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Single nucleotide polymorphisms and mRNA expression of *VEGF-A* in papillary thyroid carcinoma: Potential markers for aggressive phenotypes

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Abstract

Background and objectives: Polymorphisms of the *VEGF* gene are known to affect the biological behaviour of cancers but have seldom been studied in thyroid cancer. The aim of the current study is to evaluate the prevalence and relevance of *VEGF-A* polymorphisms and mRNA expression in papillary thyroid carcinoma (PTC).
Materials and methods: Genomic DNA and total RNA were isolated from paraffin-embedded tissue from 91 PTC (51 conventional PTC and 40 follicular variant) and 78 control thyroid tissues. Three DNA polymorphisms (+936C > *T*, +405C > *G* and −141A > *C*) in the 3′ and 5′ untranslated region (3′-UTR, 5′-UTR) of *VEGF-A* were studied using PCR and RFLP. Also, the mRNA expression of *VEGF-A* in these tissues was studied by real-time PCR.
Results: Distribution of polymorphisms in the 5′-UTR (*VEGF-VEGF -141*A > *C* and +405C > *G*) and 3′-UTR (*VEGF* +936C > *T*) were all significantly different in PTC and benign thyroid tissue ($p = 0.0001$, 0.001 and 0.028 respectively). The *VEGF* -141 C allele was more common in PTC with lymph node metastases ($p = 0.026$). *VEGF* + 405 *G*alleles and*VEGF* +936 CC genotype were more common in PTC of advanced pathological staging ($p = 0.018$ and 0.017 respectively). Also, increased expression of *VEGF-A* mRNA was noted in PTC compared to control ($p = 0.009$). Within the group of patients with conventional PTC, those with lymph nodal metastases had a higher level of *VEGF-A* mRNA expression than other patients ($p = 0.0003$).
Conclusion: These findings suggest that *VEGF* polymorphisms and mRNA expression may predict the aggressiveness behaviour of thyroid cancer.

Keywords: Papillary thyroid carcinoma; VEGF-A; Polymorphism; SNP; mRNA expression

Introduction

Vascular endothelial growth factor (VEGF) is a key mediator of cancer-associated neo-angiogenesis and progression.[1] The VEGF family includes five members (VEGF-A, -B, -C, -D and -E) and three different tyrosine kinase receptors.[2] They provide targets that have been explored extensively for cancer therapy.[3]

Papillary thyroid carcinoma (PTC) comprised many histological subtypes of different clinicopathological behaviour.[4,5] The two major histological subtypes are conventional PTC (CPTC) and follicular variant of PTC (FVPTC). Increased expression of *VEGF-C* mRNA and protein has been noted in PTC.[6] In addition, VEGF protein expression in PTC correlated with serum levels of the protein. Also, serum levels of VEGF were seen to decrease significantly after surgical removal of PTC.[7] Elevated serum VEGF levels correlated to the recurrence of PTC and the presence of lymph node metastases.[8]

Polymorphisms in *VEGF* may contribute to the variation in tumour angiogenesis. Several single nucleotide polymorphisms (SNPs), such as *VEGF*+405C > *G*, +936C > *T* and −141A > *C* have been described within the promoter, 3′ and 5′ untranslated regions of the *VEGF*-A gene. *VEGF* +405C > *G* and +936C > *T* have been found in cancers and correlated with plasma VEGF production.[9–13] *VEGF* -141A > *C*, has been implicated in the pathogenesis of

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nephrotic syndrome and diabetes.[14,15] The contribution of this polymorphism to cancer has rarely been investigated.

In thyroid cancer, there is only one study that has described the role of *VEGF* SNPs in thyroid cancer.[16] In this study, we aim to determine whether the variation of phenotypic characteristics and clinical behaviours in patients with thyroid cancer was due to the presence of a polymorphic sequence within the 3′ or 5′-untranslated region (3′-UTR or 5′-UTR) of the *VEGF-A* gene. Furthermore, we examined for the first time in thyroid cancer if there was any correlation between these polymorphisms and the mRNA expression of *VEGF-A*.

Materials and methods

Tissue samples collection

Surgically resected thyroid cancers and benign thyroid tissues were obtained from collaborating hospitals in Australia with full ethical approvals. Benign thyroid tissues were collected from the patients diagnosed with nodular hyperplasia of thyroid and without any history of thyroid cancer. The histological slides of the thyroid tissues were retrieved and reviewed by the author (AKL). The thyroid cancers were classified with reference to the criteria defined in the World Health Organization classification of tumours.[17] On pathological examination, the size of the cancer and associated histological features were noted. The thyroid cancers were staged according to the American Joint Committee on Cancer/International Union Against Cancer tumour-node-metastasis (TNM) staging system.[18]

Paraffin blocks from 91 PTCs (51 CPTC and 40 FVPTC), 13 lymph nodes with thyroid cancer metastases and 78 benign thyroid tissues were included. Of the 91 patients (63 women, 28 men) with PTC, the mean age of the patients was 44 years (range, 18–75 years). Of the 78 patients (56 women, 22 men) with benign thyroid lesions, the mean age was 53 years (range, 19–86 years). No significant differences in age and gender distribution were found between the cancer and the control groups.

From the chosen tissue block, 4 μm sections were stained with haematoxylin and eosin to collect the area of interest for DNA and RNA extraction. For the extraction, five 10 μm sections were cut. For the DNA polymorphism study, we used sections from 91 PTCs and 78 benign thyroid lesions. For the mRNA expression study, we involved sections from 91 PTCs, 13 lymph nodes with thyroid cancer metastases and 13 benign thyroid tissues.

Methods

DNA extraction was performed according to the manufacturer's protocol for Qiagen DNA extraction kits (Qiagen, Hilden, NRW, Germany). DNA content was quantified by spectrophotometric absorption (Nanodrop Spectrophotometer, BioLab, Scoresby, VIC, Australia). Polymerase chain reaction (PCR) was performed using an iCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA). Amplifications were carried out and a fragment was generated using the specific forward and reverse primer.

Primers were designed and selected using Primer3, version 0.4.0 (http://frodo.wi.mit.edu/primer3/). For −141A > *C*, the primers were - forward 5′-CCCCTGCCCCCTTCAATA-3′, reverse 5′-AGCCTCAGCCCCTCCACA-3′. For +405C > *G*, the primers were forward 5′- ACTTCCCCAAATCACTG TGG-3′, reverse-5′- CTGTCTGTCTGTCCGTCAGC -3′. For +936C > *T*, the primers were forward 5′- AAGG AAGAGGAGACTCTGCGCAGAGC -3′, reverse 5′-TAAA TGTATGTATGTGGGTGGGTGTGTCTACAGG-3′.

MasterAmp 2X PCR premix *G* (MasterAmp™ PCR Optimization Kits, Epicentre, Madison, WI, USA) and 3 units of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) have been used for amplification of the 3 SNPs. The annealing temperature and restriction enzymes for the primers, *VEGF -141*A > *C*, *VEGF* +405C > *G*, *VEGF* +936C > *T* were 60° C, 60° C and 58° C and HhaI, BsmFI and NlaIII respectively.

The products were submitted to electrophoresis. The expected products of *VEGF -141*, *A* > *C*, *VEGF* +405C > *G* and *VEGF* +936C > *T* were 263 bp, 242 bp and 208 bp respectively. *VEGF* promoter region amplicons were cut with the appropriate restriction enzyme (New England BioLabs, Ipswich, MA, USA) at the polymorphism site and one universal restriction site, to enable visualisation of complete cutting.

Total RNA were extracted and purified with Qiagen miRNeasy FFPE Kits (Qiagen Pty. Ltd., Hilden, NRW, Germany), which were specially designed for purifying total RNA and miRNA from formalin-fixed, paraffin-embedded tissue sections. RNA quality was assessed by using Experion electrophoretogram instrument (Bio-Rad). Integrity of RNA was assessed by observing total size of RNA transcripts along with 18S and 28S peaks. Purity of RNA was obtained by checking the optical density (OD) 260/280 ratios. RNA was converted to cDNA using miScript Reverse Transcription kit according to the manufacturer's instructions (Qiagen). Each cDNA sample was diluted to 30 ng/μl for providing uniformly concentrated samples.

Primers were designed for the analysis of expression of *VEGF-A* mRNA, (GenBank accession number NM_001 025366.1,) and GAPDH mRNA (GenBank accession number NM_002046). GAPDH was used as the ubiquitous control gene. For *VEGF-A*, the primers were: forward-5′-tcttcaagc-catcctgtgtg −3′; reverse-5′-tctgcatggtgatgttggac −3′) whereas for *GAPDH*, the primers were: forward- 5′-tgcaccaccaactgct-tagc −3′; reverse-5′-ggcatggactgtggtcatgag −3′.

Real-time quantitative PCR was performed for detection of *VEGF-A* gene expression by recruiting IQ5 Multicolour Real-Time PCR Detection system (Bio-Rad, Hercules, CA, USA). PCR was performed containing iQ SYBR green supermix (Bio-Rad), and all the samples (unknown and standards) were run in duplicate and accompanied by a non template control. Thermal cycling conditions included 40 cycles of

30 s at 60 °C. The melting curves of all final real-time PCR products were analysed for determination of genuine products and contamination by non-specific products and primer dimers. All samples were also subject for separation on 2% agarose gel electrophoresis for ensuring the correct product amplification.

A standard curve was constructed from a set of known concentrations of cDNA generated from universal human reference RNA (Stratagene, Cedar Creek, TX, USA) for the determination of PCR efficiency. The dilution series were prepared for obtaining ΔCt of *VEGF-A* and *GAPDH*. The fold changes in the target and housekeeping genes for the results of quantitative amplification were calculated for each sample using $2^{-\Delta\Delta Ct}$ method.[19]

Statistical analysis

Statistical analysis was performed using Student's *t*-test and ANOVA using Bonferroni and LSD correction for continuous variables and chi-square or Fisher exact tests for categorical variables. In addition, Pearson correlation (2-tailed) test was used for correlation analysis. Significance level was taken at $p < 0.05$. Statistical analysis was performed with the Statistical Package for Social Sciences for Windows (version 17.0, SPSS Inc., Chicago, IL, USA).

Results

VEGF-A SNPs

The *VEGF* gene polymorphism −141A > *C*, +405 C > *G* and +936C > *T* were successfully amplified in 97.7% ($n = 89/91$), 95.5% (87/91) and 100% (91/91) of the thyroid cancer cases respectively. The variations of these polymorphisms were significantly different in thyroid cancer compared with benign thyroid tissue (Table 1).

For *VEGF* +936 C/T, the CC genotype thyroid cancers were significantly more common in older patients than CT or TT genotypes thyroid cancers (mean age = 47 versus 40, $p = 0.03$). The polymorphism was not related to the gender of the patients and pathological features (histological variant, presence of calcification, psammoma bodies and osseous metaplasia) of the cancer. Although the polymorphism was not related to the presence of lymph nodal metastases, the CT and TT genotypes were significantly more common in cancers of lower pathological stages (stages 1 & 2 versus 3 & 4, $p = 0.017$). Thus, *VEGF* +936 CC genotype was more common in PTC of advanced pathological staging.

The frequency for the AA or AC genotypes in *VEGF*-141 A/C was higher in female patients ($p = 0.044$). The presence of this polymorphism was not related to the age of the patients or histological features (histological variant, presence of calcification, psammoma bodies and osseous metaplasia) in PTC. Within CPTC samples, the A allele of *VEGF* -141A/C was significantly more common in cancers without lymph node metastasis ($p = 0.026$).

Table 1
Association between vascular endothelial growth factor polymorphisms and thyroid cancer.

Genotype	Thyroid cancer	Benign thyroid	*P* value
−141 A/C			
AA	32 (36%)	3 (5%)	0.0001
AC	43 (48%)	54 (80%)	
CC	14 (16%)	10 (15%)	
+405C/*G*			
CC	17 (19%)	23 (32%)	0.001
CG	31 (36%)	36 (51%)	
GG	39 (45%)	12 (17%)	
+936 C/T			
CC	54 (59%)	56 (74%)	0.028
CT	31 (34%)	20 (26%)	
TT	6 (7%)	0 (0%)	

The *VEGF* polymorphism +405 C/*G* was not associated with age, gender of the patients with PTC or histological features (histological variant, presence of calcification, psammoma bodies and osseous metaplasia). Within PTCs, the *G* allele of VEGF +405 was significantly more common in PTC of advanced *T* stages (T3 and T4) ($p = 0.018$) (Table 2).

VEGF-A mRNA expression results (Figure. 1)

VEGF-A mRNA levels in PTC were found to be higher than that in non-cancer thyroid tissue ($p = 0.009$). In PTC, the expression of mRNA was not related to the patients' age, gender or the histological variant. There was also no relationship identified between *VEGF-A* mRNA and presence of calcification, psammoma bodies, osseous metaplasia, or any other clinicopathological feature, with one exception. That exception was that, within the group of patients with CPTC, those with lymph nodal metastases had a higher level of *VEGF-A* mRNA expression than other patients ($p = 0.0003$) (Table 3).

Correlation between SNP and mRNA expression of VEGF-A

None of VEGF gene polymorphisms −141 A/C, +936 C/T and +405 C/*G* in the 3′ and 5′ UTR of *VEGF-A* had any statistically significant correlation with the *VEGF-A* mRNA expression (Table 4).

Discussion

The role of *VEGF-A* SNPs in thyroid cancer have only been investigated by Hsiao et al. dealing with the role of the +936 C/T polymorphism in PTC.[16] The study was performed on peripheral blood of patients with thyroid cancer. In the current study, we studied three polymorphisms, the +936 C/T, the +405C/*G* and the −141 A/C in PTC. In addition, thyroid cancer tissue was used to directly study the effect of *VEGF-A*

Table 2
Clinicopathological relevance of VEGF polymorphisms in thyroid cancer.

Parameters	VEGF -141 A/C			VEGF +405 C/*G*			VEGF +936 C/T			
	AA, AC	CC	*p*-value	CC	CG, GG	*p*-value	CC	CT	TT	*p*-value
Gender										
Male	19 (22%)	7 (8%)	0.04	3 (4%)	22 (26%)	0.28	14 (16%)	9 (10%)	3 (3%)	0.51
Female	54 (63%)	6 (7%)		13 (15%)	46 (55%)		37 (42%)	22 (25%)	3 (3%)	
Size										
<40 mm	60 (70%)	11 (13%)	0.83	15 (18%)	54 (64%)	0.17	42 (48%)	25 (28%)	6 (7%)	0.50
≥40 mm	13 (15%)	2 (2%)		1 (1%)	14 (17%)		9 (10%)	6 (7%)	0 (%)	
Type										
CPTC	41 (48%)	9 (10%)	0.38	12 (14%)	38 (45%)	0.16	31 (35%)	17 (20%)	3 (3%)	0.80
FVPTC	32 (37%)	4 (5%)		4 (5%)	30 (36%)		20 (23%)	14 (16%)	3 (3%)	
Psammoma bodies										
Present	24 (28%)	6 (7%)	0.35	5 (6%)	24 (29%)	0.76	19 (22%)	10 (11%)	2 (2%)	0.80
Absent	49 (57%)	7 (8%)		11 (13%)	44 (52%)		32 (36%)	21 (24%)	4 (5%)	
Calcification										
Present	31 (36%)	6 (7%)	0.80	6 (7%)	31 (37%)	0.55	21 (24%)	13 (15%)	4 (5%)	0.48
Absent	42 (49%)	7 (8%)		10 (12%)	37 (44%)		30 (34%)	18 (20%)	2 (2%)	
Osseous metaplasia										
Present	2 (2%)	0 (0%)	0.54	0 (0%)	2 (2%)	0.48	0 (0%)	2 (2%)	0 (0%)	0.16
Absent	71 (83%)	13 (15%)		16 (19%)	66 (79%)		51 (58%)	30 (34%)	5 (6%)	
T stage										
1 + 2	51 (59%)	9 (10%)	0.96	15 (18%)	43 (51%)	0.01	34 (39%)	24 (27%)	4 (5%)	0.57
3 + 4	22 (26%)	4 (5%)		1 (1%)	25 (30%)		17 (19%)	7 (8%)	2 (2%)	
Lymph node metastasis										
Present	23 (27%)	5 (6%)	0.62	3 (4%)	25 (30%)	0.17	17 (19%)	7 (8%)	4 (5%)	0.09
Absent	50 (58%)	8 (9%)		13 (15%)	43 (51%)		34 (39%)	24 (27%)	2 (2%)	
TNM pathological stage										
Stage 1 and 2	59 (69%)	8 (9%)	0.12	15 (18%)	50 (60%)	0.08	37 (42%)	29 (33%)	3 (3%)	0.01
Stage 3 and 4	14 (16%)	5 (6%)		1 (1%)	18 (21%)		14 (16%)	2 (2%)	3 (3%)	

CPTC: Conventional papillary thyroid carcinoma, FVPTC: Follicular variant of papillary thyroid carcinoma.

SNPs in PTC. Thus, our analyses have taken into account any loss of heterozygosity or spontaneous mutation events that may cause the polymorphic regions in the cancer tissue to differ from the variation inherited from the germline, for this reason no Hardy–Weinberg equilibrium was performed.Also, we limited the thyroid cancer population to utilise only 2 PTC subtypes to avoid confounding effects from multiple rare subtypes with known differences in clinical behaviour.[20]

In the general population, the C allele of *VEGF* +936 C/*T* is more common, and CC genotypes predominate, at a prevalence of around 60–80% depending on ethnic group.[21] Our data showed that the frequency of the CT and TT genotypes of the *VEGF* +936 C/T SNP were increased in younger patients with PTC and also patients with lower stages PTC. Thus, our results imply that patients carrying *T* allele of *VEGF* +936 polymorphism have less aggressive PTC while patients with CC genotype presented with more aggressive PTC. Since age is a component of the staging for thyroid cancers, it is likely that these two observations are connected. This would support VEGF +936 CC as a potential marker of aggression since cancers in younger patients are known to be less aggressive. The polymorphism may be affecting the capacity of the cancers to invade surrounding tissue, perhaps by changes to the efficiency of the recruitment of tissue remodelling factors, or more extensive angiogenic response.

For the *VEGF* -141 A/C polymorphism, there was a tendency for A allele to be more common in female patients with PTC. It is worth noting that PTC is more common in women and that progesterone exposure has been linked to altered *VEGF* expression.[22,23] Thus, the gender difference in allele frequency of *VEGF* -141 SNP may be a result of differential biological responses mediated by the A allele of the SNP that manifest in the presence of female hormones. The effect may also be directly related to the mechanisms behind the gender differences in the characteristics of patients with PTC. Hsiao et al. noticed a male specific effect for the −2578 C/A SNP in increasing risk of thyroid cancer development and presence of lymph node metastasis.[16] The study was performed using peripheral blood and our study used human thyroid tissue. Nevertheless, both studies revealed that gender has a specific biological effect for a particular genotype of *VEGF*.

The prevalence of the A allele of the *VEGF* -141 A/C was increased in patients without lymph node metastasis. Thus, patients with the A allele of *VEGF* -141 potentially have better prognosis, in view of the lower proportion of lymph node metastases. Also, in view of the finding that A allele is more common in women, it may concur with the fact that thyroid cancer in females usually has a better prognosis.

There is no previous study on the role of +405 C/*G* SNP in PTC. In this study, the *G* allele of +405 C/*G* SNP in

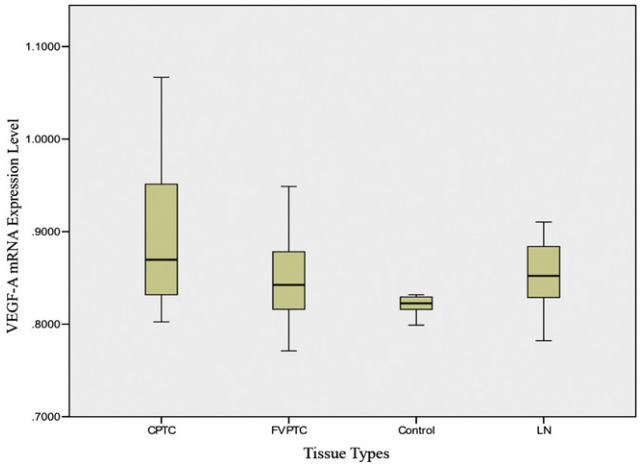

Figure 1. mRNA expression levels of *VEGF-A*. *VEGF-A* mRNA levels in PTC and their lymph node metastases were found to be higher than that in non-cancer thyroid tissue. CPTC: conventional papillary thyroid carcinoma; FVPTC: follicular variant of papillary thyroid carcinoma; control: non-cancer thyroid tissue. LN: lymph nodes metastases of papillary thyroid carcinoma.

VEGF was associated with higher *T* staging. The most obvious explanation is that the *G* allele results in increased angiogenesis, allowing the cancers to grow larger and opening channels in the disrupted blood systems prevalent in tumours to allow invasion into new areas. Thus, patients with *G* allele of +405 C/*G* SNP in *VEGF* have a tendency for a higher pathological staging.

None of the 3 polymorphisms are present in the coding region of *VEGF-A*, so they do not directly cause any amino acid substitutions, ruling out changed protein properties as a mechanism of action. Nor is there any possibility of preferential tRNA effects, since they do not bind to tRNA. The next likely candidate for the mediation of the relationships noted is through effects on *VEGF-A* mRNA expression. However, our analysis of mRNA levels in the same tissues shows no correlation between *VEGF-A* mRNA levels and any of the alleles for the tested SNPs. It is likely that *VEGF* gene polymorphism +936C allele were presented in cancer with higher expression of *VEGF-A*. However, the difference did not reach statistically significant level. This implies that none of the tested SNPs have any significant effect on the VEGF mRNA expression. At its simplest, it may be possible that we do not have sufficient numbers to accurately detect relatively minor effects on *VEGF-A* expression, but there may be some potential mechanisms of action that are beyond the detection of the methods used in this study. One such mechanism which may provide an explanation of the observed relationships is the production of alternative *VEGF* isoforms.[24] It is possible that the studied polymorphisms drive the production of specific isoforms which then mediate the specific functions. Alternatively, the SNPs may be situated at microRNA binding sites, and their previously observed effects on serum VEGF modulated through this mechanism.

Studies using semi-quantitative methods have indicated that increased expression of VEGF-A protein and mRNA was present in thyroid cancers with a higher risk of recurrence

Table 3
The relationship between clinicopathological feature and *VEGF-A* expression in thyroid cancer.

Parameters	VEGF-A expression			
	Under expression	No Change	Overexpression	*p*-Value
Age				
<45 years old	3 (3%)	22 (25%)	25 (28%)	
≥45 years old	4 (5%)	12 (14%)	22 (25%)	0.43
Gender				
Male	1 (1%)	10 (9%)	16 (18%)	
Female	6 (7%)	25 (24%)	31 (35%)	0.49
Size				
<40 mm	5 (6%)	28 (32%)	40 (45%)	
≥40 mm	2 (2%)	6 (7%)	7 (8%)	0.66
Type				
CPTC	2 (2%)	20 (23%)	29 (33%)	
FVPTC	5 (6%)	14 (16%)	18 (20%)	0.25
Psammoma bodies				
Present	1 (1%)	13 (15%)	17 (19%)	
Absent	6 (7%)	21 (24%)	30 (34%)	0.47
Calcification				
Present	1 (1%)	12 (14%)	25 (29%)	
Absent	6 (7%)	22 (25%)	22 (25%)	0.07
Osseous metaplasia				
Present	0 (0%)	1 (1%)	1 (1%)	
Absent	7 (8%)	38 (33%)	46 (52%)	0.88
T stage				
1 + 2	5 (6%)	23 (26%)	34 (30%)	
3 + 4	2 (2%)	11 (12%)	13 (15%)	0.89
Lymph node metastasis				
Present	1 (1%)	9 (10%)	18 (21%)	
Absent	6 (7%)	25 (28%)	29 (33%)	0.30
Lymph node metastasis in CPTC				
Present	0 (0%)	2 (1%)	12 (24%)	
Absent	2 (4%)	19 (37%)	17 (33%)	0.01
TNM pathological stage				
Stage 1 and 2	6 (7%)	27 (31%)	35 (40%)	
Stage 3 and 4	0 (0%)	7 (8%)	12 (14%)	0.35

CPTC: Conventional papillary thyroid carcinoma, FVPTC: Follicular variant of papillary thyroid carcinoma.

Table 4
Relationship of VEGF polymorphisms and *VEGF-A* mRNA expression in thyroid cancer.

Parameters	Number	Under expression	No change	Overex pression	*p*-Value
VEGF -141 A/C					
AA	32 (36%)	4 (5%)	12 (14%)	16 (18%)	
AC	41 (48%)	3 (3%)	16 (19%)	22 (26%)	
CC	13 (16%)	0 (0%)	6 (7%)	7 (8%)	0.722
AA, AC	73 (85%)	7 (8%)	28 (33%)	38 (44%)	
CC	13 (15%)	0 (0%)	6 (7%)	7 (8%)	0.491
VEGF +405 C/*G*					
CC	16 (18%)	2 (2%)	8 (10%)	6 (7%)	
CG	30 (36%)	2 (2%)	12 (14%)	16 (19%)	
GG	38 (46%)	3 (4%)	13 (16%)	22 (26%)	0.724
CC	16 (19%)	2 (2%)	8 (10%)	6 (7%)	
CG, CG	68 (81%)	5 (6%)	25 (30%)	38 (45%)	0.400
VEGF +936 C/T					
CC	51 (57%)	4 (5%)	19 (22%)	28 (32%)	
CT	31 (36%)	1 (1%)	13 (15%)	17 (19%)	
TT	6 (7%)	2 (2%)	2 (2%)	2 (2%)	0.173
CC, CT	82 (93%)	5 (6%)	32 (36%)	45 (50%)	
TT	6 (7%)	2 (2%)	2 (2%)	2 (2%)	0.057

and metastasis.[25,26] In concordance to these studies, we found that expression of *VEGF-A* mRNA is significantly increased in PTC in relation to non-malignant thyroid tissue. Furthermore, the expression of *VEGF-A* mRNA is significantly higher in conventional PTC with lymph nodal metastases than those without. Thus, quantitative analysis of *VEGF-A* mRNA may be useful for the detection of lymph nodal metastases, and may predict the clinical aggressiveness of PTC.

Araujo-Filho et al., based on immunochemical findings on 76 patients with PTC, noted that VEGF-A protein expression was increased in CPTC compared to the FVPTC.[27] However, we did not find any significant differences in expression of *VEGF-A* between the 2 variants. This may be a function of the differences in methodology used. The differences in *VEGF-A* expression observed by Araujo-Filho et al. may be controlled post-transcriptionally by miRNA interactions, which may not be detectable with our PCR based methodology.

Nowak et al.[28] detailed the inhibitory role of VEGF isoforms such as VEGF165b and the role of C terminal splicing in VEGF biology. This may explain the lack of apparent effect on the SNPs interrogated on *VEGF* expression in our study. It is possible that while one change in the allelic location increase or decrease the expression of one isomer, the other can be unaffected, and eventually affect the role and expression of *VEGF-A* mRNA. Also, the simple relationship between *VEGF-A* expression and pathological characteristics could be further complicated by isoform proportions and the array of receptors available within the cancer.

In conclusion, our work has indicated that the molecular status of *VEGF-A* may play a significant role in the progression of papillary thyroid carcinoma. Over expression of *VEGF-A* mRNA was noted in thyroid cancer and more so in thyroid cancer with lymph node metastases. Furthermore, distributions of *VEGF* SNPs were altered in PTCs with pathological staging and the presence of lymph node metastases. These findings imply that continued research into *VEGF* mRNA expression and polymorphisms will be an important source of information on the pathogenesis and prediction of clinical behaviour of thyroid cancer.

Conflict of interests

None declared.

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