RESEARCH ARTICLE

Vascular endothelial growth factor G+405C polymorphism may contribute to the risk of developing papillary thyroid carcinoma

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Background: Papillary thyroid carcinoma (PTC) is the most common endocrine malignancy. Vascular endothelial growth factor (VEGF) is a mediator implicated with cell proliferation, differentiation and migration, and monocyte/macrophage chemotaxis. In present study, we aimed to investigate the relationship between VEGF gene polymorphisms (G+405C, T-460C, and A-2578C) and PTC susceptibility.

Methods: DNA was isolated from peripheral blood leukocytes of 127 patients with PTC and 203 healthy controls. Genotyping was performed by real-time PCR. Association of genotypes with susceptibility of PTC was analyzed with multivariate logistic regression adjusted for age, gender and smoking status.

Results and Conclusion: In G+405C polymorphism, the frequencies of C allele (related with increased VEGF production) and combined CG+CC genotype were found to be higher (3.5 and 5-fold, respectively) among patients with PTC than controls ($P < .001$). However, VEGF T-460C and A-2578C polymorphisms are not associated with PTC risk. There was no relationship between VEGF polymorphisms and clinical/laboratory parameters of PTC. Haplotype analysis demonstrated that there was a strong linkage disequilibrium (LD) between -460/-2578 ($D' = .89$, $r^2 = .79$), weak LD between +405/-460 ($D' = .422$, $r^2 = .035$), and +405/-2578 ($D' = .43$, $r^2 = .038$) locuses. Additionally, the +405/-460/-2578 GTA haplotype was found to be protective, whereas CTA haplotype to be related with increased PTC risk. As a conclusion, we suggest that VEGF G+405C polymorphism is associated with increased risk of PTC.

KEYWORDS

papillary thyroid cancer, single nucleotide polymorphism, vascular endothelial growth factor

1 | INTRODUCTION

Papillary thyroid carcinoma (PTC) is the most common type of endocrine malignancy and accounts for more than 80%-85% of all thyroid cancers.¹ Although PTC is a cancer with relatively good prognosis, recurrence is not uncommon during long-term follow-up. New blood vessel development (angiogenesis and lymphangiogenesis) have been shown to be underlying molecular mechanisms actively involved to account for the occurrence of lymph node and distant metastasis respectively by both the primary and recurrent tumors.^{2,3} The etiopathogenesis of PTC has not been elucidated yet. Accumulating data have shown that chronic inflammation, endothelial damage, as well

as various cytokines and growth factors play important roles in the etiopathogenesis.^{4,5}

Vascular endothelial growth factor (VEGF) is a mediator implicated with cell proliferation, differentiation, migration and growth, and monocyte/macrophage chemotaxis.⁶ In addition, VEGF plays an important role in angiogenesis via inhibition of endothelial cell apoptosis and increasing vascular permeability. The presence of VEGF in normal thyroid epithelial cells as well as in thyroid gland tumors, goiter, Graves' disease, and autoimmune thyroiditis⁷⁻⁹ reveals that VEGF may be important for thyroid function and development of thyroid gland disease. Increased serum VEGF levels and VEGF expression in thyroid tissue from patients with thyroid cancer were reported as well.^{4,7-9} Increased

VEGF expression is related to uncontrolled growth and proliferation of cancer cells, local invasion, and leads to development of near or distant metastasis.^{3,10}

Genetic predisposition has been proposed as one of the risk factors for thyroid cancer. It was reported that the G+405C, T-460C and A-2578C single nucleotide polymorphisms (SNPs) of VEGF gene alter VEGF production and serum levels.¹¹⁻¹⁴ It seems possible that there is a relationship between above mentioned polymorphisms and thyroid cancer. There are only two publications on the same topic with controversial results.^{3,10} Therefore, the aim of present study was to investigate the relationship between above mentioned polymorphisms and PTC, and to evaluate the possible relationships between genotypes and clinical/laboratory characteristics of PTC.

2 | MATERIALS AND METHODS

A total of 127 patients with the diagnosis of PTC and 203 healthy controls were included in the present study. The study was approved by the Institutional Review Board at Şişli Etfal Research and Training Hospital. Informed consent was obtained from each subject. PTC had been either diagnosed or suspected in these patients for clinical or epidemiological reasons, including the results of fine-needle aspiration cytology and/or histological analysis. None of the patients and controls had received chemotherapy, radiotherapy, or surgery before admission to our department. All of the patients underwent total or near-total thyroidectomy. The subjects in control group were matched for age and sex. None of the controls had personal or family history of thyroid disease and goiter on examination, they had normal thyroid functions and were negative for thyroid autoantibodies. Exclusion criteria (for both study group and controls) were the existence of any comorbid cardiac, infectious, musculoskeletal or malignant disease and a recent history of operation or trauma. None of the patients and controls is consuming alcohol. Height (m) and weight (kg) were measured after fasting, and body mass index (BMI) was calculated by dividing the weight by the height squared.

Overnight (12 hours) fasting blood samples were taken from patients (before thyroidectomy) and controls. Peripheral venous blood samples were collected in plain tubes for routine biochemical analysis, and in EDTA-K₃ for genotype analysis. Serum TSH, free T₃, free T₄, anti-Tg (anti-thyroglobulin antibody) and anti-TPO (anti-thyroid peroxidase antibody) were measured on Modular EEE Electrode Elecsys Roche autoanalyzer (Roche Diagnostics, Mannheim, Germany).

Genomic DNA was isolated from peripheral blood leukocytes by using High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany). For detection of the mentioned polymorphisms, Light SNiP assay was used. This assay is based on simple probe melting curve analysis. They consist of pre-mixed primers and probes. They were developed and optimized according to NCBI "rs" numbers of studied SNPs by Tib MolBiol (Berlin, Germany). The detection of polymorphisms was performed in a LightCycler (Roche Diagnostics). Melting curves were evaluated by two independent observers who were blinded to the analysis of the clinical data. In addition,

10% of randomly selected samples were repeated independently to verify genotyping results and 100% concordance was found.

Mann-Whitney *U* and Kruskal-Wallis tests were used for the evaluation of clinical and biochemical parameters. Univariate analysis of variance (General Linear Model) was used for constructing the model to explain the variation in TSH. Age, gender and smoking status were used as covariates. Differences in genotype distributions and allele frequencies in the cases and the controls were compared for statistical significance using the chi-square (χ^2) test. The statistical significance for deviations from Hardy-Weinberg Equilibrium (HWE) was determined using the Pearson χ^2 -test. Multivariate logistic regression analysis was used to estimate age, gender and smoking status adjusted odds ratio (aOR) with the corresponding 95% CI. All statistical analyses were performed with SPSS statistics for Windows (version 21; SPSS Inc., Chicago, IL, USA). Linkage disequilibrium (LD) and haplotype frequencies were estimated using the Haploview software 4.1 and compared between cases and controls using a contingency χ^2 -test.¹⁵ In addition, the NCSS 2000 statistical package (Kaysville, UT, USA) was used to evaluate the power analysis. We had a 91% power to detect an effect size (*W*) of .20 using a 2 *df* (α =.05).

3 | RESULTS

In the present study, the G+405C, T-460C, and A-2578C SNPs in the VEGF gene were investigated in 127 patients with PTC and 203 healthy controls. The clinical characteristics of controls and patients with PTC were presented in Table 1. There were no significant differences among study and control groups in terms of mean age and sex distribution. No significant differences were observed between female and male patients with respect to clinical parameters and free T₃ and free T₄. The TSH levels in patients with PTC were higher than that of in controls. To check whether the factors such as age, gender and smoking status influence the TSH levels, we performed analysis of covariance. When stratified by age, gender and smoking status, the significant difference in TSH disappeared (Table 2).

The distribution of VEGF genotypes was in accordance with the HWE in either controls and cases. The genotypic and allelic distributions of investigated SNPs for cases and controls are shown in Table 3. We did not find any associations between PTC and variant alleles of VEGF -460 (aOR: 1.0, 95% CI=0.73-1.38) and -2578 (aOR: 0.95, 95% CI=0.69-1.30). With regard to +405 SNP, in patients with PTC, the C allele genotypes (CG and CC) were significantly associated with the increase of risk of PTC comparing to GG genotype ($P<.001$ and $P=.002$, respectively (Table 3), with aOR=4.45 (2.34-8.46) and 3.61 (1.62-8.05) respectively, after adjustment against age, gender, and smoking status. Individuals carrying combined CG+CC genotype had nearly 5-fold increased risk for developing PTC compared to wild homozygotes ($P<.001$, 95% CI=2.65-9.21). Moreover, the C allele was found to be 3.5-fold higher among patients with PTC than controls ($P<.001$, 95% CI=2.43-5.23). There was no relationship between VEGF polymorphisms and clinical/laboratory parameters of PTC (Tables 4-6).

TABLE 1 Characteristics of controls and patients with papillary thyroid cancer (PTC) and control group

	Control (n=203)	PTC (n=127)
Age (y)		
Mean±SD	45.0±8.9	47.5±11.2
Range	28-79	28-79
Gender		
Male, n (%)	48 (23.6)	31 (24.4)
Female, n (%)	155 (76.4)	96 (75.6)
Family history, n (%)	—	46 (36.2)
Smoking, n (%)	60 (29.5)	36 (28.3)
Exposure to radiation history	None	None
Ethnic differences	None	None
TNM stage		
I	—	88 (69.3)
II	—	17 (13.4)
III	—	17 (13.4)
IV	—	5 (3.9)
Tumor size		
≥1 cm	—	97 (76.4)
<1 cm	—	30 (23.6)
CLT	—	14 (11.0)
BMI (kg/m ²) (mean±SD)	25.45±4.41	27.55±4.69
Anti-TPO (IU/mL) (mean±SD)	—	18.69±28.57
Anti-Tg (IU/mL) (mean±SD)	—	22.88±13.33
TSH (mIU/L) (mean±SD)	1.65±0.9	6.3±6.65*
FreeT ₃ (pmol/L) (mean±SD)	3.5±0.37	2.92±0.86
FreeT ₄ (pmol/L) (mean±SD)	1.47±0.23	1.23±0.43

BMI, body mass index; anti-Tg, anti-thyroglobulin antibody; anti-TPO, anti-thyroid peroxidase antibody; CLT, concurrent chronic lymphocytic thyroiditis; TSH, thyroid-stimulating hormone.
Mann-Whitney *U* test, **P*<.05.

TABLE 2 General Linear Model for thyroid-stimulating hormone (TSH) taking age, gender and smoking status as covariates

General linear model	F-value	P-value
Age	0.943	.333
Gender	0.447	.505
Smoking status	0.096	.757
TSH	3.372	.068

Levontin's standardized disequilibrium coefficient (*D'*) was calculated as a measure for LD between the studied SNPs in the VEGF gene (Figure 1). There was a strong LD between -460/-2578 (*D'*=.89, *r*²=.79), and weak LD between +405/-460 (*D'*=.422, *r*²=.035), and +405/-2578 (*D'*=.43, *r*²=.038) locuses. Haplotype frequencies are shown in Table 7. The +405/-460/-2578 GTA haplotype was significantly overrepresented in the controls, meanwhile CTA haplotype was higher in the patients (<.001).

TABLE 3 Distribution of genotypes and allele frequencies for patients with papillary thyroid cancer (PTC) and control group

	Controls n (%)	PTC n (%)	aOR (95% CI)*	P-value
VEGF G+405C (rs 2010963)				
GG	152 (74.9)	49 (38.6)	1.0 ^a	—
CG	47 (23.2)	65 (51.2)	4.45 (2.34-8.46)	<.001
CC	4 (1.9)	13 (10.2)	3.61 (1.62-8.05)	.002
CG+CC	51	78	4.94 (2.65-9.21)	<.001
G allele	0.86	0.64	1.0 ^a	
C allele	0.14	0.36	3.55 (2.43-5.23)	<.001
VEGF T-460C (rs 833061)				
TT	75 (36.9)	49 (38.6)	1.0 ^a	—
CT	90 (44.3)	52 (40.9)	0.80 (0.42-1.54)	.51
CC	38 (18.8)	26 (20.4)	1.00 (0.54-1.36)	.61
CT+CC	128	78	0.81 (0.44-1.48)	.49
T allele	0.59	0.59	1.0 ^a	
C allele	0.41	0.41	1.00 (0.73-1.38)	.99
VEGF A-2578C (rs 699947)				
AA	68 (33.5)	50 (39.4)	1.0 ^a	—
AC	100 (49.3)	51 (40.1)	0.70 (0.30-1.36)	.29
CC	35 (17.2)	26 (20.5)	0.97 (0.65-1.46)	.89
AC+CC	135	77	0.77 (0.42-1.42)	.40
A allele	0.58	0.59	1.0 ^a	
C allele	0.42	0.41	0.95 (0.69-1.30)	.74

Each *P*-value was based on chi-square (χ^2) analysis.

*Adjusted for age, gender and smoking status.

^aReference values for aOR.

This study has some limitations. We were not able to measure plasma VEGF concentration in all of the subjects that were involved, particularly the patients with PTC, therefore we could not test the effect of studied polymorphisms on VEGF production.

4 | DISCUSSION

The results of present study demonstrated that: (1) TSH levels in PTC patients were higher than that of controls, but this difference disappeared after adjustment for age, gender and smoking status; (2) VEGF T-460C and A-2578C polymorphisms are not associated with PTC risk; (3) the frequencies of VEGF +405 C allele and combined CG+CC genotype were found to be higher (3.5 and 5-fold, respectively) among patients with PTC than controls; (4) there was a strong LD between -460/-2578, weak LD between +405/-460, and +405/-2578 locuses; (5) the +405/-460/-2578 GTA haplotype was found to be protective, whereas CTA haplotype to be related with increased PTC risk.

Among the preoperative test markers for thyroid cancer (TC), TSH levels are still under evaluation for the prediction of malignancy in thyroid nodules. While some studies showed an increased

TABLE 4 Thyroid hormonal status and anti-thyroid antibodies in patients with papillary thyroid cancer (PTC) in accordance with their genotypes of the VEGF +405 polymorphism [mean (range)]

	GG	CG	CC	C allele carriers (CG+CC)
BMI (kg/m ²)	26.3 (19-39)	27.9 (20-39)	30.1 (24-41)	28.21 (20-41)
Free T ₃ (pmol/L)	3.0 (1.0-5.2)	2.8 (0.1-5.4)	3.2 (2.6-4.6)	2.9 (0.1-5.2)
Free T ₄ (pmol/L)	1.2 (0.05-2.1)	1.2 (0.08-2.3)	1.4 (1.1-2.0)	1.3 (0.1-2.3)
TSH (mIU/L)	4.5 (0.01-39.40)	8.7 (0.01-110)	3.3 (0.01-0.8)	7.3 (0.01-110)
Anti-TPO (IU/mL)	19.5 (10-202)	19.3 (10-173)	12.2 (10-21.5)	18.3 (10-173)
Anti-TG (IU/mL)	25.7 (20-119)	21.7 (20-82.8)	20 (20-82.8)	21.4 (20-82.8)

BMI, body mass index; TSH, thyroid-stimulating hormone; Anti-TPO, anti-thyroid peroxidase; Anti-Tg, anti-thyroglobulin.

Kruskal-Wallis (when GG, CG and CC genotypes were compared) and Mann-Whitney U (when C allele carriers were compared to GG) tests.

TABLE 5 Thyroid hormonal status and anti-thyroid antibodies in patients with papillary thyroid cancer (PTC) in accordance with their genotypes of the VEGF -460 polymorphism [mean (range)]

	TT	CT	CC	T allele carriers (TT+CT)
BMI (kg/m ²)	28.4 (21-41)	27.0 (19-39)	26.9 (20-39)	27.0 (19-39)
FreeT ₃ (pmol/L)	2.9 (1.6-4.8)	2.9 (0.1-5.4)	3.0 (1.0-5.2)	2.9 (0.1-5.4)
Free T ₄ (pmol/L)	1.3 (0.8-2.0)	1.2 (0.1-2.3)	1.1 (0.1-1.9)	1.2 (0.1-2.3)
TSH (mIU/L)	2.73 (0.01-31.00)	9.02 (0.01-110)	7.43 (0.03-39.40)	8.6 (0.01-110)
Anti-TPO (IU/mL)	14.2 (10-53.2)	18.8 (10-173)	30.2 (10-202)	21.6 (10-202)
Anti-TG (IU/mL)	23 (20-82)	20.5 (20-39)	30.1 (20-119)	22.8 (20-119)

BMI, body mass index; TSH, thyroid-stimulating hormone; Anti-TPO, anti-thyroid peroxidase; Anti-Tg, anti-thyroglobulin.

Kruskal-Wallis (when TT, CT and CC genotypes were compared) and Mann-Whitney U (when T allele carriers were compared to CC) tests.

TABLE 6 Thyroid hormonal status and anti-thyroid antibodies in patients with papillary thyroid cancer (PTC) in accordance with their genotypes of the VEGF -2578 polymorphism [mean (range)]

	AA	AC	CC	C allele carriers (AC+CC)
BMI (kg/m ²)	28.3 (21-41)	27.0 (19-39)	27.1 (20-39)	27.1 (19-39)
Free T ₃ (pmol/L)	2.9 (1.6-4.8)	2.9 (0.1-5.4)	3.1 (1.0-5.2)	2.9 (0.1-5.4)
Free T ₄ (pmol/L)	1.3 (0.8-2.0)	1.3 (0.1-2.3)	1.0 (0.1-1.9)	1.2 (0.05-2.3)
TSH (mIU/L)	2.80 (0.01-31.00)	8.91 (0.01-110.0)	7.37 (0.03-39.40)	8.4 (0.01-110)
Anti-TPO (IU/mL)	14.2 (10-53.2)	19.1 (10-173)	27.8 (10-202)	21.4 (10-202)
Anti-TG (IU/mL)	22.6 (20-82)	20.5 (20-39)	29.8 (20-119)	23.1 (20-119)

BMI, body mass index; TSH, thyroid-stimulating hormone; Anti-TPO, anti-thyroid peroxidase; Anti-Tg, anti-thyroglobulin.

Kruskal-Wallis (when AA, AC and CC genotypes were compared) and Mann-Whitney U (when C allele carriers were compared to AA) tests.

risk of malignancy in a thyroid nodule with high serum TSH,¹⁶⁻¹⁸ other studies show no relationship.¹⁹⁻²¹ Despite the biological relevance of TSH as a risk factor for TC, existing results remain controversial and inconsistent. The lack of consistency, reflects the impact of ethnic difference, phenotypic heterogeneity among populations, limited statistical power in different studies. In present study, although TSH levels in PTC patients were found to be higher than that of controls, this difference disappeared after adjustment for age, gender and smoking status. The reason for the observed tumor-specific difference in the TSH level is unknown. Different carcinogenic processes may be involved in the genesis of various differentiated TC because of the presence of different concentration of serum TSH.

Vascular endothelial growth factor is a potent angiogenic factor which is a mitogen for vascular endothelium, and leads to the development of near and distant metastases.⁴ VEGF plays an important role in angiogenesis via inhibiting endothelial cell apoptosis and increasing vascular permeability.⁶ The microvascular density in thyroid cancer tissue is increased when compared to normal thyroid tissue.²² Accumulating data have shown that increased VEGF expression promotes cancer cell growth, subsequent lymph node metastasis, local invasion, and distant metastasis, whereas the inhibition of VEGF signaling results in suppression of the tumor growth.²³⁻²⁵ In addition, preoperative serum VEGF levels were shown to be elevated in PTC patients and correlated with different clinicopathological features corresponding to their expected biological effects.⁴ In addition,

FIGURE 1 Haplotype analysis and LD patterns (estimated as r^2 and D') of VEGF+405, VEGF-460 and VEGF-2578. There was a strong linkage disequilibrium (LD) between -460/-2578 ($D'=.89$, $r^2=.79$), and weak LD between +405/-460 ($D'=.422$, $r^2=.035$), and +405/-2578 ($D'=.43$, $r^2=.038$) locuses. Number of boxes indicates decimal places of D' . Haplotype frequencies and haplotype association analyses were estimated using Haplowiew Software

increased serum VEGF levels in PTC patients returned to normal after therapy.⁴ Moreover, serum VEGF may be a marker of progression in the follow-up of patients with PTC.²³

The human VEGF gene is a 14 kb DNA fragment located on chromosome region 6p21. The regulatory region of VEGF gene contains a number of transcription factor-binding sites, and transcriptional regulation of this gene appears to be extremely complex, with levels of control at the transcriptional and translational level.²⁶ The expression level of VEGF in various tissues such as liver, lung, breast, and kidney¹⁴ is dependent on its promoter activity. The polymorphisms in the promoter region (loci -2578 and -460) or 5' untranslated region (+405) have been related different levels of expression, VEGF production and its serum levels.¹¹⁻¹⁴ Indeed, +405C, -460T, and -2578C alleles appear to correlate with increased expression and serum levels.¹¹⁻¹⁴

Vascular endothelial growth factor polymorphisms have been implicated in the susceptibility to several cancers including breast,²⁷⁻²⁹

cervical,³⁰ ovarian,³¹ colorectal³² and lung³³ cancers. There are only two studies in literature investigating the -2578, +405, +936, and -141 locuses of VEGF gene.^{3,10} In a study performed by Hsiao et al.³ which has been conducted in Taiwanese population, VEGF -2578, +405, and +936 locuses have been analyzed, and -2578A allele was found to be related with increased risk for PTC. In another study carried out in Australian population,¹⁰ the -141, +405, and +936 locuses were examined, and the -141A, +405G, and +936C allele frequencies have been found to be higher in patients with PTC than controls. There is no previous study on the role of T-460C polymorphism in PTC. The results of present study show that there were no notable differences in allele or genotype frequencies for -460 and -2578 SNPs between PTC patients and controls. With regard to +405 locus, combined CG+CC genotype was related with a fivefold increased risk for developing PTC according to GG genotype. Meanwhile, the frequency of +405C allele was 3.5-fold increased in PTC patients when compared to controls. Previously, the +405 CC genotype was found to be related with a significantly lower overall survival in colorectal cancer,³² presence of ascites in higher volumes in ovarian cancer,³¹ high tumor aggressiveness in breast cancer²⁹ and increased risk of lung cancer.³³ We suggest that the increased susceptibility to PTC is probably related with increased VEGF production in +405C allele carrying subjects.¹¹⁻¹⁴

In our study, haplotype analysis demonstrated that the +405/-460/-2578 GTA haplotype was found to be protective, and CTA haplotype to be related with increased PTC risk. In some studies, the +405/-460 GT haplotype has been shown to be associated with reduced risk of breast cancer²⁷ and epithelial ovarian cancer³¹ and the +405/-2578 GA haplotype was significantly associated with low histologic grade breast cancer.²⁹ The +405/-460/-2578 CTC-, CGA-, and CTC-haplotype were reported to be associated with cervical,³⁰ breast,²⁸ and lung- cancers,³³ respectively. The variable results obtained from various studies may be due to ethnical differences among populations. Also, this variability might arise from the difference in genetic background among different populations and regional geographical variations.

As a conclusion, we found that the +405 polymorphism of VEGF gene may predispose the risk of development of PTC.

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TABLE 7 Haplotype analysis of the VEGF polymorphisms in patients with papillary thyroid cancer (PTC) and control subjects

Haplotype +405/-460/-2578	Frequency of haplotype	Frequency in PTC	Frequency in controls	P-value
GTA	.396	.255	.474	<.001
GCC	.341	.343	.339	.925
CTA	.165	.321	.080	<.001
CCC	.046	.040	.049	.614
GTC	.022	.018	.024	.655
GCA	.021	.018	.023	.701

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