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Effect of polymorphisms of vascular endothelial growth factor on prognosis in osteosarcoma patients

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ABSTRACT. We investigated the association between vascular endothelial growth factor (VEGF) gene +1612G/A, -634C/G, and +936G/C and the clinical outcome of osteosarcoma. Genomic DNA was isolated from blood samples, and 3 VEGF gene polymorphisms (+1612G/A, -634C/G, and +936G/C) were analyzed using polymerase chain reaction-restriction fragment length polymorphism. Of the 194 patients, 82 patients (42.27%) showed a good response to chemotherapy, while 73 (37.63%) died during the follow-up period. When comparing good and poor responders, we observed no significant association between the VEGF +1612G/A, -634G/C, and +936T/C polymorphisms and clinical outcome of osteosarcoma patients. According to Cox regression analysis, the VEGF +1612A/G, -634G/C, and +936T/C polymorphisms did not statistically significantly increase the risk of overall survival of patients with osteosarcoma. This study showed that VEGF +1612A/G, -634G/C, and +936T/C polymorphisms were not

related to the response to chemotherapy and clinical outcome of osteosarcoma patients.

Key words: Chemotherapy; Clinical outcome; Osteosarcoma; Vascular endothelial growth factors

INTRODUCTION

Human osteosarcoma is one of the most common primary malignant bone tumors, often originating in the metaphyses of long bones and occurring in children and adolescents (Ries et al., 1999; Savage et al., 2007; Duong and Richardson, 2013). It is estimated that there are 4-5 cases per million worldwide, and is a leading cause of cancer-related deaths in children and young adults (Ries et al., 1999). Because of the high incidence of systemic spread, the prognosis of patients with osteosarcoma is unsatisfactory (Longhi et al., 2006). Through combinatorial chemotherapy, the 5-year survival rate of patients with osteosarcoma has been greatly improved to 60-70% in recent years (Longhi et al., 2001). However, 40% of osteosarcoma patients still show poor response to chemotherapy, with a high risk of local recurrence and distant metastasis even after receiving curative resection of the primary tumor and intensive chemotherapy (Collins et al., 2013). Moreover, the molecular events initiating and propagating osteosarcomagenesis remain unknown. Therefore, the identification of genetic factors that may influence chemotherapy toxicity and clinical outcome of osteosarcoma can be helpful for improving osteosarcoma treatment.

The vascular endothelial growth factors (VEGF) gene is one of the most potent endothelial cell mitogenes and consists of 8 exons located on chromosome 6p21.3; the gene exhibits alternative splicing to form a family of proteins (Vincenti et al., 1996). VEGF is a potent regulator of angiogenesis and thus plays a role in the development and prognosis of solid tumors (Kapahi et al., 2014; James et al., 2014). Several common single-nucleotide polymorphisms (SNPs) in the VEGF gene have been reported to have a role in gene expression. Three potentially functional SNPs of the VEGF gene, VEGF +1612G/A, -634C/G, and +936T/C, influence plasma VEGF levels. VEGF gene polymorphisms are associated with various cancers, such as lung, colorectal, breast, oral, ovarian, gastric, and bladder cancers (Kim et al., 2013; Leng et al., 2013; Fu et al., 2013; Oh et al., 2013; Jaiswal et al., 2013; Kushlinskii et al., 2014).

Although several studies reported an association between VEGF polymorphisms and cancer risk, few studies have examined the association between the 3 gene polymorphisms and clinical outcome of patients with osteosarcoma. Only one study investigated the association between VEGF polymorphisms and risk of osteosarcoma (Wang et al., 2014). The aim of this study was to investigate the association between VEGF gene +1612G/A, -634C/G, and +936T/C and the clinical outcome of osteosarcoma.

MATERIAL AND METHODS

All patients in our study were histologically confirmed to have osteosarcoma between January 2008 and December 2010 at Zhejiang Provincial Corps Hospital. These patients were newly diagnosed within 1 month. Written informed consent was obtained from each patient before inclusion in the study. The protocol of this study was approved by the ethics committee board of the Zhejiang Provincial Corps Hospital.

All patients in this study received chemotherapy before surgery, including doxorubicin, methotrexate, and cisplatin. After surgery, all patients received adjuvant chemotherapy, including methotrexate, cisplatin and vincristine, and cyclophosfamide. Response to chemotherapy was divided into 2 groups according to criteria from the European Organization for Research and Treatment of Cancer, including good responders and poor responders. Overall survival (OS) was calculated to evaluate the association between VEGF polymorphisms and osteosarcoma prognosis. OS was assessed from the date of entrance into the study until the date of death or the last follow-up. All patients selected were followed up until the December 31, 2012.

DNA extraction and polymerase chain reaction (PCR) amplification

All patients were asked to provide 5 mL venous blood, and genomic DNA was isolated using a Qiagen Blood Kit (Qiagen, Hilden, Germany) according to manufacturer instructions. Polymorphisms of VEGF +1612G/A, -634C/G, and +936T/C were assessed by PCR-restriction fragment length of polymorphism. Primers of VEGF +1612G/A, -634C/G, and +936T/C were designed using the Sequenom Assay Design 3.1 software (San Diego, CA, USA), as shown in Table 1. The PCR reaction was performed in 50 μL reaction solution containing 25 mM $MgCl_2$, 2 mM 4X dNTPs, 20 μM primer, and 5 U/μL *Taq* DNA polymerase. The PCR reaction was performed under the following conditions: beginning at 94°C for 5 min, followed by 35 cycles of denaturing at 94°C for 45 s, annealing at 62°C for 60 s, and extension at 72°C for 60 s; final extension was performed at 72°C for 10 min. The PCR products were visualized by 1.0% agarose gel electrophoresis with staining using ethidium bromide staining and ultraviolet light. For quality control, a group randomly chosen of 10% of the cases and control subjects were selected, and the results of repeated samples showed 100% concordance.

Table 1. Primers of VEGF genetic polymorphisms for PCR-RFLP analysis.

SNPs	Primer sequences	Restriction enzymen	PCR products (bp)
+1612G/A	5'-CACATGCTGCACGCGCATCTC A-3' 5'-ACCCCAGGAAGG GGAGCAGGA-3'	*Mnl*I	AA: 206 AG: 206, 180, 26 GG: 180, 26
-634C/G	5'-GTAGCAAGAGCTCCAGAGAGAAGT-3' 5'-TGGACGAAAAGTTTCAGTGCGACG-3'	*Bsm*FI	CC: 197 CG: 197, 157, 40 GG: 157, 40
+936T/C	5'-CTC GGT GATTTAGCAGCAAG-3' 5'-CTCGGTGATTTAGCAGCAAG-3'	*Nla*III	TT: 122, 86 TC: 208, 122, 86 CC: 208

Statistical analysis

Continuous variables were expressed as means ± standard deviation, and categorical variables were expressed as N (%) of study participants. The odds ratios (OR) and corresponding 95% confidence intervals (CIs) were calculated by unconditional logistic regression analysis and utilized to assess the potential association between genotypes frequencies and response to chemotherapy of osteosarcoma patients. Survival distributions were assessed using the Kaplan-Meier method and a log-rank test. HRs and their CIs were used to analyze the association between VEGF +1612G/A, -634C/G, and +936T/C and survival time using a multivariate Cox proportional hazards model. All tests were 2-sided and $P < 0.05$ was considered to be statistically significant.

RESULTS

The demographic and clinical distributions of cases included are shown in Table 2. A total of 194 patients were included in our study. The mean age at diagnosis of all patients was 17.8 ± 12.5 years. Of the 194 osteosarcoma patients, 143 (73.71%) were less than 20 years old, 130 (67.01 %) were male, and 100 (51.55%) showed tumor location at the femur, 66 (34.02%), in the tibia/fibula, 138 (71.13%) did not show metastasis, 114 (58.76%) received limb salvage, 112 (57.73%) showed poor response to chemotherapy, and 73 (37.63%) died during the follow-up period.

Table 2. Demographic and clinical characteristics of osteosarcoma patients.

Indexes	Patients, N	%
Age at diagnosis, y	17.8 ± 12.5	
≤20	143	73.71
>20	51	26.29
Gender		
Male	130	67.01
Female	64	32.99
Tumor location		
Femur	100	51.55
Tibia/fibula	66	34.02
Arm	15	7.73
Central	13	6.70
Metastasis		
No	138	71.13
Yes	56	28.87
Therapy		
Amputation	80	41.24
Limb salvage	114	58.76
Chemotherapy response		
Poor	112	57.73
Good	82	42.27
Death		
No	121	62.37
Yes	73	37.63

Of the 194 patients, 82 patients (42.27%) showed a good response to chemotherapy (Table 3). When comparing good and poor responders, we observed no significant association between VEGF +1612G/A, -634G/C, and +936T/C polymorphisms and clinical outcome of osteosarcoma patients.

Table 3. Association between VEGF polymorphisms and tumor response to chemotherapy.

Genotype		Patients N = 194	%	Tumor response				OR (95%CI)	P value
				Good	%	Poor	%		
+1612G/A	GG	71	36.60	31	38.2	40	35.71	1.0 (Ref.)	-
	GA	90	46.39	36	44.3	54	48.21	0.86 (0.44-1.70)	0.64
	AA	33	17.01	14	17.5	19	16.96	0.95 (0.38-2.37)	0.91
-634G/C	CC	74	38.14	34	41.3	40	35.71	1.0 (Ref.)	-
	CT	100	51.55	41	50.2	59	52.68	0.82 (0.43-1.57)	0.51
	TT	20	10.31	7	8.5	13	11.61	0.63 (0.19-1.95)	0.38
+936T/C	TT	96	49.48	43	52.6	53	47.32	1.0 (Ref.)	-
	TC	76	39.18	32	38.6	44	39.29	0.90 (0.47-1.72)	0.72
	CC	22	11.34	7	8.8	15	13.39	0.58 (0.18-1.67)	0.27

Table 4 shows the potential association between VEGF genetic polymorphisms and overall survival of patients with osteosarcoma. After adjusting for potential confounding factors, we did not find that VEGF +1612G/A, -634G/C, and +936T/C polymorphisms statistically significant increased the risk of overall survival of patients with osteosarcoma.

Table 4. Cox regression analysis of VEGF polymorphisms and overall survival of patients with osteosarcoma.

Genotype		Patients N = 194	%	Death Event	%	Alive Event	%	HR (95%CI)[1]	P value
+1612G/A	GG	71	36.6	24	32.88	47	38.84	1.0 (Ref.)	-
	GA	90	46.39	35	47.95	55	45.45	1.25 (0.62-2.52)	0.51
	AA	33	17.01	14	19.18	19	15.70	1.44 (0.56-3.65)	0.4
-634G/C	CC	74	38.14	25	34.25	49	40.50	1.0 (Ref.)	-
	CT	100	51.55	38	52.05	62	51.24	1.20 (0.61-2.37)	0.57
	TT	20	10.31	10	13.70	10	8.26	1.96 (0.63-5.99)	0.18
+936T/C	TT	96	49.48	33	45.21	63	52.07	1.0 (Ref.)	-
	TC	76	39.18	30	41.10	46	38.02	1.25 (0.64-2.43)	0.49
	CC	22	11.34	10	13.70	12	9.92	1.59 (0.55-4.49)	0.33

[1]Adjusted for gender, age, tumor location, metastasis, and therapy methods.

DISCUSSION

VEGF is a growth factor that regulates angiogenesis, and is considered to be the most potent stimulatory cytokine modifying tumor angiogenesis and regulating tumor metastasis, survival, and tumor spread (Salven et al., 1997). The present study investigated the potential association between VEGF genetic polymorphisms and prognosis of osteosarcoma in a Chinese population. Our study found that VEGF +1612G/A, -634G/C, and +936T/C polymorphisms did not significantly influence the response to chemotherapy, and were not associated with the clinical outcome of patients with osteosarcoma. Our study showed that the VEGF +1612G/A, -634G/C, and +936T/C polymorphisms cannot be used as predictive markers for the response to chemotherapy and clinical outcome of patients with osteosarcoma.

Angiogenesis is an important factor in the development and metastasis of tumors, of which VEGF plays a key role and may enhance endothelial cell proliferation and regulate the extracellular matrix in blood vessels (Roy et al., 2006; Kushner and Bautch, 2013). An increasing number of studies have shown that VEGF expression is associated with an increased risk of solid tumors, and increased VEGF expression and microvessel density in tumor tissues are correlated with advanced stage disease and worse prognosis of these solid tumors (Ferrara, 2002; Iordache et al., 2010; Simonetti et al., 2013). Three common SNPs (VEGF +1612G/A, -634G/C, and +936T/C) in the VEGF gene, located in the 5'- and 3'-untranslated regions of VEGF have been commonly reported to influence protein translation efficiency, circulating plasma concentrations, and tumor tissue expression of VEGF (Watson et al., 2000; Renner et al., 2000; Koukourakis et al., 2004). Several previous studies investigated the role of VEGF polymorphisms and clinical outcome of multiple types of solid tumors, including breast cancer, colorectal cancer, gastric cancer, bladder cancers, and prostate cancer (Jain et al., 2009). However, we found no significant association between VEGF gene polymorphisms and the clinical outcome of osteosarcoma, which disagrees with the results of a previous study (Jain et al., 2009). The inconsistent role of these results may be caused by other functional SNPs in the VEGF gene or other SNPs of the angiogenesis pathway. Further studies are needed to

elucidate the potential role of VEGF polymorphisms in tumor biology.

This study has several limitations. First, cases were selected from 1 hospital, which may not be representative of the general population. There was still a certain risk of selection bias, as the patients were not a random sample of the osteosarcoma population and may not well-represent the actual situation of patients with osteosarcoma. Second, because of the rarity of osteosarcoma, we examined a small number of cases. The relative small number of cases may have limited the statistical power for identifying difference between groups. Third, other SNPs of the angiogenesis pathway may affect the clinical outcome of osteosarcoma besides the *VEGF* gene. Therefore, further large sample and well-designed studies are needed to investigate the association between *VEGF* gene polymorphisms and clinical outcome of osteosarcoma.

In summary, we found that the VEGF +1612G/A, -634G/C, and +936T/C polymorphisms did not affect the response to chemotherapy and clinical outcome of osteosarcoma patients. Further studies in Chinese osteosarcoma patients including larger sample sizes are needed.

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