Vascular endothelial growth factor gene polymorphisms in thyroid cancer

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Abstract

Vascular endothelial growth factor (VEGF) is a potent stimulator for angiogenesis. It has been implicated in growth and metastasis of thyroid cancer. Three functional single nucleotide polymorphisms (SNPs) of VEGF (−2578C/A, −634G/C, and +936C/T) are known to be related with VEGF expression. We conducted a case–control study to evaluate the genetic effects of these three functional SNPs on the development of thyroid cancer and lymph node metastasis. A total of 332 cases and 261 controls were recruited for this study. The genotypes were determined by the TaqMan 5′-nuclease assay. Hardy–Weinberg equilibrium (HWE) was tested for each SNP, and genetic effects were evaluated by the χ^2 -test and multiple logistic regression. We used Bonferroni correction to account for multiple testing, and a two-tailed *P* value <0.017 was considered statistically

significant. All three SNPs were in HWE. The A allele of −2578C/A (i.e. SNP rs699947) increased a risk for thyroid cancer (adjusted OR = 1.36, 95% CI = 1.02–1.81, *P* = 0.039). Haplotype analysis yielded a less significant result (an empirical *P* value of 0.07). There was a tendency of increasing the frequency of the risk allele from controls, patients without lymph node metastasis to patients with lymph node metastasis (*P*_{trend} = 0.019). Further analysis showed that the genetic effect was only in men (adjusted OR = 1.97, 95% CI = 1.16–3.37, *P* = 0.013) but not in women (adjusted OR = 1.15, 95% CI = 0.81–1.62, *P* = 0.435). The other two SNPs did not show significant results. The A allele of the SNP rs699947 increased the risk of thyroid cancer development and regional lymph node metastasis in men.

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Introduction

Thyroid cancer accounts for <1% of all human cancers but is the most common endocrine neoplasia (Schlumberger & Torlantino 2000). The incidence of thyroid cancer (0.5–10/100 000) increases with age, and it reaches a plateau after the age of 50 years (DeLellis *et al.* 2004). Thyroid cancer is two to four times more frequent in females than males. Radiation exposure, hormonal factors, and family history are the risk factors for thyroid carcinoma (Kondo *et al.* 2006). Genetic predisposition has been proposed as one of the risk factors for thyroid cancer (Malchoff & Malchoff 2006). Different histological types of thyroid cancer have distinct clinical features. More than 90% thyroid tumors are well-differentiated carcinoma, including papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC). For these two types of thyroid cancer, the treatment can be effective and prognosis is often favorable for patients identified in the early stage.

In contrast, anaplastic thyroid cancer is aggressive, responds poorly to treatment, and is associated with worse prognosis (Kondo *et al.* 2006). Follicular thyroid carcinoma tends to spread via the blood stream, whereas papillary thyroid tumor spreads occur via the lymphatic system (Turner *et al.* 2003).

Angiogenesis is a process of new blood vessel development from preexisting vasculature. The vascular endothelial growth factor (VEGF) is one of the most potent endothelial cell mitogens and plays a crucial role in both angiogenesis and lymphogenesis (Carmeliet & Jain 2000). The microvascular density is increased in the thyroid malignancy compared with normal thyroid tissue and benign thyroid tumors (Segal *et al.* 1996, Akslen & Livolsi 2000). Growing evidence from *in vitro* and *in vivo* experiments have shown that increased VEGF expression promotes thyroid 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 (Lin & Chao 2005).

A previous study reported that plasma VEGF levels in advanced thyroid cancer patients were significantly elevated (Lin *et al.* 2003). A 5-year cohort study showed that expression of VEGF was strongly associated with a higher frequency of local and distant recurrence of papillary thyroid carcinoma (Kilicarslan *et al.* 2003).

Some polymorphisms in the promoter and 3'-untranslated region are known to regulate VEGF expression (Brogan *et al.* 1999, Awata *et al.* 2002). Three single nucleotide polymorphisms (SNPs; -2578C/A, -634G/C in the promoter region and +936C/T in the 3'-untranslated region) are common in the Han Chinese population (<http://www.ncbi.nlm.nih.gov/SNP/>) and are related to VEGF protein production (Rogers & D'Amato 2006). The current SNP nomenclature for the above three SNPs is rs699947 for -2578C/A, rs2010963 for -634G/C, and rs3025039 for +936C/T. VEGF polymorphisms have been implicated in the susceptibility to several cancers including prostate cancer (Sfar *et al.* 2006), breast cancer (Kataoka *et al.* 2006), lung cancer (Koukourakis *et al.* 2004), gastric cancer (Tzanakis *et al.* 2006), and renal cell carcinoma (Kawai *et al.* 2007). However, the effect of these polymorphisms on thyroid cancer has not been reported. On the basis of the biological and pathologic significance of VEGF, it is possible that functional genetic variations in the VEGF gene may contribute to the development and progression of thyroid cancer. To test the hypothesis, we conducted a case-control study to evaluate the effects of these three functional polymorphisms on the development of thyroid cancer and lymph node metastasis.

Materials and Methods

Subjects

We initially recruited 332 patients with thyroid cancer (only PTC or FTC was enrolled) and 261 controls at the Kaohsiung Medical University Memorial Hospital in Taiwan. The initial diagnostic year of thyroid cancer was from 1980 to 2006. The diagnosis with thyroid cancer and lymph node metastasis were both confirmed by pathological examination. The presence of regional lymph node metastasis was detected while the patient receiving thyroidectomy. The control subjects were patients from the Department of Endocrinology and Otorhinolaryngology for other reasons. None of the controls had known thyroid disease or any type of cancer upon enrollment. All of the control subjects received thyroid physical examination by an endocrinologist or otolaryngologists. Twenty-one control subjects with simple goiter have been further evaluated by thyroid sonography and fine needle aspiration to exclude any malignant change. The female versus male ratio was higher in the cases than controls. To reduce the impact of unbalanced sex ratio in this case-control study, we subsequently enrolled another 156 female controls and genotyped the 'interesting' SNP based on the results from

the initial study subjects. These 156 female controls were recruited from the healthy individuals who came to the Department of Preventive Medicine for annual health checkup. None of them had reported cancer or thyroid disease. Information on demographic characteristics, family history of cancer, and exposure to ionizing radiation were collected. The study was proved by the Institutional Review Board of Kaohsiung Medical University Hospital and written informed consent was given by each subject or custodian (if the age of the participant was <18 years old).

Genotyping

Genomic DNA was extracted from peripheral blood by a standard method. Genotyping was carried out by the TaqMan technology. Briefly, PCR primers and TaqMan minor groove binder probes were designed and reactions were performed in 96-well microplates with ABI 9700 thermal cyclers (Applied Biosystems, Foster City, CA, USA). The information about probe sequences and PCR can be found in Supplementary Table 1 which can be viewed online at <http://joe.endocrinology-journals.org/content/vol195/issue2/>. Fluorescence was measured with an ABI 7500 Real-Time PCR System and analyzed with its System SDS software version 1.2.3.

Statistical analysis

SPSS for Windows 13.0 version was used for statistical analysis. Continuous variables were analyzed by independent *t*-test and were presented as mean $\pm$ S.D. Allele frequencies were estimated by direct gene counting. Observed numbers of each genotype were compared with those expected for Hardy-Weinberg equilibrium (HWE) using the χ^2 -test. Genetic effects were first assessed by χ^2 -test or Fisher's exact test. Multiple logistic regression analysis was performed to adjust for the effects of age and sex while assessing the genetic effects. A trend test (P_{trend}) assuming a dose-response with increasing number of a risk allele was also performed. Haploview was applied to calculate linkage disequilibrium (LD) and haplotype blocks. Haplotype analysis was conducted using the Haplustering program (Tzeng *et al.* 2006). We used Bonferroni correction to account for multiple testing, and a two-tailed *P* value <0.017 (=0.05/3 SNPs) was considered statistically significant.

Results

Table 1 shows the baseline characteristics of the subjects. The genotyping call rate for each SNP ranged from 88 to 92%. The age of study group ranged from 11 to 82 years old. No significant difference of age was found between the case and control groups. Females were predominant among our cases ($P<0.001$), which is in agreement with the gender distribution in patients with thyroid cancer. Histological

Table 1 Baseline demographics of study characteristics

	Case (n=332)	Control (n=261)	P value
Age (mean $\pm$ s.d., range, y/o)	43.3 $\pm$ 13.1 (11–82)	42.6 $\pm$ 15.0 (11–81)	0.583
Sex (male: female)	1:4.75	1:1.24	0.001
Pathology ^a			
Papillary cancer	255 (85.3%)	–	–
Follicular cancer	44 (14.7%)	–	–
Lymph node metastasis			
Yes ^b	76 (26.4%)	–	–
No	212 (73.6%)	–	–

^aMissing information of histological type in 33 thyroid cancer patients.^bLymph node metastasis (+): papillary cancer, n=68; follicular cancer, n=8.

classification of thyroid tumor was as follows: 85.3% (255 patients) papillary cancer and 14.7% (44 patients) follicular cancer. Regional lymph node metastasis was detected in 26.4% (76 patients) of all thyroid cancer patients while receiving thyroidectomy. All the cases denied previous exposure to ionizing radiation sources (either accidental or therapeutic).

The distribution of VEGF genotypes was in HWE in either cases or controls. The minor A allele of rs699947 was overrepresented in the patients with lymph node metastasis than controls (33.6 vs 23.3%, $P=0.014$). However, the difference of A allele frequencies between all patients and controls was borderline significant (28.1% in cases vs 23.3% in controls, $P=0.070$; Table 2). The multivariate logistic regression model yielded an adjusted OR of 1.36 (95% CI=

1.02–1.81, $P=0.039$) for the risk A allele, while age and sex were adjusted for and an additive mode of inheritance was assumed. We also found that the frequency for either the AC or AA genotype was highest in patients with lymph node metastasis, followed by patients without lymph node metastasis, and lowest in controls. The proportion of the A allele carriers from these three types of subjects yielded a trend test P value of 0.019 (57.1 vs 45.0 vs 40.5%), which was close to Bonferroni-adjusted significance. When we analyzed each type of thyroid cancer, the results were less significant but the pattern of association was still present (Supplementary Tables 2 and 3 which can be viewed online at <http://joe.endocrinology-journals.org/content/vol195/issue2/>). For SNPs rs2010963 and rs3025039, we did not find any significant association between genotypes and phenotypes under dominant, recessive,

Table 2 Association between vascular endothelial growth factor polymorphisms and thyroid cancer

	Thyroid cancer, <i>n</i> (%)			Control, <i>n</i> (%)	$\chi^2 P$ value	<i>P</i> _{trend}
	Total	LN meta (+)	LN meta (−)			
Genotype						
rs699947(−2578C/A)						
CC	155 (52.2%)	30 (42.9%)	109 (55.0%)	148 (59.5%)	<i>P</i> ₁ = 0.202	0.019
AC	117 (39.4%)	33 (47.1%)	73 (36.9%)	86 (34.5%)	<i>P</i> ₂ = 0.043	
AA	25 (8.4%)	7 (10.0%)	16 (8.1%)	15 (6.0%)		
Allele						
C	427 (71.9%)	93 (66.4%)	291 (73.5%)	382 (76.7%)	<i>P</i> ₁ ' = 0.070	0.454
A	167 (28.1%)	47 (33.6%)	105 (26.5%)	116 (23.3%)	<i>P</i> ₂ ' = 0.014	
rs2010963(−634G/C)						
GG	104 (33.2%)	20 (28.6%)	68 (32.4%)	67 (29.1%)	<i>P</i> ₁ = 0.438	0.454
CG	160 (51.1%)	40 (57.1%)	108 (51.4%)	119 (51.7%)	<i>P</i> ₂ = 0.606	
CC	49 (15.7%)	10 (14.3%)	34 (16.2%)	44 (19.1%)		
Allele						
G	368 (58.8%)	80 (57.1%)	244 (58.1%)	253 (55.0%)	<i>P</i> ₁ ' = 0.213	0.867
C	258 (41.2%)	60 (42.9%)	176 (41.9%)	207 (45.0%)	<i>P</i> ₂ ' = 0.655	
rs3025039(+936C/T)						
CC	171 (59.4%)	43 (65.2%)	101 (55.2%)	145 (62.5%)	<i>P</i> ₁ = 0.739	0.867
CT	105 (36.5%)	21 (31.8%)	73 (39.9%)	77 (33.2%)	<i>P</i> ₂ = 0.862	
TT	12 (4.2%)	2 (3.0%)	9 (4.9%)	10 (4.3%)		
Allele						
C	447 (77.6%)	107 (81.1%)	275 (75.1%)	367 (79.1%)	<i>P</i> ₁ ' = 0.562	0.867
T	129 (22.4%)	25 (18.9%)	91 (24.9%)	97 (20.9%)	<i>P</i> ₂ ' = 0.621	

LN meta: lymph node metastasis. P_1 and P_1' : total thyroid cancer versus control. P_2 and P_2' : thyroid cancer with LN meta (+) versus control. Trend test: genotypes versus LN meta (+) $\rightarrow$ LN meta (–) $\rightarrow$ control.

or additive genetic model. When these two SNPs were analyzed for the patients with lymph nodes metastasis, neither of them reached to the statistical significance.

The sex distribution was very different between cases and controls. We genotyped SNP rs699947 in additional 156 female controls and found that the A allele frequency in the control increased from the original 23.3 to 24.7%. These controls did not change the potential effect of SNP rs699947. We further divided the original study subjects by sex and examined the sex-specific genetic effect (Table 3). We found that the association between SNP rs699947 and the risk of thyroid cancer existed only in men (adjusted OR = 1.97, 95% CI = 1.16–3.37, $P=0.013$) but not in women (adjusted OR = 1.15, 95% CI = 0.81–1.62, $P=0.435$). The other two SNPs still did not show significant sex-specific effect. The two SNPs rs699947 and rs2010963 were in strong LD ($D'=0.96$), but both SNPs had low LD ($D'<0.15$) with the third SNP rs3025039. Haplotype analysis using the two SNPs in the same block demonstrated a less significant result (an empirical P value of 0.07).

Discussion

In the present study, we examined the relationship between three functional SNPs of the VEGF gene and the risk of thyroid cancer and regional lymph node involvement. Our results suggest a potential role of the VEGF promoter SNP rs699947 (i.e. –2578 C/A) in the development of thyroid cancer and lymph node metastasis. The A allele of SNP rs699947 increases a risk for thyroid cancer development, and may also facilitate lymph node metastasis. The effect of this risk allele is present in men but not in women. The association between genetic variation in VEGF and thyroid cancer is biologically plausible for several reasons: first, VEGF has been identified as a critical factor in angiogenesis required for tumor growth (Bunone *et al.* 1999). Second, VEGF expression has been related to tumor progression (Viglietto *et al.* 1995, Lewy-Trenda & Wierchniewska-Lawska 2002) and metastasis (Fellmer *et al.* 1999, Klein *et al.* 1999). The promoter polymorphism of the VEGF gene in our study in

part explains the variation of individual susceptibility to thyroid cancer and regional lymph node involvement. However, our positive result is based on a small number of male subjects. Further studies to replicate our result are necessary. Before any further replication, our result should be considered potentially intriguing but tentative.

It is of particular interest that the genetic risk of rs699947 only exists in males. Although the prevalence of thyroid cancer in women is in excess of men, being male is regarded as one of the high risk factors for thyroid cancer recurrence and cancer death (Liska *et al.* 2005). A growing body of evidence suggests that sex hormone plays a role in regulating VEGF expression. Studies showed that androgens can stimulate VEGF expression (Haggstrom *et al.* 1999, Stewart *et al.* 2001, Woodward *et al.* 2005). Androgens can activate hypoxia inducible factor-1 (Mabjeesh *et al.* 2003) which in turn translocates into nucleus to enhance VEGF expression (Josko & Mazurek 2004). On the other hand, studies also demonstrate that both estrogen and progesterin can increase VEGF expression. Garvin *et al.* (2005) showed that estrogen acts as an angiogenic switch in breast cancer cells. Furthermore, progesterone-response elements had been identified in the promoter of the VEGF gene (Mueller *et al.* 2003). Mutation in the Sp-1 region of VEGF promoter can abolish progesterin induction of VEGF synthesis (Wu *et al.* 2005). The role of sex hormone in regulation of VEGF expression in thyroid cancer has not been explored and our finding of sex-specific VEGF genetic effect has yet to be replicated by other studies.

Twenty-nine cases with genotypes at SNP rs699947 do not have information regarding lymph node metastasis. If all these 29 cases have a systemic bias towards the group with lymph node metastasis, the result is still significant after adding all of them into statistical models. On the other hand, if the systemic bias is towards the group without lymph node metastasis, the statistical result remains significant. For the patients without evidence of lymph node metastasis, it is possible that the surgeons did not remove the involved lymph nodes leading to an underestimation of the percentage of lymph node metastasis. However, this bias would be

Table 3 Sex-specific association between rs699947(–2578C/A) and thyroid cancer

	Thyroid cancer, <i>n</i> (%)			Control, <i>n</i> (%)	Adjusted OR ^a (95% CI)	Sig.	<i>P</i> _{trend} ^b
	Total	LN meta (+)	LN meta (–)				
Genotype							
Male							
CC	23 (41.1%)	7 (30.4%)	11 (42.3%)	70 (62.5%)	1.97	$P=0.013$	0.002
AC+AA	33 (58.9%)	16 (69.6%)	15 (57.7%)	42 (37.5%)	(1.16–3.37)		
Female							
CC	132 (55.0%)	23 (48.9%)	98 (57.0%)	77 (56.6%)	1.15	$P=0.435$	0.489
AC+AA	108 (45.0%)	24 (51.1%)	74 (43.0%)	59 (43.4%)	(0.81–1.62)		

^aComparing total cases with controls by multiple logistic regression where age was adjusted and an additive mode of inheritance was assumed.
^b*P*_{trend}: genotypes in LN meta (+)→LN meta (–)→control.

independent of genotypes and should not result in a different conclusion. Retrospectively recruiting post-operative patients could lead to survival bias. To test for this potential bias and its impact on our conclusion, we divided the subjects into two groups by the follow-up period ≥ 5 and < 5 years. We found that the frequency of the risk A allele of SNP rs699947 was 28.0% in the long follow-up group and 28.5% in the short follow-up group. Therefore, the ascertainment of this retrospective case-control study did not have survival bias.

In conclusion, we found that the -2578 C/A SNP in the promoter region of the VEGF gene may predispose the risk of development of thyroid cancer and regional lymph node metastasis. Our data also suggested a sex-specific effect and males are under stronger genetic influence than females.

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