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Abstract

Vascular endothelial growth factor (VEGF) is one of the most potent endothelial cell mitogens and plays a critical role in angiogenesis. Polymorphisms in this gene have been evaluated in patients with several types of cancer. The objectives of this study were to determine if there was an association of the -1154G/A polymorphism of the *VEGF* gene with head and neck cancer and the interaction of this polymorphism with lifestyle and demographic factors. Additionally, the distribution of the *VEGF* genotype was investigated with respect to the clinicopathological features of head and neck cancer patients. The study included 100 patients with histopathological diagnosis of head and neck squamous cell carcinoma. Patients with treated tumors were excluded. A total of 176 individuals 40 years or older were included in the control group and individuals with a family history of neoplasias were excluded. Analysis was performed after extraction of genomic DNA using the real-time PCR technique. No statistically significant differences between allelic and genotype frequencies of -1154G/A *VEGF* polymorphism were identified between healthy individuals and patients. The real-time PCR analyses showed a G allele frequency of 0.72 and 0.74 for patients and the control group, respectively. The A allele showed a frequency of 0.28 for head and neck cancer patients and 0.26 for the control group. However, analysis of the clinicopathological features showed a decreased frequency of the A allele polymorphism in patients with advanced (T3 and T4) tumors (OR = 0.36; 95%CI = 0.14-0.93; P = 0.0345). The -1154A allele of the *VEGF* gene may decrease the risk of tumor growth and be a possible biomarker for head and neck cancer. This polymorphism is associated with increased VEGF production and may have a prognostic importance.

Key words: Head and neck cancer; Polymorphism; Genetic; *VEGF* gene

Introduction

Head and neck squamous cell carcinoma is a significant cause of morbidity and mortality with 481,100 new cases reported annually worldwide. Two-thirds occur in developing countries, with an overall 5-year mortality rate of approximately 50% (1,2). In São Paulo State, Brazil, the occurrence of 4,510 new cases was estimated for 2008 (3).

The growth of solid tumors depends on angiogenesis, the process by which new blood vessels develop from the endothelium of the preexisting vasculature. Tumors promote angiogenesis by secreting or activating angiogenic factors that stimulate endothelial migration, proliferation, and capillary morphogenesis. Newly formed blood vessels supply the tumor with nutrients and oxygen, dispose of the metabolic waste products of tumor cells, generate paracrine stimuli, and provide potential routes for tumor dissemination. Thus, tumor-induced angiogenesis plays a pivotal role in cancer progression and metastasis (4,5).

Vascular endothelial growth factor (VEGF) is one of the most potent endothelial cell mitogens and plays a critical role in angiogenesis. VEGF binds specifically to two transmembrane VEGF receptor tyrosine kinases on endothelial cells to initiate intracellular signal transduction pathways that mediate angiogenesis and vascular permeability (6). The properties of angiogenesis correlate with tumor aggressiveness, with intratumor microvessel density and they have been identified as an independent prognostic factor (7).

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VEGF forms the new blood vessels of the tumor by acting as an endothelial cell mitogen, thereby it promotes the growth and invasion of the tumor. VEGF functions to increase vascular permeability, which causes edema in the extracellular matrix and supports the spouting and splitting of new blood vessels by making fibrin freely available. VEGF also allows the intravascular circulating tumor cells to easily penetrate the basement membrane of blood vessels (8).

Several lines of compelling evidence from *in vitro* and *in vivo* experiments have shown that increased *VEGF* expression is associated with tumor growth and metastasis, whereas the inhibition of VEGF signaling results in suppression of both tumor-induced angiogenesis and tumor growth (6).

The *VEGF* gene is located on chromosome 6p21.3 and consists of 8 exons. At least 30 single-nucleotide polymorphisms have been described in this gene. Among these, there is evidence that the -634G/C, -1154G/A, and -2578C/A *VEGF* polymorphisms have been shown to be associated with increased VEGF production (9-11).

VEGF polymorphisms have been evaluated in patients with skin (12), bladder (13), lung (14,15), prostate (16,17), stomach (18), and breast (19,20) cancers. Several studies have focused on the potential prognostic importance of *VEGF* polymorphisms (13,19,20). The -1154GG *VEGF* genotype is associated with higher VEGF production and is located in the promoter region of the gene (11). The -1154A/A genotype has been shown to be associated with a decreased risk for prostate cancer and less advanced melanoma (14-16).

Six studies have investigated *VEGF* polymorphisms in head and neck cancers. In one study, an association of -460C/T polymorphism was reported in oral cancer. The distribution of the -460TT homozygote in the patient group was greater than that in the control group and the results showed that with a high -460TC ratio there is a higher risk for oral cancer in the patients (21).

A second study showed an association between +960C/T *VEGF* polymorphism and oral cancer (22). This study clearly indicates that the low production of VEGF by the T allele is associated with increased risk for oral cancer. Cheng et al. (24) also identified an association between +960C/T polymorphism and vascular invasion in oral squamous cell carcinoma, whereas recently Vairaktaris et al. (23) were not able to demonstrate an association between this polymorphism and the disease in the logistic regression models. However, the genotype *VEGF* -460CT was associated with early stage tumors.

One study that analyzed the polymorphism *VEGF* -2578C/A has suggested that carriers of *VEGF* -2578C allele may play a role in susceptibility to nasopharyngeal carcinoma (25).

In the -1154G/A polymorphism of the *VEGF* gene, the -1154G/G genotype of the *VEGF* gene appears to increase the risk for laryngeal squamous cell carcinoma (26). The polymorphism -1154G/A *VEGF* has been shown to be associated with differential expression of VEGF *in vitro* and has been implicated in the risk for several types of tumors and other diseases with putative angiogenic components. However, some of the data are contradictory. It is possible that functional variations of the *VEGF* gene may contribute to the progression of head and neck cancer. To test this hypothesis, the aims of the present study were to investigate the association of the -1154G/A polymorphism of the *VEGF* gene with head and neck cancer and to determine whether the interaction of this polymorphism with lifestyle and demographic factors may be associated with a higher risk. In addition, the VEGF genotype distribution was investigated in relation to the clinicopathological features of head and neck cancer patients.

Material and Methods

A prospective study of 91 male and 9 female patients diagnosed with head and neck cancer (average age: 57 years; range: 36-80 years) was conducted at Hospital de Base, São José do Rio Preto, São Paulo, Brazil. Diagnosis was made from pathological specimens obtained during total excision surgery or biopsy. All tumor cells were squamous cell carcinomas and the samples were collected before the beginning of treatment. The primary anatomic sites included the oral cavity (N = 34), oropharynx (N = 24), hypopharynx (N = 8), supraglottis (N = 11), and glottis (N = 18). The primary site of the tumor was unknown for 5 of the 100 patients.

A control group contained 176 blood donors with no familial history of cancer for three generations.

Alcoholism was defined as the intake of alcohol at a frequency determined without a quantitative analysis and individuals who had an average of four drinks a week were considered to be alcohol consumers. The smoking habit was defined as the consumption of at least 5 cigarettes/day.

The patients were staged according to the tumor-node-metastasis (TNM) classification of the International Union against Cancer (27).

The study protocol was approved by the Ethics Committee of the Medical School of São José do Rio Preto, São Paulo (FAMERP) and all participants gave written informed consent to participate in the study.

DNA isolation and genotyping assay

DNA was isolated from peripheral blood leukocytes by the method of Miller et al. (28). DNA concentration was measured using a Cary spectrophotometer (Varian, USA). Allelic discrimination of the -1154 *VEGF* polymorphism was achieved with the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, USA) using the fluorogenic 5' nuclease assay with Taqman Minor Groove Binder (MGB) probes. The wild-type Taqman MGB probes were FAM (allele G) labeled and the mutants were VIC (allele A) labeled.

The final volume of each reaction was 5 µL, consisting of 1.5 µL Taqman Universal PCR Master Mix (Applied Biosystems), 0.9 µM of each primer, 0.2 µM of each Taqman probe, and 5 ng genomic DNA. PCR sequencing involved an initial denaturation step at 95°C for 10 min followed by 40 cycles at 92°C for 15 s and 60°C for 1 min. Fluorescent signals were measured at 60°C. The primers and probes for the G-1154A were purchased from Applied Biosystems (ID - C_1647379-10).

The SDS computer program version 2.0 was used to analyze the fluorescence emitted in real-time and at the end of PCR (endpoint read).

Statistical analysis

Demographic and lifestyle data were analyzed by descriptive statistics and compared by the chi-square test.

The chi-square test and Hardy-Weinberg equilibrium (HWE) were used for statistical analysis of the allele and genotype distributions of this polymorphism. A P value <0.05 was used to establish statistical significance. Multiple logistic regression models were used to determine the effect of the variables on head and neck squamous cell carcinoma. The model included age (reference: <54 years - mean of the groups), gender (reference: female), smoking habit (reference: non-smokers), and drinking habit (reference: non-drinkers). Data are reported as odds ratio (OR) and 95% confidence intervals (95%CI).

Clinicopathological features were analyzed by multiple logistic regression. The anatomic primary site of the tumor was divided into three head and neck cancer regions: oral cavity, pharynx and larynx. The clinical staging of head and neck cancer was performed according to the TNM classification of the International Union against Cancer (27). Tumor classification was divided into low T (T1,T2) and high T (T3,T4) categories. N classification was dichotomized into negative (N0) and positive involvement of lymph nodes (N1, N2, N3). Stage grouping was divided into early stage (Stage I or II) and advanced stage (Stage III or IV) categories. The

analysis of M classification was not performed since all patients were classified as M0.

Disease-free survival was defined as the time from the date of diagnosis to the date of first local or distant recurrence or last contact. Overall survival analysis was defined as the time from the date of diagnosis to death if the patient died from head and neck cancer, or to last contact. Survival curves were plotted using the Kaplan-Meier method and the differences between groups were calculated by the log-rank test.

Statistical analyses were performed using the computer program Minitab for Windows (version 12.22). A value of P < 0.05 was considered to be statistically significant.

Results

Demographic data and lifestyle factors

Table 1 shows the distribution of the variables of patients and controls. There were statistically significant differences between patients and controls for age, gender, and smoking and drinking habits.

VEGF genotype

The genotype distribution of the polymorphism under study did not deviate from HWE for patients (P = 0.9521) or controls (P = 0.0675).

The allele and genotype frequencies of the *VEGF* gene of head and neck cancer patients and of the control group are shown in Table 2. There was no statistically significant difference in the allele distribution of the -1154 *VEGF* gene G/A polymorphism between controls and head and neck cancer patients. The differences in genotype frequencies between patients and controls were also not significant.

The potential interaction between the distribution of the *VEGF* genotype and exposure to risk factors for head and neck cancer are shown in Table 3, with no statistical difference being detected using multiple logistic regression.

Table 1. Distribution of the characteristics of patients and controls.

Variable	Patients (N = 100)	Controls (N = 176)
Male	91 (91%)	119 (68%)*
Female	9 (9%)	57 (32%)*
Age (years)	57 ± 10	52 ± 12*
Smoking habit (%)	91 (91%)	99 (56%)*
Drinking habit (%)	80 (80%)	61 (35%)*

Data are reported as number with percent in parentheses or means ± SD. The smoking habit was defined as the consumption of at least 5 cigarettes/day. The drinking habit was defined as the consumption of an average of four drinks a week. *P < 0.05 compared to patients (chi-square test).

Table 2. Distribution of the -1154G/A *VEGF* polymorphism among head and neck cancer patients and controls.

	Patients (N = 100)	Controls (N = 176)
Genotypes		
GG	51 (51%)	100 (57%)
GA	41 (41%)	59 (34%)
AA	8 (8%)	17 (9.7%)
Alleles		
G	0.72	0.74
A	0.28	0.26

Data are reported as number with percent in parentheses. There were no statistically significant differences between allele distribution or genotype frequencies between patients and controls (chi-square test).

The associations of the clinicopathological features of head and neck cancer patients with *VEGF* genotypes are shown in Table 4. Only patients with complete pathological data were considered for this analysis. The analysis showed that the polymorphic A allele was less frequent in patients with T3 and T4 tumors. No significant association of genotype frequency was found for the other clinical parameters. An analysis of metastasis classification was not performed since all patients were classified as M0.

There was no statistical difference between survival curves by genotype. There was also no association be-

tween the polymorphism and overall survival (Figure 1A) or evidence for an association between the time of recurrence of the disease and genotype (Figure 1B).

Discussion

The present study confirms the well-known fact that head and neck cancer development is strongly associated with lifestyle factors, such as alcohol intake and cigarette smoking (29). Furthermore, the significant association between age and gender and this disease was confirmed (29,30).

Table 3. Odds ratio of head and neck cancer related to *VEGF* genotypes by gender, age, smoking, and drinking habits.

Characteristic	GG (Patients/Controls)	OR (95%CI)	GA and AA (Patients/Controls)	OR (95%CI)
Gender				
Female	4/32	1.00 (ref)	5/25	2.22 (0.46-10.55)
Male	47/68	1.00 (ref)	44/51	1.25 (0.64-2.41)
Age (years)				
<54	16/56	1.00 (ref)	15/42	1.50 (0.59-3.82)
>54	35/44	1.00 (ref)	34/34	1.33 (0.61-2.88)
Smoking habits				
Non-smokers	5/38	1.00 (ref)	4/39	1.08 (0.24-4.90)
Smokers	46/62	1.00 (ref)	45/37	1.40 (0.72-2.71)
Drinking habits				
Use of alcohol	41/38	1.00 (ref)	39/23	1.29 (0.61-2.72)
Non-use of alcohol	10/62	1.00 (ref)	10/53	1.63 (0.56-4.71)

OR data were adjusted for age, gender, smoking, and drinking habits. None of the differences between groups were statistically significant by multiple logistic regression analysis.

Table 4. Clinicopathological features and *VEGF* polymorphism.

Variables	GG genotype	OR (95%CI)	GA and AA genotype	OR (95%CI)
Site of tumor (N = 95)				
Oral cavity	22	1.00 (ref)	20	0.81 (0.35-1.89)
Pharynx	16	1.00 (ref)	11	0.73 (0.29-1.85)
Larynx	14	1.00 (ref)	12	0.91 (0.36-2.31)
Tumor extension (N = 95)				
T1/T2	11	1.00 (ref)	15	1.00 (ref)
T3/T4	40	1.00 (ref)	29*	0.36 (0.14-0.93)
Lymph node involvement (N = 99)				
No	26	1.00 (ref)	16	1.00 (ref)
Yes	25	1.00 (ref)	32	2.20 (0.94-5.14)
Stage (N = 82)				
I/II	7	1.00 (ref)	9	1.00 (ref)
III/IV	36	1.00 (ref)	30	1.26 (0.33-4.71)

OR data were adjusted for age, gender, smoking, and drinking habits. *P < 0.05 compared to GG (multiple logistic regression analysis).

In the present study, the allele and genotype distribution of the -1154G/A *VEGF* polymorphism in patients with head and neck cancer was in close agreement with that reported for healthy Caucasian individuals (31). However, when submitted to univariate and multiple logistic regression analyses, our data showed no significant differences in the allele and genotype distribution for patients with head and neck cancer compared to controls.

Five studies have reported the association of at least one *VEGF* polymorphisms with head and neck cancer. Ku et al. (21) reported the association between -460T/T *VEGF* genotype and oral cancer, Yapijakis et al. (22) showed an elevated frequency of heterozygotes with the +936C/T polymorphism and oral cancer, and Cheng et al. (24) identified an association between +960C/C *VEGF* genotype and vascular invasion in oral squamous cell carcinoma. Nasr et al. (25) reported an association between carriers of *VEGF* -2578C allele polymorphism and aggressiveness of the tumor in nasopharyngeal carcinoma and Ünal et al. (26) showed an association between the *VEGF* -1154G/G genotype and larynx cancer.

The *VEGF* genotype can be divided into low (homozygote AA), intermediate (heterozygote AG) and high expression (homozygote GG). Thus, Unal et al. (26) hypothesized that patients with high expression of the *VEGF* genotype have more aggressive and metastatic tumor behavior than low expression or heterozygote genotypes.

In lung cancer, the -1154A/A *VEGF* polymorphism was shown to be significantly associated with less VEGF expression compared to the GG polymorphism, while GA seemed to be related to an intermediate level of expression (14).

In the analysis of clinicopathological features, the decreased frequency of the polymorphic A allele (-1154AA and -1154GA genotypes) in T3 and T4 tumors supports the hypothesis that this allele is associated with decreased angiogenesis. Evidence has shown that the -1154G/A *VEGF* polymorphism is associated with increases in VEGF production (9-11). The -1154AA genotype is associated with a decreased prostate cancer risk and less advanced melanomas and these data suggest that the polymorphisms involved in the angiogenic pathway may affect the progression or aggressiveness of the tumor (11,16).

Howell et al. (12) reported that the *VEGF* -1154A/A genotype was associated with the thinner primary vertical growth phase of malignant cutaneous melanomas while *VEGF* -1154GG was associated with thicker primary tumors. McCarron et al. (16) observed that the *VEGF* -1154AA genotype is associated with a reduced risk for prostate cancer development. These results indicate that the *VEGF* genotype may influence tumor growth, possibly via the effects of differential VEGF expressions on tumor angiogenesis. Nasr et al. (25) showed an association between -2578C allele carriers and the aggressive forms of nasopharyngeal carcinoma defined by large tumor size and advanced tumor stages.
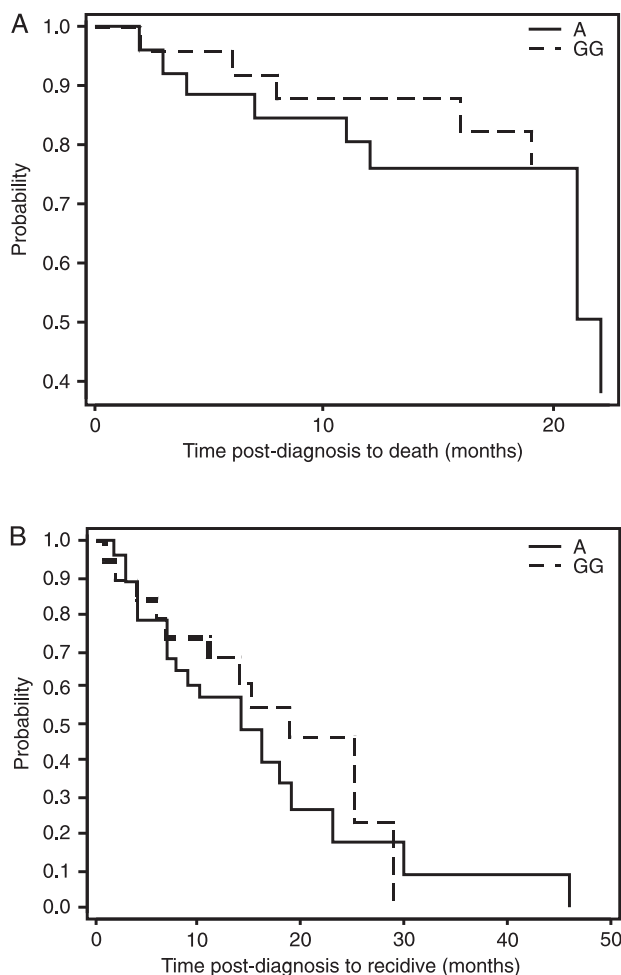

Figure 1. Kaplan-Meier curves for the overall survival (P = 0.1521) (A) and relapse time (P = 0.4502) (B) for patients according to the -1154G/A *VEGF* polymorphism.

Several studies have reported that VEGF expression is significantly higher in patients with regional lymph node involvement (32,33) and therefore may promote the progression of oral cancer. In the present study, no association was observed between lymph node involvement and -1154G/A *VEGF* polymorphism. Moreover, no significant differences between the different tumor stages and the distribution of -1154G/A *VEGF* polymorphism were observed. In a study of +936C/T *VEGF* polymorphism, Yapijakis et al. (22) reported an increase in the frequency of the *VEGF* 936T polymorphic allele, which is associated with lower circulating levels of VEGF.

A clear correlation between the *VEGF* gene and clinical parameters has not been demonstrated in squamous cell head and neck carcinomas (34). The present study suggests that the *VEGF* -1154A allele can reduce the risk of tumor growth and thus supports views about the role of

VEGF in head and neck cancer reported by others. Our result is not surprising because VEGF, as a key mediator of angiogenesis, is more likely to alter the aggressiveness of the tumor than susceptibility to cancer.

The expression of the *VEGF* gene is also regulated by a variety of hormones, growth factors, and cytokines, including luteotrophic hormone, PDGF, EGF, TGF-b, and IL-6, suggesting that other angiogenic factors may also regulate VEGF expression (31). The process of virtualization is not totally known, but VEGF seems to be the predominant vascular growth factor in most tumors (35). High expression of VEGF is correlated with some solid tumors such as breast cancer (36), colorectal (37), and oral squamous cell carcinoma (7,38). For the last one, positive immunoreactivity of VEGF was associated with a poor prognosis (7,37) and reduced survival (7).

The -1154A allele of the *VEGF* gene decreases the risk of tumor growth and may be an important biomarker in head and neck cancer.

Acknowledgments

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