF gene between cases and controls.

VEGF	Genotype	Osteosarcoma group	%	Control group	%	HWE	OR (95%CI) <sup>1</sup>	P value
VEGF -634G/C	CC	48	26.67	138	38.33	0.95	1.0 (Ref.)	-
	CG	90	50.00	170	47.22		1.52 (0.98-2.36)	0.05
	GG	42	23.33	53	14.44		2.28 (1.31-3.96)	0.002
Allele	C	186	51.67	446	61.94		1.0 (Ref.)	-
	G	174	48.33	276	38.06		1.51 (1.16-1.97)	-
VEGF +936C/T	CC	66	36.67	148	41.11	0.16	1.0 (Ref.)	-
	CT	92	51.11	175	48.61		1.18 (0.79-1.77)	0.4
	TT	22	12.22	37	10.28		1.33 (0.69-2.53)	0.35
Allele	C	224	62.22	471	65.42		1.0 (Ref.)	-
	T	136	37.78	249	34.58		1.15 (0.87-1.51)	0.3
VEGF +1612G/A	CC	77	42.78	163	45.28	0.58	1.0 (Ref.)	-
	CT	80	44.44	155	43.06		1.09 (0.73-1.63)	0.65
	TT	23	12.78	42	11.67		1.16 (0.62-2.13)	0.62
Allele	C	234	65.00	481	66.81		1.0 (Ref.)	-
	T	126	35.00	239	33.19		1.08 (0.82-1.43)	0.55

HWE = Hardy-Weinberg equilibrium. <sup>1</sup>Adjusted for gender, age and family history of cancer.

### Interaction between the VEGF -634G/C polymorphism and the demographic characteristics of osteosarcoma

In addition, we assessed the association between the VEGF -634G/C polymorphism and the demographic characteristics of patients with osteosarcoma, including age, gender, and family history of cancer (Table 3).

**Table 3.** Stratification analysis on the association between the VEGF -634G/C gene polymorphism and demographic characteristics in osteosarcoma risk.

Variables	VEGF -634G/C						OR (95%CI)	P	OR (95%CI)	P
	Cases			Controls						
	CC	CG	GG	CC	CG	GG				
							CG vs CC	GG vs CC		
Age (years)										
≤20	47	90	42	138	170	53	1.54 (0.89-2.68)	0.1	2.84 (1.39-5.37)	0.001
>20	31	59	33	90	111	35	1.58 (0.73-3.46)	0.21	1.50 (0.49-4.39)	0.42
Gender										
Male	28	55	27	81	104	35	1.53 (0.86-2.74)	0.12	2.23 (1.09-4.55)	0.02
Female	19	35	15	57	66	18	1.59 (0.78-3.28)	0.17	2.6 (1.02-6.42)	0.03
Family history of cancer										
No	4	10	3	4	5	2	2.00 (0.25-16.01)	0.44	1.5 (0.10-27.08)	0.72
Yes	43	80	39	134	165	51	1.51 (0.96-2.40)	0.06	2.38 (1.34-4.23)	0.001

We found that the GG genotype of VEGF -634G/C was associated with a significantly increased risk of osteosarcoma in both genders, and in younger patients and in those with a family history of cancer. However, we did not find significant interaction between the VEGF -634G/C gene polymorphism itself and age, gender, or family history of cancer in the risk of osteosarcoma.

## DISCUSSION

In the present study, we investigated the influence of SNPs in the VEGF gene on the

risk of osteosarcoma, and examined the effect of gene-environmental interaction on the development of this cancer in a Chinese population.

It is well known that angiogenesis is correlated with the development of several tumors, and that VEGF can regulate angiogenesis in human cancer (Qin et al., 2014; Rahoui et al., 2014). The expression of VEGF can promote endothelial cell proliferation and remodel the extracellular matrix of the blood vessels; alterations in VEGF expression can play a role in the development of cancers (Roy et al., 2006; Kushner and Bautch, 2013). As functional SNPs in the *VEGF* gene can affect the expression of this protein in cancer cells, these SNPs can thus promote the angiogenic activity of tumors and accelerate the process of carcinogenesis (Ajaz et al., 2011; Sáenz-López et al., 2013). *VEGF* -634G/C is located in the 3'-untranslated region of the gene, and has been reported to have a role in influencing plasma VEGF levels (Stathopoulou et al., 2013).

Our study found that the *VEGF* -634G/C polymorphism was associated with an increased risk of osteosarcoma. Several previous studies have reported the association between the *VEGF* -634G/C polymorphism and the risk of several cancers (Hsiao et al., 2007; Sfar et al., 2009; Sa-Nguanraksa et al., 2013; Deng et al., 2014), but the results have been inconsistent. Sa-Nguanraksa et al. (2013) reported that the *VEGF* -634G/C polymorphism had a significant role in breast cancer susceptibility and aggressiveness. However, other studies did not find that *VEGF* -634G/C played a role in the development of cancer. Deng et al. (2014) conducted a case-control and a meta-analysis study in a Chinese population, and found that the *VEGF* -634G/C polymorphism had no association with lung cancer risk. Hsiao et al. (2007) did not find significant association between the *VEGF* -634G/C polymorphism and the risk of thyroid cancer development and regional lymph node metastasis in men. The discrepancy between these results might be caused by differences in ethnicities, study design, tumor types, and sample size.

Several previous studies have reported an association between *VEGF* polymorphisms and the development and prognosis of osteosarcoma (Dong-Ju et al., 2014; Wang et al., 2014). Wang et al. (2014) conducted a case-control study on *VEGF* genetic polymorphisms and osteosarcoma susceptibility in a Chinese population, and found that the T allele and the TT genotype of the +936C>T variant could be a risk factor that increased the susceptibility to osteosarcoma. However, our study reported results inconsistent with these. Further large sample studies are needed to confirm the association between *VEGF* polymorphisms and the risk of osteosarcoma.

In summary, we found that the *VEGF* -634G/C gene polymorphism is associated with an increased risk of osteosarcoma. Due to the limitations of our study, further well designed, multicenter studies with larger sample sized are needed to confirm our findings.

## Conflicts of interest

The authors declare no conflict of interest.

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