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Vascular endothelial growth factor genetic polymorphisms and haplotypes in female patients with bisphosphonate-related osteonecrosis of the jaws

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OBJECTIVE: To investigate the polymorphisms of the vascular endothelial growth factor (VEGF) gene in relation to female patients who developed bisphosphonaterelated osteonecrosis of the jaws (BRONJ). METHODS: Test subjects were 30 Italian female patients with BRONJ (Group A). Control subjects were 30 female patients with a history of intravenous bisphosphonate use without any evidence of osteonecrosis (Group B) and 125 unrelated healthy volunteers (Group C). Three singlenucleotide polymorphisms were investigated:)634 G>C, occurring in 5¢ untranslated region (UTR); +936 C>T, occurring in 3¢ UTR; and)2578 C>A of the promoter region. RESULTS: The frequency of the VEGF CAC (+936/)2578/)634) haplotype was increased in patients with BRONJ, compared with female disease-negative controls [odds ratio (OR) = 2.76, 95% CI = 1.09–4.94, P = 0.039; corrected P value: Pc = 0.117], and was also increased compared with female healthy controls (OR = 2.11, 95% CI = 1.14–3.89, P = 0.024; corrected P value: Pc = 0.072). The CC homozygotes of)634G>C of VEGF gene and AA homozygotes of)2578C>A have also been significantly correlated in female patients who developed BRONJ compared with healthy controls (OR = 2.04, 95% CI = 1.12–3.70, P = 0.008; corrected P value: Pc = 0.024). CONCLUSIONS: These results suggest a possible haplotype effect of VEGF polymorphisms expression in BRONJ Italian female patients. Studies with different and larger populations possibly using TagSNP to represent all haplotypes within the VEGF gene are needed to further delineate the genetic contribution of this gene to BRONJ.

Keywords: bisphosphonate; gene polymorphisms; osteonecrosis; VEGF

Introduction

Bisphosphonates are now the standard care for the management of hypercalcaemia in patients with metastases from multiple myeloma and breast, prostate, and lung cancers (1, 2) and are also used in Paget disease of bone, osteogenesis imperfecta, and osteoporosis (3, 4). Bisphosphonate-related osteonecrosis of the jaws (BRONJ) is a significant complication in some patients, especially cancer patients treated with the intravenous nitrogenated bisphosphonates (zolendronate and pamindronate) (5–7). However, the full pathogenesis of BRONJ remains to be clearly elucidated. Bisphosphonates block 3-hydroxy- 3-methylglutaryl coenzyme A (HMG-CoA) reductase and thereby inhibit osteoclast function (8) and osteoclast development from monocytes (7). Bisphosphonates are also anti-angiogenic (9) and, in theory, their ability to inhibit angiogenesis and vasculogenesis (10) may be accentuated in bones with high vascularity and bone turnover, as are the jaw bones, particularly after trauma. Vascular endothelial growth factor (VEGF) modulates angiogenesis and vasculogenesis by acting as an essential mitogen for vascular endothelial cells (11). VEGF also regulates other biological functions in endochondral ossification (12). Furthermore, other forms of osteonecrosis such as avascular necrosis of the hip, corticosteroid-induced osteonecrosis, and

osteoradionecrosis such as of the jaws appear to result from vascular disturbances (6, 11) and it has therefore been hypothesized that the vasculature may also play a role in the pathophysiology of BRONJ (6). There is a considerable variation in VEGF expression, with at least 25 single-nucleotide polymorphisms (SNPs) recognized, although most are rare. However, three common functional SNPs have been recognized: these are)634 G>C occurring in 5¢ untranslated region (UTR) (13); +936 C>T occurring in 3¢ UTR (14); and)2578 C>A of the VEGF promoter region (15). The 5¢- UTR, a promoter region, and the 3¢-UTR of VEGF have been shown to be highly polymorphic and reported to be associated with many diseases; in particular, these three polymorphisms ()2578C>A,)634G>C, and +936C>T) in VEGF were recently genotyped in patients with osteonecrosis of the femoral head, reporting an increased susceptibility (16). Therefore, we investigated the possible association between these VEGF polymorphisms and female Italian patients with BRONJ.

Materials and methods

Participants

Unrelated Caucasian female patients with BRONJ and controls, including healthy blood donors, participated in this study approved by the ethical research committee of the University of Turin. Informed consent was obtained. Peripheral venous blood (20 ml) was obtained from 185 individuals. A total of 30 Italian female patients with BRONJ were enrolled in this study (Group A). Control subjects were 30 female patients with history of intravenous bisphosphonates taking without any evidence of osteonecrosis (Group B) and 125 unrelated healthy volunteers (Group C). The characteristics of the study population are summarized in Table 1. Participants with BRONJ (Group A; 30 patients) were recruited from subjects referred to Oral Medicine and Oral Surgery Sections, Lingotto Dental School, University of Turin, Turin, Italy. The case definition of BRONJ patients used was as follows (7): non-healing exposed bone in the maxilla or mandible that has persisted for more than 8 weeks in a patient who has received bisphosphonates but has not received local radiation therapy. We only chose patients who underwent intravenous bisphosphonate. Controls were disease controls (Group B; 30 patients), who were unrelated patients with a history of intravenous bisphosphonate use but no evidence of osteonecrosis after oral and dental examination, and healthy controls (Group C; 125 subjects), who were healthy unrelated blood donors, never exposed to bisphosphonate therapy, recruited randomly from among the healthy blood donor population in Turin. All subjects were matched with no significant age differences. Group A and Group B were also matched regarding primary disease. All patients of Group A and Group B were clinically evaluated by an expert group of oral health care providers (P.G.A., M.S., R.B., and M.M.). At the time of the first visit, those patients were included in a computerized clinical file, which also recorded information on age, gender, presence of any systemic disease, and use of other medications. Every included patient was asked for a comprehensive history concerning the use, the dose, the frequency, and the duration of therapy with bisphosphonates. The patients who had been treated with zoledronic acid received 4 mg intravenously over 15 min monthly. In patients of Group A, the clinical appearance, size and sites of oral involvement, as well as signs of secondary infection, and the putative initiating event were also recorded. All patients of group A were referred to undergo a radiological examination, which included dental panoramic radiographs and computed axial tomography scans. Patients of group B only underwent a dental panoramic radiograph, to exclude any possible bone involvement without clinical sings of BRONJ (17).

Genotyping

Genomic DNA was purified from 200 ll of whole blood using the Nucleospin Blood Quickpure kit (Machery – Nagel) according to the manufacturer's instructions. VEGF polymorphisms rs3025039 (+936 C>T), located in the 3' UTR, rs699947 (-2578 C>A), and rs2010963 (-634 G>C), located, respectively, in the promoter region and in 5' UTR, were genotyped by the TaqMan Allelic Discrimination Assay (c_16198794_10, c_8311602_ 10, c_8311614_10 assays; Applied Biosystems, Monza, Italy), as previously reported (16, 18).

Statistical analysis

Subjects with BRONJ (Group A) were compared with patients taking BP without clinical evidence of BRONJ (Group B) and also compared with the healthy control group (Group C). Genotype frequencies were compared between groups using chi-squared method. Fisher's exact tests were applied if the expected frequency was < 0.05 was considered to be significant. Odds ratio (OR) was calculated along with 95% confidence intervals (95% CI) and used to described further associations or differences. Chi-square test was also used to compare the haplotype frequencies of cases and controls. To adjust for multiple comparisons, corrected P value (Pc) for a number of comparisons (Bonferroni correction) was applied. The goodness-of-fit to Hardy– Weinberg equilibrium, calculating the expected frequencies of each genotype and comparing them with the observed values, was performed using a Pearson's chi-squared goodness-of-fit test. All the statistical analyses were performed using STATISTICA 6.0 software.

Results

Vascular endothelial growth factor genotypes were successfully detected in all 185 enrolled subjects. Italian female patients with BRONJ (Group A) were compared with disease-negative controls (Group B) and unrelated healthy volunteers (Group C). All genotype distributions of the control groups were coherent with the assumption of Hardy–Weinberg equilibrium. There were no significant differences in genotype distributions for any VEGF SNPs between patients of Group A and Group B, between Group A and C, and between Group B and C (Table 2). There was also no effect from age, sex, primary disease, and duration of therapy (data not shown). For haplotype analysis, there were eight main extended haplotypes predicted by genotyping data (Table 3); we excluded the TAC (+936/-2578/-634) and TCG (+936/-2578/-634) haplotypes because of a frequency there was also no effect from age, sex, primary disease, and duration of therapy (data not shown). Finally, we also analyzed only two polymorphisms (-2578/-634) for all cases reported (Table 4): the AC (-2578/-634) haplotype frequency was significantly increased in Group A compared with Group B (OR = 3.11, 95% CI = 1.28–3.59, P = 0.035; Pc = 0.105) and compared with healthy controls of Group C (OR = 2.04, 95% CI = 1.12–3.70, P = 0.008); the last one was also statistically significant when corrected (Pc = 0.024).

Discussion

Bisphosphonate-related osteonecrosis of the jaws remains an enigma, and although environmental factors clearly significantly increase the risk, genetic factors may also be at play. However, to date, only one study has reported genetic susceptibility – a possible association with polymorphisms of cytochrome P450 CYP2C8 (19). The present study is the first to report on VEGF SNPs in BRONJ. We selected for study three common functional VEGF gene polymorphisms, because of their known effects on VEGF production and their association with other bone diseases (16, 20). Moreover, we decided to analyze only female patients with breast cancer, to make the population studied as homogeneous as possible. Our results demonstrate that in female patients with BRONJ, the CAC (+936/-2578/-634) haplotype appears to be possibly associated with susceptibility to BRONJ. It may be that decreased VEGF expression in the non-necrotic area or in area with acute inflammation could affect angiogenesis, influencing progression of BRONJ, as previously reported in relation to osteonecrosis of the head of the femur (16). The -634G>C polymorphism has been significantly correlated with VEGF expression, being lowest in the CC homozygotes (14). Similar data have been reported for the -2578C>A polymorphism as shown in our results, and +936C>T (14, 21, 22). For the last, however, our results seemed at first to be contradictory, because VEGF expression has been reported to increase for the CC homozygotes; for this reason, we also analyzed only two polymorphisms (-2578/-634) for all cases reported finding similar results: the AC (-2578/-634) haplotype frequency was significantly increased in females of Group A compared with females of Group B and compared with female healthy controls of Group C. These findings support the hypothesis that the CC homozygotes of -634G>C of VEGF gene and the AA homozygotes of -

2578C>A could be significantly correlated with patients who developed BRONJ. Different reports indicate that normal angiogenesis is central to tissue repair and that VEGF may be a major signal in the coupling of angiogenesis and osteogenesis during bone repair (23–26) and is a cytokine that also regulates several biological functions involved in endochondral ossification of jaw growth (12, 20). There have been no systematic studies assessing the vascular pattern in BRONJ, but recent case reports of exposed bone in the mandible in cancer patients not treated with bisphosphonates but treated with bevacizumab, a recombinant human monoclonal antibody that binds to VEGF and inhibits angiogenesis, suggest a considerable role of the vasculature in osteonecrosis (27). Moreover, it has recently been reported that zoledronic acid was not associated with a reduced immunohistochemical expression of VEGF in vital bone at the tooth extraction site in rats (28). In conclusion, this is the first report describing VEGF polymorphisms expression in BRONJ, and although the results are not conclusive, suggest a possible haplotype effect at least in this specific population. Studies with larger sample sizes and different populations, using TagSNP to represent all haplotypes within the VEGF gene, are needed to further delineate the genetic contribution of this gene to BRONJ.

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Table 1 Some of the main characteristics of the BRONJ patients (Group A) and control subjects (Group B and Group C)

	Group A	*Group B*	*Group C*	*Total*
Definition	*BRONJ*	*IV bisphosphonates but no BRONJ*	*Healthy blood donors*	*All subjects*
Number of subjects	30	30	125	185
Females, *n* (%)	30 (100)	30 (100)	125 (100)	
Age at enrollment (years): mean (±SD)	64.7 (±11.9)	64.2 (±9.9)	64.3 (±15.3)	
Primary disease				
Breast cancer, *n* (%)	20 (66.6)	20 (66.6)		
Multiple myeloma, *n* (%)	10 (33.3)	10 (33.3)		
Bisphosphonate used				
Zoledronic acid, *n* (%)	30 (100)	30 (100)		
Duration therapy (months): mean (range)	17.1 (6–48)	11.4 (6–36)		

Table 2 Distribution of VEGF genotype frequencies (+936, -2578, -634)

SNP	VEGF Genotypes	Group A (BRONJ) (n = 30), n (%)	Group B (Disease controls) (n = 30), n (%)	Group C (Healthy controls) (n = 125), n (%)	P	OR (95% CI)
+936	CC	22 (73.3)	20 (66.6)	100 (80)		1.00[a,b,c]
	CT	8 (29.7)	10 (33.4)	22 (17.6)	0.28[a]	0.5 (0.18–1.37)[a]
					0.94[b]	1.14 (0.36–3.68)[b]
					0.32[c]	0.5 (0.26–1.6)[c]
	TT	0 (0)	0 (0)	3 (2.4)	–	–
	TT + CT	8 (29.7)	10 (33.4)	25 (20)	0.40[a]	0.57 (0.21–1.54)[a]
					0.94[b]	1.14 (0.36–3.68)[b]
					0.57[c]	0.67 (0.25–1.75)[c]
−2578	AA	5 (16.6)	5 (16.6)	22 (17.6)		1.00[a,b,c]
	CA	15 (50.0)	18 (60.0)	53 (42.4)	0.97[a]	0.8 (0.23–2.76)[a]
					0.77[b]	0.94 (0.22–4.09)[b]
					0.99[c]	0.83 (0.25–2.8)[c]
	CC	10 (33.4)	7 (23.4)	50 (40)	0.97[a]	1.3 (0.34–4.9)[a]
					0.95[b]	0.67 (0.13–3.45)[b]
					0.91[c]	1.16 (0.31–4.31)[c]
	AC + CC	25 (83.3)	25 (83.3)	103 (82.4)	0.78[a]	0.99 (0.31–3.18)[a]
					0.91[b]	0.84 (0.21–3.42)[b]
					0.83[c]	0.95 (0.3–3.03)[c]
−634	GG	11 (36.6)	10 (33.4)	39 (31.2)		1.00[a,b,c]
	CG	15 (50.0)	18 (60.0)	61 (48.8)	0.80[a]	1 (0.36–3.79)[a]
					0.92[b]	1.15 (0.34–3.89)[b]
					0.83[c]	1.03 (0.37–2.82)[c]
	CC	6 (13.4)	2 (6.6)	25 (20)	0.88[a]	0.9 (0.26–2.79)[a]
					0.37[b]	0.28 (0.04–1.88)[b]
					0.93[c]	0.77 (0.22–2.67)[c]
	CG + CC	21 (70)	26 (86.6)	86 (68.8)	0.86[a]	0.96 (0.37–2.53)[a]
					0.94[b]	0.88 (0.27–2.81)[b]
					0.90[c]	0.95 (0.37–2.45)[c]

BRONJ, bisphosphonate-related osteonecrosis of the jaws. OR, odds ratio; VEGF, vascular endothelial growth factor. [a]Group A (BRONJ positive) vs. Group B (BRONJ negative); [b] Group A vs. Group C (healthy controls); [c] Group B (ONJ-BP negative) vs. Group C (healthy controls).

Table 3 Haplotype frequencies of VEGF gene (+936, -2578 and -634)*

VEGF Haplotype	Gender	Group A (BRONJ) %	Group B (Disease controls) %	Group C (Healthy controls) %	OR (95% CI)	P
CAC	♀	19.2	9.1	8.1	2.76 (1.09–4.94)[a]	0.039[a†]
					2.11 (1.14–3.89)[b]	0.024[b‡]
					1.22 (0.67–2.21)[c]	0.628[c]
CAG	♀	26.9	30.1	31.1	0.58 (0.33–1.03)[a]	0.081[a]
					0.63 (0.38–1.05)[b]	0.095[b]
					1.09 (0.75–1.58)[c]	0.737[c]
CCC	♀	27.1	23.9	29.3	1.47 (0.83–2.59)[a]	0.236[a]
					0.88 (0.55–1.41)[b]	0.682[b]
					0.90 (0.39–1.61)[c]	0.216[c]
CCG	♀	22.1	20.8	21.1	1.16 (0.64–2.12)[a]	0.737[a]
					1.08 (0.65–1.81)[b]	0.862[b]
					0.93 (0.60–1.45)[c]	0.843[c]
TCC	♀	1.7	3.1	3.0	0.32 (0.04–2.75)[a]	0.500[a]
					0.37 (0.05–2.89)[b]	0.537[b]
					1.18 (0.41–3.39)[c]	0.983[c]
TAG	♀	7.2	8.0	4.9	0.86 (0.33–2.23)[a]	0.944[a]
					1.82 (0.74–4.48)[b]	0.291[b]
					1.69 (0.65–4.42)[c]	0.415[c]

BRONJ, bisphosphonate-related osteonecrosis of the jaws; OR, odds ratio; VEGF, vascular endothelial growth factor. *a Group A (BRONJ positive) vs. Group B (BRONJ negative); b Group A vs. Group C (healthy controls); c Group B (ONJ-BP negative) vs. Group C (healthy controls). Group A compared with Group B for CAC haplotype: OR = 2.76, 95% CI = 1.09–4.94, P = 0.039; Pc = 0.039 · 3 = 0.117. Group A compared with Group C for CAC haplotype: OR = 2.11, 95% CI = 1.14–3.89, P = 0.024; Pc = 0.024 · 3 = 0.072.

Table 4 Haplotype frequencies of VEGF gene (-2578 and -634)*

VEGF Haplotype	*Gender*	*Group A (BRONJ) %*	*Group B (Disease controls) %*	*Group C (Healthy controls) %*	*OR (95% CI)*	P
_AC	♀	20.3	9.8	9.3	3.11 (1.28–3.59)[a]	0.035[a†]
					2.04 (1.12–3.70)[b]	0.008[b‡]
					1.31 (0.75–2.29)[c]	0.419[c]
_AG	♀	28.8	24.9	31.7	0.60 (0.36–1.01)[a]	0.070[a]
					0.76 (0.48–1.21)[b]	0.299[b]
					1.28 (0.89–1.84)[c]	0.217[c]
_CC	♀	28.8	25.8	33.1	1.30 (0.75–2.26)[a]	0.435[a]
					0.82 (0.51–1.31)[b]	0.475[b]
					2.76 (0.97–6.61)[c]	0.058[c]
_CG	♀	25.0	23.8	22.9	1.07 (0.61–1.89)[a]	0.939[a]
					1.12 (0.68–1.83)[b]	0.738[b]
					1.05 (0.69–1.59)[c]	0.890[c]

BRONJ, bisphosphonate-related osteonecrosis of the jaws; OR, odds ratio; VEGF, vascular endothelial growth factor. *a Group A (BRONJ positive) vs. Group B (BRONJ negative); b Group A vs. Group C (healthy controls); c Group B (ONJ-BP negative) vs. Group C (healthy controls). Group A compared with Group B for _AC haplotype: OR = 3.11, 95% CI = 1.28–3.59, P = 0.035; Pc = 0.035 · 3 = 0.105. Group A compared with Group C for _AC haplotype: OR = 2.04, 95% CI = 1.12–3.70, P = 0.008; Pc = 0.008 · 3 = 0.024.